# A COMPARATIVE STUDY OF THE CATECHIN COMPONENTS IN THE BARKS OF WATTLE SPECIES RELATED TO ACACIA MEARNSII

by

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for the

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## FOREWORD

This thesis is submitted in accordance with the regulations for the Degree of Doctor of Philosophy of Rhodes University. The work has been carried out at the Leather Industries Research Institute, Grahamstown, and is wholly original except where due reference is made in the text. It has not been submitted in whole, or in part, for any degree at any other University.

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## DEFINITION OF TERMS

*Acacia mollissima* Willd., black wattle, has recently been reclassified as *Acacia mearnsii* De Wild. (Brenan and Melville 144).

SUMMARY.

The distribution of flavonoid constituents in the barks of Acacia mearnsii De Wild.(black wattle), A. decurrens Willd.(green wattle), A. dealbata Link.(silver wattle) and A. pycnantha Benth.(golden wattle) has been studied.

Bark extracts of the four wattle species have been fractionated into low molecular weight fractions containing mainly catechins and other low molecular weight constituents, and high molecular weight fractions containing the bulk of the polymerized tannins. The low molecular weight fractions have been further fractionated by "preparative paper chromatography".

(-)-Robinetinidol, (-)-7:3':4':5'-tetrahydroxyflavan-3-ol, a new naturally occurring catechin, (+)-catechin and (+)-gallocatechin have been isolated from the barks of A. mearnsii, A. dealbata and A. pycnantha. (-)-Epicatechin and (-)-epigallocatechin have been identified in the bark extracts of A. dealbata and A. pycnantha, but appeared to be absent in the barks of A. mearnsii and A. decurrens. (-)-Epicatechin has been isolated from A. dealbata, and both (-)-epicatechin and (-)-epigallocatechin were isolated from A. pycnantha. (-)-Epicatechin gallate, (-)-epigallocatechin gallate and gallic acid were isolated from A. pyc-

nantha only. These three constituents appeared to be absent in the barks of the three other wattle species.

(-)-Epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate which were not available for direct comparison, were subsequently isolated from green tea where they are present as major phenolic constituents.

A method for the quantitative estimation of polyphenolic substances on two dimensional paper chromatograms has been developed, and a photoelectric densitometer constructed. Two spray reagents, ammoniacal silver nitrate and bisdiazotised benzidine, were found to give straight line relationships of instrument deflection against log concentration for flavonoid substances.

This estimation method for the first time supplied means for a detailed study of the concentration of catechin constituents in the bark extracts of A. mearnsii, A. decurrens, A. dealbata, A. pycnantha and of A. mearnsii x A. decurrens hybrids. The concentration of catechin constituents has been shown to vary considerably between species whereas variation within species was small. In the latter respect silver wattle is an exception.

Taxonomic significance may possibly be attached to the distribution of catechin constituents in the bark of the four Acacia species. The concentration of (-)-robinetinidol, which appears to be the characteristic compound of these

*Acacias*, progressively decreases in the sequence black-, black x green hybrid, green-, silver- and golden wattle, while the number of catechin constituents of the "phloroglucinol series" increases in the same sequence. It thus appears, that by the examination of their bark components, a differentiation between species of a subgenera may be possible.

Two tannins, constituents D and B, which are related to the leuco-anthocyanidins (flavan-3:4-diols), have been found in the barks of the four wattle species.

One of the two, constituent D, was isolated in a pure form from the barks of *A. mearnsii* and *A. pycnantha*. Constituent D was found to generate robinetinidin and an orange pigment, the structure of which has not yet been fully identified. Compound D and its acetyl- and methoxyl derivatives did not crystallize. From the results of alkaline-, acidic- and enzymatic degradations, colour reactions and light-absorption studies, combustion analysis of the compound and its derivatives and molecular weight estimations, constituent D is surmised to be a dimer of 7:3':4':5'-tetrahydroxyflavan-3:4-diol (leuco-robinetinidin). The isolation of this complex leuco-anthocyanidin tannin represents the first isolation of a flavonoid tannin from commercial vegetable tannin sources.

The second tannin obtained from the bark of *A.*

mearnsii, "constituent B" appears to consist of two overlapping substances, which have not yet been separated. The tannin (B) was found to have an average molecular weight of 676 and it is considered likely that both substances may be dimolecular. On heating with mineral acid robinetinidin, fisetinidin and an orange pigment are generated, the pigment being identical with the pigment generated from constituent D. It may therefore be assumed that "constituent B" consists of a mixture of complex leuco-robinetinidins and leuco-fisetinidins.

The distribution of complex leuco-anthocyanidins in the bark extracts of Acacia mearnsii, A. decurrens, A. dealbata and A. pycnantha has been examined. A correlation between the distribution of leuco-anthocyanidins in the bark of the four wattle species, and accepted systematics, does not, apparently, exist.

CONTENTS

# CHAPTER 1.

## INTRODUCTION.

Vegetable tannins are widely distributed in nature. Structural elucidation of vegetable tannins proved to be extremely difficult due to the complexity of the mixtures, and it has been only in the last decade that some structures have been resolved.

When vegetable tannins are heated with diluted mineral acid some are hydrolysed to give gallic acid and glucose as main degradation products, while others form highly condensed "tanners' reds" and on prolonged heating insoluble "phlobaphenes". These phenomena led Freudenberg (1) to suggest a subdivision into two main classes : hydrolysable tannins and condensed tannins.

It was shown in recent years that this classification is not absolute, and that a classification based on structural differences would be more accurate.

Hydrolysable Tannins.

1. Chestnut; Bark, wood, leaves and twigs of *Castanea* sp. fam. *Fagaceae*.
2. Myrobalans; Fruit of *Terminalia chebula*. fam. *Combretaceae*.
3. Valonia; Acorn cups of *Quercus aegilops*. fam. *Fagaceae*.

4. Oak; Wood of Quercus sp. fam. Fagaceae.
5. Sumach; Leaves of Rhus coriaria, R. typhinus and other spp.,fam. Anacardiacea.
6. Divi-divi; Pods of Caesalpinia coriaria, fam. Leguminosae.

The hydrolysable tannins have been studied in recent years by Schmidt at Heidelberg (2-14), Grassmann at Munich (15a, 15b), Hathway at B.L.M.R.A. Egham (16), by White at Harpenden (17-21) and by Haworth at Sheffield (22-25). The work of these authors established conclusively that hydrolysable tannins are based on two substances only, gallic acid and glucose.

Condensed Tannins.

1. Quebracho; Wood of Schinopsis lorentzii (syn. S. quebracho-colorado) and S. balansae, fam. Anacardiaceae.
2. Mimosa or Wattle; Bark of Acacia mearnsii De Wild., (144) (A. mollissima Willd., syn. A. decurrens (Wendl.) Willd., var mollis Lindl.) A. decurrens Willd., A. dealbata Link., and A. pycnantha Benth., fam. Leguminosae.
3. Mangrove (syn. Borneo cutch); Bark of various Rhizophoraceae spp.
4. Spruce; Bark of Picea abies and other spp., fam. Pinaceae.

5. Hemlock; Bark of Tsuga canadensis. fam. Pinaceae.
6. Gambier; Leaves and twigs of Uncaria gambir fam. Rubiaceae.
7. Burma cutch; Wood of Acacia catechu, fam. Leguminosae.
8. Myrtan; Wood, bark and leaves of Eucalyptus redunca, fam. Myraceae.
9. Oak; Bark of various Quercus spp,,fam. Fagaceae.
10. Tizerah; Wood and roots of Rhus pentaphylla, fam. Anacardiaceae.
11. Urunday; Wood of Astronium balansae, fam. Anacardiaceae.

Until recent years little was known about this important class of vegetable tannins due to the extreme complexity of the tannin mixtures. Various attempts, however, have been made to explain their chemistry on the basis of a single interpretation fitting the whole group. Of these theories three gained widest recognition: the Catechin Hypothesis of Freudenberg (26-30), the Flavpinacol Hypothesis of Russel (31-35) and the Leuco-anthocyanidin Hypothesis of Bate-Smith and Swain (36). Russels concept, which was based on the idea that 4-hydroxyflavans were dimerised to form flavpinacols, was heavily criticised by Freudenberg, Karimullah and Steinbrunn (37) and by Finch and White (38), who showed that Russel had misinterpreted his experimental

results. The two remaining concepts, of Freudenberg and of Bate-Smith and Swain, will be discussed in a special paragraph on "Leuco-anthocyanidins and Catechins as Tannins".

Two groups of constituents, catechins (flavan-3-ols) and leuco-anthocyanidins (flavan-3:4-diols), were found to be present in relative high concentration in a great number of tannin extracts, (not necessarily commercial tannin extracts) which give the characteristic "phlobaphene reaction" of condensed tannins. In spite of this fact, which may lead to the conclusion that all condensed tannins are polymers or condensates of flavonoid bodies, there are at least two groups of condensed tannins, those from spruce bark (Grassmann et al. 29-47) and those from the wood of Eucalyptus species (Hathway and Seakins 48), where polyhydroxystilbenes form the major part of the tannin mixture.

In tannin extracts from the bark and also from the wood of wattle species, however, only flavonoid constituents and their polymers have been identified so far (Roux et al. 49-63) (White 20) (Hillis 64) (Keppler 65, 66). Simple and complex leuco-anthocyanidins apparently form the bulk of the tannin mixture.

Naturally occurring catechins.

When Freudenberg commenced work on catechins after the first world war, catechin itself had been known for a century, Runge having discovered it in 1824. Freudenberg and co-workers conclusively established the constitution of catechin (26, 67-70) as 5:7:3':4'-tetrahydroxyflavan-3-ol (II), but it took another 30 years to clarify the stereochemical structures of catechin and related compounds(71-75). (+)-Catechin is the main crystalline principle in gambier extracts from leaves and twigs of Uncaria gambir Roxb., of the family of Rubiaceae (Kostanecki & Tambor, 76), whereas the heartwood of Acacia catechu Willd. (family Leguminosae) yields mainly (-)-epi-catechin.

A related catechin, (-)-gallocatechin*, 5:7:3':4':5'-pentrahydroxyflavan-3-ol (III), was found in 1929 by Tsujimura in green tea (77). The second catechin of this series, (+)-gallocatechin, was isolated from the bark of Casuarina equisitifolia Linn. ten years later by Oshima (78) and named "casuarin". Oshima's casuarin was shown to consist of two components, (+)-catechin and (+)-gallocatechin, by Roux in 1957 (79), who isolated both (+)-catechin and (+)-gallocatechin from Casuarina equistifolia. His findings were proved to be correct when, in 1957, Mayer and

* Tsujimura's (-)-gallocatechin was later shown to be (-)-epigallocatechin (Bradfield & Bate-Smith 83).

Bauni (80) isolated (+)-gallocatechin from the bark of oak and sweet chestnut and compared its physical and chemical properties with values reported by Oshima. The values for casuarin were shown to be incorrect.

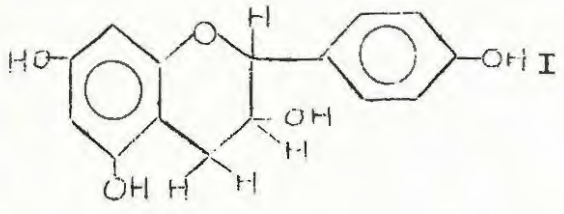
Bradfield and Penny in 1947-1948 (81,82) isolated (-)-epicatechin, so called (-)-gallocatechin, (±)-gallocatechin [shown to be (+)-gallocatechin (84)], (-)-epicatechin gallate (IV) and a (-)-gallocatechin gallate (V) from the polyphenolic fraction of green tea. Bradfield and Bate-Smith subsequently (83) suggested that (-)-gallocatechin and (-)-gallocatechin gallate might more accurately be designated as (-)-epigallocatechin (III) and (-)-epigallocatechin-gallate (V) respectively, on the basis of their paper chromatographic behaviour. From chromatographic evidence Roberts (84) showed that green tea does not contain the racemic (±)-gallocatechin, but only (+)-gallocatechin, and in 1960 confirmed this by the isolation of crystalline (+)-gallocatechin from green tea (85).

In 1955 King, Clark-Lewis and Forbes (72) obtained another catechin of the "phloroglucinol series" from the wood of Afzelia species, (-)-5:7:4'-trihydroxyflavan-3-ol, which closely resembles (-)-epicatechin and was named (-)-epiafzelechin (I).

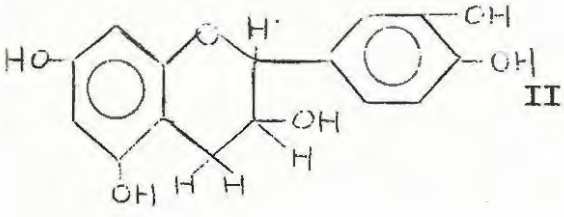
In 1958 Roux and Maihs (54,57) reported the

isolation of crystalline (-)-robinetinidol, (-)-7:3':4':5'-tetrahydroxyflavan-3-ol (VII), from the bark of Acacia mearnsii De Wild., and confirmed its structure by comparison with synthetic (-)-robinetinidol (Weinges 74). This was the first catechin of the "resorcinol series" to be isolated from a natural source. The second catechin of this series, (-)-fisetinidol, (-)-7:3':4'-trihydroxyflan-3-ol (VI), was recently isolated in crystalline form from the wood of Acacia mearnsii De Wild. by Roux and Paulus, (61) who also synthesized (-)-fisetinidol via hydrogenation of (+)-7:3':4'-trihydroxyflavan-3:4-diol. The synthetic product and the isolated component proved to be identical.

Naturally occurring catechins of the phloroglucinol series;

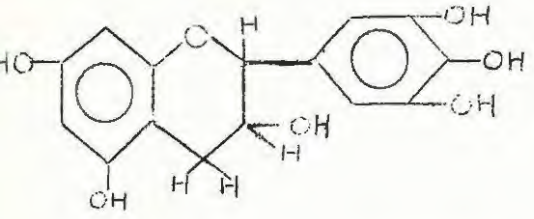


I (-)-epiafzelechin.



II (+)-catechin

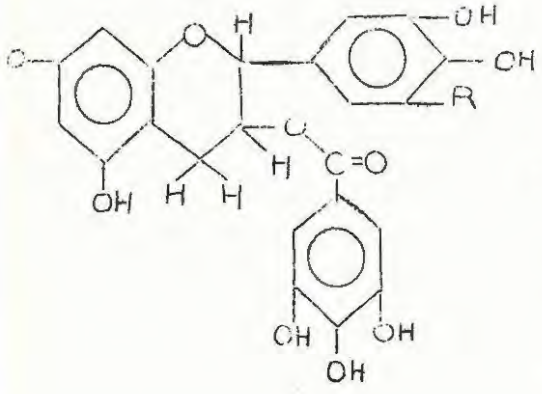
(-)-epicatechin



(+)-gallocatechin

(-)-epigallocatechin

III



R = H

(-)-epicatechin gallate

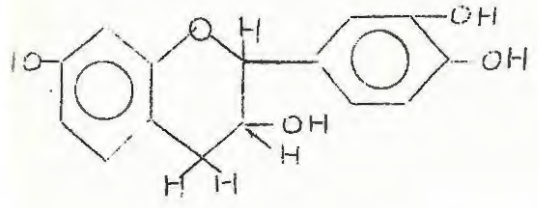
IV

R = OH

(-)-epigallocatechin gallate

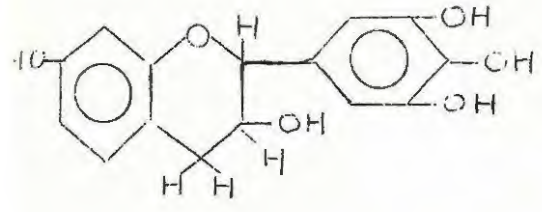
V

Naturally occurring catechins of the resorcinol series.



(-)-fisetinidol

VI



(-)-robinetinidol

VII

Naturally occurring leuco-anthocyanidins.

Leuco-anthocyanidins were found to be present in numerous plants since their recognition by Rosenheim (86) in 1920. Rosenheim, who identified leuco-anthocyanidins by the formation of their corresponding anthocyanidin chlorides on heating with hydrochloric acid, was also the first to name

these colourless substances, from which anthocyanidins are generated, leuco-anthocyanidins. Ten years later, Robinson and Robinson, who suggested a 2:3:4-trihydroxyflavan structure for the heterocyclic ring of these flavonoid bodies, showed their widespread distribution in nature (111-114). In 1953 Pigman, Anderson, Fischer, Buchanan and Browning (87) improved the methods of generating anthocyanidins from leuco-anthocyanidins, and for the first time showed the presence of leuco-anthocyanidins in commercial mimosa, quebracho and mangrove extracts. The authors were, however, unable to identify the anthocyanidins generated from these commercial tanning extracts, but showed that the absorption maxima of anthocyanidins generated from mimosa and quebracho extracts were lower ( $\lambda_{max.}$ 525-530 mμ) than those of the phloroglucinol series, i.e. pelargonidin (XIX) $\lambda_{max.}$ 530 mμ; cyanidin (XX) $\lambda_{max.}$ 545 mμ; and delphinidin (XXI) $\lambda_{max.}$ 555 mμ.

In 1954 King and Bottomley (88) achieved the first isolation of a naturally occurring leuco-anthocyanidin, 7:8:3':4'-tetrahydroxyflavan-3:4-diol, from the wood of Acacia melanoxylon and called it melacacidin (XV). Although amorphous, the above substance formed crystalline derivatives, e.g. a hexaacetyl-melacacidin and an O-tetramethyl-melacacidin, which was later (89,90) identified by

comparison with synthetic (±)-7:8:3':4'-tetramethoxyflavan-3:4-diol. Melacacidin, on treating with boiling hydrochloric acid, afforded a deep cherry red solution due to an anthocyanidin, which by paper chromatography was shown to be 3:7:8:3':4'-pentahydroxyflavylium chloride (XXII) [(King and Clark-Lewis (89), Bottomley (91), Robinson and Vasey (92)].

Another member of this class of polyphenols, (+)-leuco-fisetinidin, (+)-7:3':4'-trihydroxyflavan-3:4-diol (IX), was isolated simultaneously, in 1957, in crystalline form from the wood of Acacia mearnsii by Keppler (66) and called mollisacacidin, and by Mitsuno (93) from the wood of Gleditsia japonica and named gleditsin. Gleditsin and mollisacacidin proved to be identical (94).

When Roux (95), in 1958, isolated (-)-leuco-fisetinidin from the heartwood of Schinopsis quebracho-colorado and showed that (+)- and (-)-leuco-fisetinidins were enantiomorphous (Clark-Lewis and Roux, 98,99), it was the first report of the occurrence of an enantiomorphous pair of flavonoid compounds in Nature (for further reference see also Roux and Freudenberg, 96; Freudenberg and Weinges, 97; and Roux and Evelyn, 56). Clark-Lewis and Roux, in subsequent work (98), found these two leuco-fisetinidins to have a 2:3-trans-3:4-cis-configuration.

The isolation of another leuco-anthocyanidin of the "resorcinol series", (+)-leuco-robinetinidin, (+)-7:3':4':5'-tetrahydroxyflavan-3:4-diol (X), from the wood of Robina pseudacacia, was reported by Weinges in 1958 (74). Racemic leuco-robinetinidin had previously been synthesized by Freudenberg and Roux (100) via catalytic reduction of (±)-dihydro-robinetin.

The third member of the series of flavan-3:4-diols containing resorcinol, guibourtacacidin, 7:4'-dihydroxyflavan-3:4-diol (VIII), was isolated and identified by Roux (101) in 1959. The substance was isolated from the heartwood extractives of Guibourtia coleosperma, G.tessmanii and G.demeusei and Roux therefore proposed the trivial names guibourtacacidin and guibourtinidin for the leuco-anthocyanidin (VIII) and the anthocyanidin (XVI) generated from it, respectively. A crystalline leuco-anthocyanidin, related to guibourtacacidin, was synthesized by Kulkarni et al. (110) in 1959.

In 1958 Ganguli and Seshadri (104) reported the isolation of the first representative of the "phloroglucinol series" of flavan-3:4-diols, (-)-leuco-pelargonidin, (-)-5:7:4'-trihydroxyflavan-3:4-diol (XI), from the gum of

Eucalyptus calophylla. A racemic leuco-pelargonidin was synthesized one year later by Freudenberg and Weinges (30,105) via reduction of tribenzyldihydrokaempferol

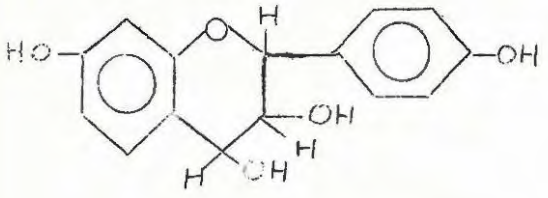
A second naturally occurring leuco-anthocyanidin of the same series, leuco-delphinidin, 5:7:3':4':5'-pentahydroxyflavan-3:4-diol (XIII), was found to be present in plants in two optical active forms by Ganguli, Seshadri and Subramanian (106) also in 1958. (-)-Leuco-delphinidin was isolated from the bark of Cleistanthus collinus (karada bark) exhibiting a negative rotation of $-53.8^{\circ}$, while (+)-leuco-delphinidin, from the gum of Eucalyptus piluaris, has a rotation of $+72.9^{\circ}$. The two substances and their derivatives show different melting points. Hathway (107) reported the isolation of a crystalline leuco-delphinidin from oak bark in the same year, but did not give its optical rotation.

In 1959 Ganguli and Seshadri (102) isolated the third member of the "phloroglucinol series" of leuco-anthocyanidins, (+)-leuco-cyanidin, (+)-5:7:3':4'-tetrahydroxyflavan-3:4-diol (XII), from the gum of Butea frondosa. A (+)-leuco-cyanidin had been previously synthesized by Swain (103) and also by Freudenberg and Weinges (97,204) via reduction of taxifolin (3:5:7:3':4'-pentahydroxyflavan-4-on) from Douglas fir bark.

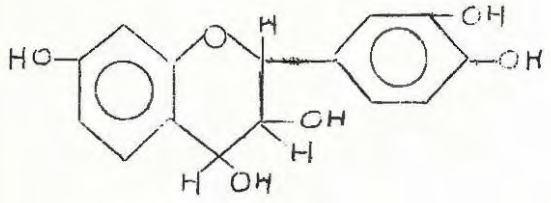
In a very recent paper (in 1960), Clark-Lewis and Mortimer (108) announced the isolation of crystalline (-)-melacacidin (XV) from Acacia excelsa and A. harpophylla. An amorphous isomelacacidin was isolated from three Acacia species (A.melanoxylon, A.excelsa and A.harpophylla) in low yield by the same authors.

The second naturally occurring leuco-anthocyanidin of the "pyrogallol series," (-)-teracacidin, (-)-7:8:4'-trihydroxyflavan-3:4-diol (XIV), and a small amount of isoteracacidin was isolated from the heartwood of Acacia intertexta by Clark-Lewis, Katekar and Mortimer in 1961 (109). Teracacidin, which has a 2:3-cis-3:4-cis-configuration, and isoteracacidin appear to be related to each other in the same way as melacacidin and isomelacacidin, and the authors suggest that the epimers differ only in configuration at the 4-position.

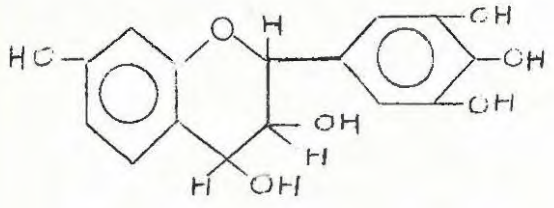
Naturally occurring leuco-anthocyanidins of the resorcinol series:



VIII guibourtacacidin

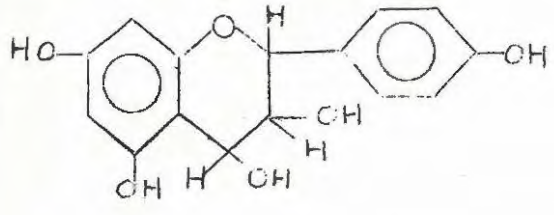


IX (+) or (-)-leuco-fisetinidin

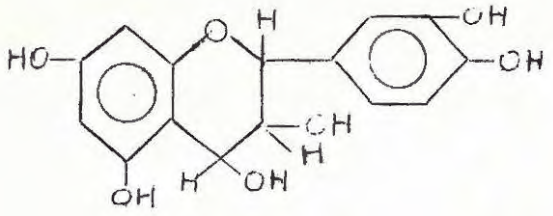


X (+)-leuco-robinetinidin

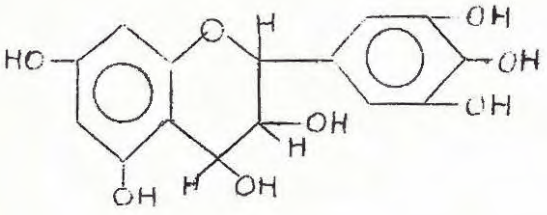
Naturally occurring leuco-anthocyanidins of the phloroglucinol series:



XI (-)-leuco-pelargonidin

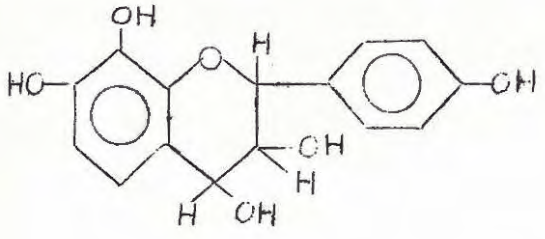


XII (+)-leuco-cyanidin

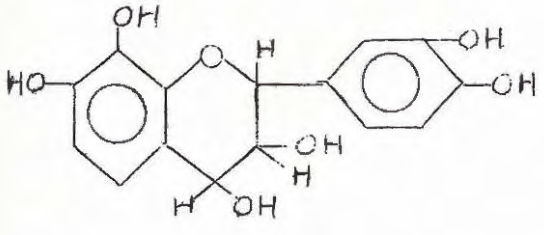


XIII (+)- or (-)-leuco-delphinidin.

Naturally occurring leuco-anthocyanidins of the pyrogallol series:

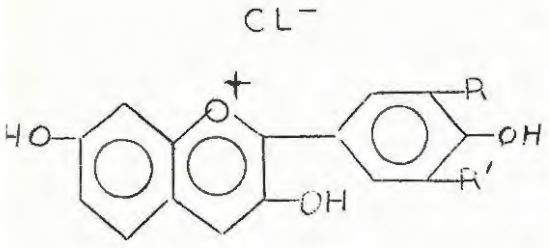


XIV (-)-teracacidin



XV (-)-melacacidin

Anthocyanidins (flavylium chlorides) of the resorcinol series:

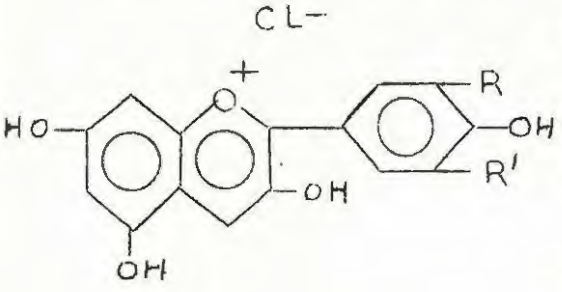



XVI R,R'=H, 3:7:4'-trihydroxyflavylium chloride (guibourtinidin)

XVII R = OH, R' = H fisetinidin

XIIX R, R' = OH, robinetinidin

Anthocyanidins of the phloroglucinol series:

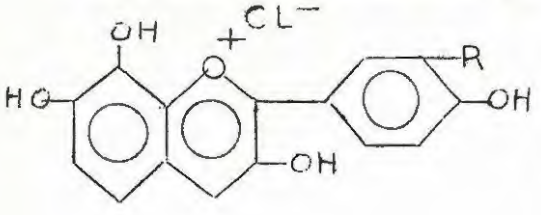



XIX R, R' = H, pelargonidin

XX R = OH, R' = H, cyanidin

XXI R, R' = OH, delphinidin

Anthocyanidins of the pyrogallol series:




XXII R = H, 3:7:8:4'-tetrahydroxyflavylium chloride

XXIII R = OH, 3:7:8:3':4'-pentahydroxyflavylium chloride

Leuco-anthocyanidins and catechins as tannins.

The widespread distribution of catechins in high proportion in plant sources used for the manufacturing of tanning extracts, e.g. Burma cutch from the wood of Acacia catechu, and cube gambier from the leaves and twigs of Uncaria gambir, and their similarity with condensed tannins in forming insoluble "phlobaphenes" on treatment with hot dilute mineral acid , led Freudenberg to suggest that condensed tannins may be polymers of catechins (Freudenberg 26-28, 30) (Freudenberg and Maitland 29) (Freudenberg and Weinges 115). Freudenberg and his school at Heidelberg investigated various possible reaction mechanisms of catechin condensation and found that only flavans, hydroxylated in the 7- and 4'-position,are capable of self-condensation (29, 37, 67, 69, 70, 117, 115, 118, 119). Additional hydroxylation in the 3- or in the 3- and 4-position (catechins and leuco-anthocyanidins respectively) was shown to increase the reactivity toward self-condensation considerably, but is not essential (30,117). To support their concept, Freudenberg and Maitland (118) synthesized (±)-epi-7:3':4'-trihydroxyflavan-3-ol (VI), a catechin they considered to be the prototype of quebracho tannins. Although Freudenberg was unable to isolate this component from quebracho extracts,it is of special interest in connection

with the isolation of a closely related compound, (-)-7:3':4'-trihydroxyflavan-3:4-diol, by Roux in 1958 from the sapwood of Schinopsis quebracho colorado, where it forms about 10% of the total phenolic extractives (95,99,120). Freudenberg and his school at Heidelberg (29,30,37,117,118) also developed a theory of acid-induced selfcondensation of catechins. In more recent years, (1957-1959) Mayer and Merger (122) isolated a reaction product of acid-induced condensation of (+)-catechin and phloroglucinol and Freudenberg, Stocker and Porter (123), Freudenberg and Weinges (124) and Freudenberg and Alonso de Lama (117) reported the isolation of a dimeric amorphous catechin in 10% yield from the reaction products of (+)-catechin and HCl in dioxane. The dimeric catechin and its acetyl derivative are optically active. The dimeric catechin also precipitates gelatine and is therefore a tannin.

While Freudenberg supports the idea of a plant acid induced selfcondensation of catechins, especially in the case of heartwood tannins (30), Hathway and Seakins, (125) from their experiments with mushroom-, potato- and tobacco-phenoloxidases, drew the conclusion that tannins present in gambier and Burma cutch extracts are formed by aerobic oxidation of catechins by polyphenoloxidases in the detached leaves and heartwood, respectively.

Bate-Smith *et al.* (36,126-130) found that when leuco-anthocyanidins, prepared from the wood of *Pinus maritimus* and from cacao beans (Forsyth 131-133), or when plant tissues, known to contain leuco-anthocyanidins (Robinson and Robinson 111-114), were heated with dilute mineral acid they not only produce deeply coloured anthocyanidin chlorides, but also form water insoluble phlobaphenes as do the condensed tannins and catechins (36). Isolated leuco-anthocyanidins from *Pinus* species and from cacao beans (the latter shown to be dimeric by Forsyth (134-136) were found to be sorbed by hide powder to a greater extent than (+)-catechin, to precipitate gelatine and alkaloids ("tannin reaction"), to give absorption spectra and colour reactions similar to condensed tannins and also catechins and to be astringent in taste. From these findings Bate-Smith and Swain (36) suggested that both classes of compounds, catechins (flavan-3-ols) and leuco-anthocyanidins (flavan-3:4-diols), should be regarded as prototypes of condensed tannins.

Pigman *et al.* (87) were the first to surmise the presence of leuco-anthocyanidins in commercial tanning extracts, i.e. mimosa extract from the bark of *Acacia mearnsii*, quebracho extract from the heartwoods of *Schinopsis balansae* and *S. quebracho colorado*, mangrove extract from

the bark of various Rhizophora spp., hemlock extract from the wood of Tsuga heterophylla and spruce bark extract from the bark of black spruce Picea mariania. Hillis (137,138) found that leuco-anthocyanidins are also present in the heartwoods of various Eucalyptus spp. and in the bark of Pinus radiata, both being known sources of condensed tannins, and expressed the view that the leuco-anthocyanidins in the xylem originate from the leaves of plants and that they are the immediate precursors of bark and heartwood extractives (tannins).

Roux, following a study of synthetic and naturally occurring anthocyanidins (51,52), has shown that, under the conditions of Pigman et al.(87), mainly two anthocyanidins, robinetinidin (XVII) and fisetinidin (XVI) are generated from both high- and low-molecular weight fractions of wattle bark and quebracho heartwood extracts (Roux 120, 139; Roux and Evelyn 55,56). For quebracho extracts Roux and Evelyn (55) demonstrated the even distribution of polymeric leuco-anthocyanidins in almost all those areas on two-way paper chromatograms, where reducing tannins are located. When fractionated according to $R_F$ and molecular weight, each fraction of commercial quebracho extract contains leuco-fisetinidins in almost equal concentration.

Leuco-anthocyanidins in wattle extracts (fresh

ark extracts of Acacia mearnsii) were shown by the same uthors to be of greater complexity compared with quebracho xtracts. Three pigments, robinetinidin, fisetinidin and . yellow pigment of unknown structure, were generated on eating with dilute hydrochloric acid in alcoholic solu- ion under pressure. Two dimensional paper chromatography howed that, whereas the leuco-fisetinidins of quebracho xtract are fairly evenly distributed throughout the poly- henolic fraction, leuco-robinetinidins and leuco-fisetini- lins of fresh black wattle bark extract were present in liscrete spots (mobile fraction of medium $R_F$ and of a verage molecular weight of 649-1033), but also evenly dis- tributed in the low $R_F$ tannin fraction of high molecular weight (1033-3240). The overall proportion of anthocyani- dins generated from the complex wattle tannins, (molecular weight 550-3250 compared with a molecular weight of 790- 2350 for quebracho extract) was shown by Roux and Evelyn (55) to be of the same order as those generated from que- bracho tannins.

The presence of both, monomeric leuco-robinetini- din and leuco-fisetinidin in low concentration in fresh bark extracts of Acacia mearnsii, was shown by Roux (120) in 1958. This was the first occasion on which leuco-robinetinidin (previously synthesized by Freudenberg and Roux in 1954, 100)

had been identified in natural sources.

The apparent bio-chemical transformation of monomeric leuco-anthocyanidins (non-tannins) into polymeric leuco-anthocyanidin tannins was shown by Roux for the heartwood of Schinopsis spp. (56,58,120,139,140,141). Radial sampling (obtained by drilling) from the outer sapwood to the centre of the heartwood showed that the concentration of monomeric leuco-fisetinidin, which forms 10% of the sapwood, gradually decreases towards the centre of the heartwood, while the average molecular weight of the extractable polyphenols, and the ratio of tannins to nontans progressively increases in the same direction (outer sapwood to inner heartwood). King and White (142,143) simultaneously observed the same decrease in the concentration of leuco-fisetinidin towards the middle of the heartwood (of quebracho logs) and maintained that (+)-catechin showed a similar behaviour.

The extracts of various Acacia species grown in South Africa for commercial tanning purposes.

Until two decades ago little was known on the fundamental side of wattle extracts, in spite of the fact that the use of wattle bark as a tanning agent has been known since 1824, as reflected by articles published in Australian and Tasmanian journals. The afforestation of wattle in South Africa started after the seeds of Acacia mearnsii De Wild. (144) had been brought from Australia in 1864. The seeds of other wattle species were also brought to South Africa and, as early as 1884, large-scale planting of wattle trees was practised in South Africa, mainly in Natal. In all, four species of Acacia were associated with the local wattle industry. These are:

Acacia mearnsii De Wild., (syn. A.decurrens (Wendl.) Willd., var. mollis Lindl.) (144), black wattle.
Acacia decurrens Willd., green wattle,
Acacia dealbata Link., silver wattle
Acacia pycnantha Benth., golden wattle.

The bark extracts of all four species have been used in South Africa for tanning purposes but, when the superior quality of black wattle bark became evident from trial tannages, the afforestation of other wattles (mainly silver wattle and green wattle) was discontinued. Today

the sale of silver wattle bark under the name "black wattle" is prohibited by government proclamation.

The commercial "black wattle" extract is guaranteed by the manufacturer to contain not less than 60% tannins. A typical analysis of present day production is:

| | |
|---|---|
| Tannins | 61.7% |
| Non-tannins | 19.1% |
| Insolubles | 1.2% |
| Moisture | 18.0% |

The tannin constituents of black wattle bark.

Early fundamental work on wattle extracts by Jakimoff (145) and by Moeller (146) dealt with the purification of commercial black wattle extract to increase its tannin content. Corbett (147) investigated the effect of ultrafiltration and obtained a highly purified phenolic fraction but in low yield. Stephen (148) increased the tannin content, by acetone extraction, from 61 to 81%. Analytical figures of Stephen's purified extract were:

| % C | % H | Empirical formula: |
|---|---|---|
| 60.8 | 4.4 | $C_{15}H_{13}O_{6.5}$ |
| 62.8 | 4.6 | $C_{15}H_{13}O_{6}$ |

Acetyl value : 35.8 - 39.2%.

Methoxyl value : 31.6%.

Williams (149) purified wattle extracts by successive solvent extraction with acetone, ethyl acetate and amyl acetate and claimed to have obtained 95% tannins. These claims were found to be inaccurate by Roux (150), who found the purity achieved by Williams only to be of the order of 80% tannins. Douglas and Humphreys (151) and Rich (152) obtained products of more than 91% tannin content by electrodialysis, but the obtained polyphenolic product represented only a fraction of the total polyphenols of the black wattle extract. Roux (153) achieved a polyphenol content of 95 - 98% by three different purification methods: a). Hide powder method: b). Lead salt method and c). Combined salting out and solvent extraction. The official hide powder (shake) method showed a purity (=tannin content) of 94 - 95% for Roux's purified extracts, but the author pointed out that a number of lower molecular weight polyphenols (phenolic non-tans) are not reflected by this method, and that the true polyphenol content of his purified extracts was therefore in the order of 95-98%.
Roux (154) reported the following analytical figures for a lead salt purified black wattle extract of 96-98% polyphenol content: Found C, 61.4 - 61.8; H, 4.7 - 4.8%; Acetyl value : 37.6%; Methoxyl value : 36.5%v $C_{15}H_{13}O_6$ requires: C, 62.3; H, 4.5%.

Degradation of black wattle tannins.

The chemistry of the tannin fraction was investigated by several authors, and provided valuable information of the chemical nature of the complex mixture of black wattle polyphenolic constituents.

The following components have been isolated from amongst the degradation products of wattle tannins and their methoxyl derivatives.

Alkali fusion of tannins:

| Compound: | Investigator: | % Yield: |
|---|---|---|
| Resorcinol | Stephen (155) | 3.5 |
| | Williams (149) | less than 1.0 |
| | Russel (35) | " |
| | Roux (154) | 10.5 |
| Phloroglucinol | Roux (154) | low yield |
| Pyrogallol | Roux (154) | 1.0 |
| β-Resorcylic acid | Roux (154) | 4.2 |
| Gallic acid | Stephen (155) | no yield reported |
| | Roux (154) | 11.0 |
| Protocatechuic acid | Roux (154) | low yield |

Alkali fusion of methylated tannins:

| | | |
|---|---|---|
| Methoxyresorcinol, | Heugh (203) | 2.0 |

Oxidative nitration of tannins:

| Compound: | Investigator: | % Yield: |
|---|---|---|
| Styphnic acid | Jablonski & Einbeck (202) | 1.0 |
| | Stephen (155) | no yield reported |
| | Kirby (239) | 2.0 |
| Oxalic acid | Stephen (155) | No yield reported |

Oxidative nitration of methylated tannins:

| | | |
|---|---|---|
| 5:4-Dinitro-β-resorcylic acid | Kirby (239) | no yield reported |

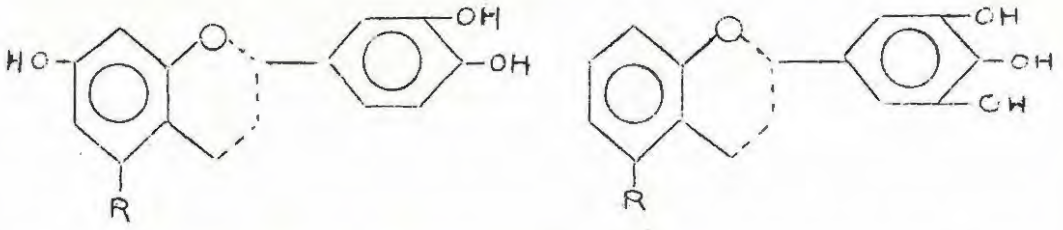
Permanganate oxidation of methylated tannins:

| | | |
|---|---|---|
| -Trimethylgallic acid | Corbett (147) * | 4 - 8 |
| | Heugh (203) * | " |
| | Roux (154) | ** |
| Veratric acid | Crobett (147) * | 4 - 8 |
| | Heugh (203) * | " |
| | Roux (154) | ** |

*Figures taken from "Wattle Tannin and Mimosa Extract". Grocott & Sherry Printers, Grahamstown 1955, page 52-53.

* * Roux reported a 20% yield of a mixture of trimethoxy-gallic acid and veratric acid.

Roux (154) pointed out that "the yields of these degradation products (from black wattle tannins), their proportions and their behaviour on permanganate oxidation point to the presence of the following nuclear types in the tannin mixture": In the following formula R = H.



The low proportion of phloroglucinol isolated by Roux (154) made this author suggest that also a low proportion of a basic nuclear type where R=OH (in the above formula) is present. Roux,however,pointed out that "many alternative structures are also possible".

Acetylation and methylation studies of black wattle tannins.

To establish the degree of hydroxylation of black wattle polyphenols, acetylation and methylation studies have been carried out on purified wattle extracts by several authors. Their results were governed by three factors: the purity of the starting material (phenolic fraction of black wattle extract), the methods for the acetylation and methylation and the methods for the determination of the acetyl- and methoxyl-content.

Stephen (148) acetylated acetone extracted tannins of 81% purity (=tannin content) with acetic anhydride and pyridine, or acetic anhydride and sodium acetate,and reported acetyl values (determined by the transesterification method of Matchett and Levine, 156) of 35.8 - 36.3 % for once acetylated tannins, and acetyl values of 38.7-39.2 %

for twice acetylated tannins. Tannins methylated with methyl sulphate/KOH reflected values of 31.6% $-OCH_3$, while tannins methylated with diazomethane, only reflected a methoxyl content of below 30.0% (both values obtained by the Zeisel method). Stephen also investigated combined methylations and acetylations of wattle tannins and found that fully methylated tannins could be acetylated to give a material with an acetyl content of 3.8 - 4.3%. Stephen (148) concluded from his findings that wattle tannins methylated with dimethylsulphate contain -OH groups which can be acetylated by acetic anhydride, and that some of the $-OCH_3$ groups are actually replaced by $-OCOCH_3$. Stephen summarized his acetylation and methylation studies with the finding that only 3.7 -OH groups in each hypothetical $C_{15}$-unit are capable of methylation by methylsulphate/KOH, whereas $4\frac{1}{4}$ -OH groups in each $C_{15}$-unit may be acetylated.

Roux (154, 157, 158) acetylated black wattle polyphenols of 97-98% purity. His acetylated tannins gave acetyl values of 37.6 - 38.4% by the transesterification method (156).

| | | | |
|---|---|---|---|
| Found: | C , 60.1% | H , 4.6 %, | $COCH_3$, 37.6 % |
| $C_{15}H_9O_2(OAc)_4$ requires | C , 60.9% | H , 4.6 % | $COCH_3$, 36.8 % |

Roux (154) reported the following figures for black wattle tannins methylated with dimethylsulphate/KOH:

| | | | |
|---|---|---|---|
| Found: | C , 65.9 % | H , 6.2 % | $-OCH_3$ , 36.5 % |
| $C_{15}H_9O_2(OCH_3)_4$ requires | C , 66.1 % | H , 6.1 % | $-OCH_3$ , 35.9% |

Acetylation of fully methylated black wattle tannins (methoxyl content 36.5%) with acetyl chloride/pyridine furnished a "mixed derivative" of 34.6% $-OCH_3$ and 3.7% $COCH_3$, the result giving evidence for some displacement of methoxyl- by acetyl-groups, but no increase in the total number of hydroxyl groups substituted (Roux 157). Roux's (154) findings showed that a constant proportion of 4.1 hydroxyl groups in the tannin fraction of black wattle extract may be methylated with diazomethane and are therefore phenolic in nature. The absence of carbonyl groups, as shown by infrared absorption spectra, made Roux suggest that the 1.9 non reactive oxygen units per $C_{15}$ unit are ether linkages.

Putnam (159, 160) developed a so called "peracetylation" technique and claimed acetyl values of 47.4 - 48.7% for peracetylated black wattle tannins. Putnam determined his acetyl values by the alkaline hydrolysis method. His values proved to be erroneously high, probably due to partial decomposition of the labile phenolic nuclei under the experimental conditions employed, resulting in the consequent formation of volatile aliphatic acids (Roux 158). Kirby and White (161) also re-examined Putnam's acetyl values and found these values too high, when using the same acetyl determination technique.

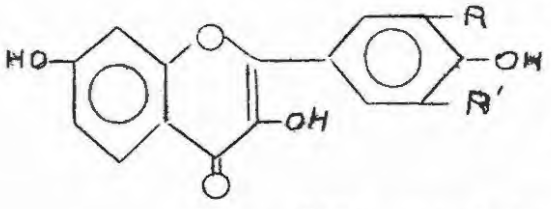
Molecular weight studies of black wattle tannins.

Investigations of the molecular weight of unpurified and purified (=tannin content enriched) black wattle extracts have been carried out by several authors with different methods. Molecular weights determined cryoscopically reflected values between 432 and 603 for unpurified extracts [(Douglas and Humphreys (151), Corbett (147) and Rich (162), and values of 485 - 1760 for purified extracts (Douglas and Humphreys (151) and Rich (162)]. Roux (163), in preliminary investigations, determined the molecular weight of purified black wattle tannins by the cryoscopic (benzene), the Rast (camphor) and the ebulliometric method (benzene), and reported an average molecular weight of 950 for the tannins. Evelyn (164, 165, 166) and Roux and Evelyn (167) improved the existing ebulliometric method and investigated, not only the molecular weight of commercial tannin extracts, but also of fresh bark extracts of all four wattle species (Evelyn 168). Evelyn reported average molecular weights of 1200 - 1300 for tannins extracted with methanol from commercial wattle extract, and average molecular weights between 600 and 1600 for fresh bark tannin fractions.

Paper chromatography of black wattle polyphenols.

Paper chromatography of black wattle extracts showed that black wattle tannins are a highly complex mixture of

polyphenols (Kirby, Knowles and White 17, 170) (Roux 49, 50, 154, 169). A number of yellow or blue fluorescing bodies are visible under U.V.-light. When black wattle tannins are separated on a two-way chromatogram (first direction water saturated sec.-butanol, second direction 2% aqueous acetic acid), three of the yellow fluorescing constituents have an $R_F$ of 0.0 in the water direction. One of these constituents, fisetin (XXIII), 3:7:3':4'-tetrahydroxyflavone ($R_F$ 0.71 in sec. butanol), was isolated and identified by Roux 49 , the two other bodies being identified by paper chromatography as robinetin (XXIV), 3:7:3':4':5'-pentahydroxyflavone ($R_F$ 0.59 in sec. butanol), and 3:7:4'-trihydroxyflavone (XXV) by Kirby and White (171), and by White (20).




XXIII fisetin R = OH
R' = H
XXIV robinetin R = OH
R' = OH
XXV 3:7:4'-trihydroxyflavone
R = H, R' = H.

Roux (50) provisionally identified (+)-catechin (II) and (+)-gallocatechin (III), in the ether extract of fresh black wattle bark, by paper chromatographic comparison with reference components.

This was confirmed, when, in 1958, Roux and Maihs (54, 57) isolated crystalline (+)-catechin, (+)-gallocatechin and a new catechin, (-)-robinetinidol (VII), from a low molecular weight tannin-enriched fraction of black wattle bark.

Roux in 1957 identified robinetinidin (XXIV) and fisetinidin (XXIII) from amongst the reaction products of black wattle extract with hot mineral acid in alcoholic solutions, by paper chromatographic comparison with synthetic anthocyanidins, and thus established the presence of leuco-robinetinidin and leuco-fisetinidin in black wattle extract. With the aid of a new spray reagent, toluene-p-sulphonic acid Roux (52) showed that leuco-anthocyanidins were distributed throughtout the whole molecular range of black wattle tannins (Roux and Evelyn, 55).

The presence of delphinidin and the surmised presence of cyanidin in reaction products of black wattle extract with hot mineral acid as reported by Hillis (64), and the presence of leuco-pelargonodin, leuco-cyanidin and leuco-delphinidin in commercial black wattle extracts reported by White (20), could not be confirmed by other authors (Roux & Evelyn, 56).

Some phenolic constituents of the wood of the black wattle tree.

In 1956 Keppler (65,66) isolated (+)-7:3':4'-trihydroxyflavan-3:4-diol (mollisacacidin) (2:3-trans:3:4-cis) from the wood of Acacia mearnsii. In recent years Roux et al. (58,60,61,63,238) isolated and identified six interrelated compounds, (+)-fustin, (+)-3:7:3':4'-tetrahydroxyflavanone (2:3-trans); (-)-fisetinidol, (-)-7:3':4'-trihydroxyflavan-3-ol (2:3-trans); (-)-butin, (-)-7:3':4'-trihydroxyflavanone

(2 S); butein, 2:3-dihydroxyphenyl-3:4-dihydroxystyryl ketone; fisetin, 3:7:3':4'-tetrahydroxyflavone and a trimeric leucofisetinidin, the first three of which are stereochemically identical, from the heartwood of A. mearnsii. Roux pointed out that because of their stereochemical identity, these substances may be formed, by interconversion, from one common precursor, (+)-7:3':4'-trihydroxyflavan-3:4-diol (2:3-trans: 3:4:cis).

Carbohydrate non-tannins of black wattle bark extracts.

Gums and sugars constitute the bulk of the water soluble non-tannin fraction of commercial black wattle extracts.

The gums represent an appreciable proportion (6-7%) of the total extract (Roux 172, Maihs and Roux 173, 174). Gums present in black wattle bark are similar to those which exude naturally from the bark on injury and are closely related to gums from other trees of the Leguminosae family (Stephen 175). Stephen hydrolysed black wattle gums (bark exudate on injury) with acid and isolated l-arabinose, l-rhamnose, d-galactose and d-glucuronic acid in the proportions of 6:1:5:1 respectively. The natural exudate is of high average molecular weight (92,000) and in common with most gums of this class readily undergoes autohydrolysis on heating to yield polymers of a molecular weight of about 30,000. Roux (49) examined black wattle gums present in commercial extract. On hydrolysis Roux (154, p.65) obtained carbohydrates similar to those found by Stephen in the natural exudate.

The second group of carbohydrate non-tannin constituents present in black wattle extract are the sugars. The exact proportions of the identified sugars i.e. sucrose, fructose and glucose in commercial black wattle extract still remain to be established, but their proportions will undoubtedly vary with season (Roux 154, p.67). The indications are, however, that the carbohydrate non-tannin fraction (sugars) constitutes about 9-13% of the total commercial extract. Roux also found that sucrose was the only sugar present in fresh bark extracts. The presence of fructose and glucose in the commercial extracts is probably due to partial hydrolysis during the extraction procedures.

The bark extracts of green, silver and golden wattle.

The three species, Acacia decurrens Willd., or green wattle, A. dealbata Link., or silver wattle and A. pycnantha Benth., or golden wattle, have many advantages but also disadvantages compared with black wattle. The main advantage of the three species is that they are capable of growing under poorer climatic and soil conditions than black wattle and that some of them are more resistant to insect pests (176, 177). The discontinuation of their afforestation in South Africa was due to the deeper colour of their extracts, and consequent reddening of leather tanned with their extracts on exposure to light and on ageing (Roux 154, p.130; Williams 176), The discontinuation of the affores-

tation of silver wattle trees was also due to a 50% lower tan content when compared with black wattle bark. Until recently, the green and black wattle species were regarded by some authorities as varietal forms of the same species A. decurrens (Williams 176). The foliage of these species is very similar in appearance, having typical feathery compound leaves, while the golden wattle tree is easily distinguished by its lanceolate phyllodes. Golden wattle is also known as the "broadleaved wattle" in Australia, and its bark is still collected for commercial tannage purposes.

The extract of green wattle bark.

Roux (150) compared two dimensional paper chromatograms of green and black wattle extracts and found a general similarity in pattern, but also some differences. The characteristic 5 areas of high concentration of black wattle extract seem to be absent in green wattle, but many of the compounds present in black wattle are also present in green wattle extracts.

Yellow fluorescent bodies, representing the flavonols (fisetin, robinetin), seem to be absent when a one dimensional chromatogram is viewed under U.V. light (Roux 154, p.130). Chromatograms of green wattle extract show a much more pronounced trail in the low $R_F$ region than black wattle extract. This trail of highly condensed material may be the

reason for the deeper colour of green wattle extracts (178, 179 cited from Roux 154, p. 139). Black and green wattle tannins yield similar phenolic degradation products. The proportion of phloroglucinol isolated is, however, significantly higher (Roux 150).

During tanning, green wattle tannins appear more astringent than black wattle tannins when compared under identical conditions. Green wattle extracts have a similar tan/non tan ratio than black wattle extracts and contain the same carbohydrates (150).

The extract of silver wattle bark.

While green wattle exhibits a general similarity when compared with black wattle, silver wattle shows greater differences.

The bark of the silver wattle is not only much thinner when compared with the bark of black wattle trees of the same age, but has approximately half the tannin content of black wattle bark. Silver wattle bark extracts exhibit a by far deeper redness than black and green wattle extracts. A general similarity with black wattle is reflected by two dimensional paper chromatograms. Many reducing spots occupy identical positions on chromatograms of both extracts. One dimensional chromatograms, when viewed under U.V. light, show the absence of fisetin in silver

wattle extracts (Roux 154, p.130). Alkali fission of silver wattle extracts furnish the same degradation products found in black wattle, e.g. gallic acid, β -resorcylic acid, protocatechuic acid, pyrogallol, resorcinol and phloroglucinol.

The extract of golden wattle bark.

Comparison of two dimensional chromatograms of golden wattle extracts and black wattle extracts by Roux (154, p.130) shows marked differences. Several reducing areas which stand out prominently on a chromatogram of golden wattle bark extract do not appear in the fresh bark extracts of other wattle species.

## CHAPTER 2.

## DISCUSSION OF EXPERIMENTAL RESULTS.

Of the four Acacia species introduced into South Africa from Australia for their tannin rich bark, A. mearnsii (black wattle) is most closely related to A. decurrens (green wattle) and A. dealbata (silver wattle), all having feathery compound leaves of similar appearance. A. pycnantha (golden wattle), with leaves in the form of lanceolate phyllodes, is known to be more distantly related to the group. The first three species may again be differentiated into the pair, A. mearnsii and A. decurrens, which were at one time regarded as varietal forms of the same species (A. decurrens) (Williams 176), and A. dealbata, which, although similar, is further removed from the A. decurrens group.

In the present study, the distribution of flavonoid constituents (i.e., flavan-3-ols and flavan-3:4-diols) in the barks of these species is compared. The distribution of known chemical compounds in the phylogenetically old and relatively unspecialised organs, e.g. barks and heartwoods, of these interrelated Acacia species is of interest, especially in view of the extensive work of Erdtman (182) on the "chemical taxonomy" of coniferous plants.

Of the total phenolic constituents in the bark extracts of the four wattle species, monomolecular catechins represent only between 1 and 3%, while the concentration of

other individual flavonoid constituents, such as flavonols, is surmised to be even lower (Roux 49). The presence of high percentages of tannins, varying in average molecular weight over the range 600 - 3000 (Roux & Evelyn, 55, 188), complicates such isolations. Means had therefore to be found for the removal of the bulk of the highly polymerised tannins from the low proportion of individual flavonoid substances, and for the further fractionation of the latter. In order to enrich the polyphenolic constituents of lower molecular weight (mono- and dimolecular flavonoid constituents), two methods, the precipitation of the higher molecular weight tannins with chloroform, or the retention of the latter by chromatography on cellulose columns, were attempted. Both methods were found equally effective. Chloroform precipitation, however, yields a solution which is easily evaporated, while the substances eluted from cellulose columns are in aqueous solution, thus enhancing the possibility of oxidation of the very sensitive phenolic wattle constituents during evaporation.

For the isolation of individual catechins and leuco-anthocyanidins from fractions previously enriched in low molecular weight constituents, the "preparative" paper chromatographic method, as outlined by Nordström and Swain (184), was used. In spite of the disadvantage that some

oxidation may occur during elution (stripping), the method has the great advantage that the fractions isolated may be linked directly to the distribution of constituents on two-way paper chromatograms.

Three flavan-3-ols, (+)-catechin, (+)-gallocatechin and (-)-robinetinidol, were found to be present in bark extracts of all four wattle species, and were subsequently isolated from A. mearnsii, A. dealbata and A. pycnantha. The polyphenolic constituents isolated were identified by their $R_F$ values in different solvent systems, colour reactions, micro-degradation, melting points and mixed melting points with reference components, combustion analysis, optical rotations, infrared- and ultra-violet-absorption curves and, where possible, by the formation of derivatives. Where no reference components were available, as in the case of the golden wattle constituents, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate, these were isolated from green tea.

(-)-Epicatechin was isolated from A. dealbata and A. pycnantha only, and seems to be absent, or present in too low a concentration in A. mearnsii and A. decurrens, to allow for its identification. (-)-Epigallocatechin was found in two wattle species, silver and golden wattle, only. The concentration of this catechin in silver wattle

bark was too low to allow for its isolation. From enriched golden wattle fractions, however, (-)-epigallocatechin crystallised with relative ease. The 3-galloyl esters of both (-)-epicatechin and (-)-epigallocatechin, and also gallic acid were isolated from bark extracts of A. pycnantha only. The two gallates and free gallic acid appeared to be absent in black, green and silver wattle, thus emphasizing the more distant relationship of A. pycnantha with the two wattle species of the A. decurrens group and with A. dealbata.

The association of (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (+)-gallocatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and gallic acid in the bark of A. pycnantha is of interest in view of the presence of an identical mixture of components in dried green tea leaves (Bradfield and Penny, 82). In golden wattle, however, the catechins are present as minor components (total 2.6% of the ethyl acetate extract) associated with high-molecular-weight tannins, whereas in tea leaves they form the major phenolic components. Of further interest is the fact that only the pairs, (+)-catechin: (-)-epicatechin, and (+)-gallocatechin: (-)-epigallocatechin, have so far been isolated from unaltered natural sources (see also Roberts and Wood; 215, 234).

The simultaneous presence of (+)-catechin and (+)-gallocatechin in the barks of all wattle species is interesting in view of their parallel occurence in the bark tannins of Casuarina equisitifolia (Roux; 197), Quercus sessifolia and Castanea vesca (Mayer and Bauni; 121, 235) and in Quercus pendunculata (Hathway; 236).

It appeared that the quantitative estimation of the individual flavonoid constituents in tannin extracts would, in many respects, be of diagnostic value. The quantitative estimation of substances on paper chromatograms by means of visual comparison or photoelectric measurement is widely employed in the protein and carbohydrate fields. These methods have, however, not been applied to polyphenolic substances. Commercial densitometers, designed for the estimation of amino acids on one dimensional, transparent strips are of little value for the estimation of phenolic substances in tannin mixtures. The complexity of these mixtures makes it necessary to resolve the constituents on relatively large (16 in. by 16 in.), two-way paper chromatograms. A densitometer was therefore designed and built for use with the latter. The photoelectric densitometer was fitted with an optical system controlling the light intensity, and with apertures of different diameters instead of a slit, thus enabling direct readings from round spots.

Two spray reagents, ammoniacal silver nitrate and

bisdiazotised benzidine were shown to give straight-line relationships between log concentrations of the flavonoid compounds and the galvanometer deflections over a sufficiently wide concentration range to allow accurate measurements. Constituents, which were well separated on two dimensional paper chromatograms, e.g. (+)-catechin, (-)-robinetinidol, (-)-epicatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate, gave accurate results with both the above spray reagents. Where, however, adequate separation was not achieved, as in the case of (+)-gallocatechin and (-)-epigallocatechin, the estimated values were erroneously high due to interference from neighbouring substances. The strong interference from these substances was at least partially eliminated by using the more selective bisdiazotised benzidine reagent. This reagent produces claret-maroon with phloroglucinol containing substances (i.e. gallocatechin), and yellow with the unknown overlapping substances. With the aid of a yellow green filter, the colour density of the yellow substances was depressed and the density of the claret-maroon increased.

The concentration of individual catechin constituents in barks of the four Acacia species investigated, was generally found to show small tree to tree variations within the species. In this latter respect silver wattle, the bark of which could be divided into two categories ('rich' or

'poor' in (-)-epicatechin), was an exception. Marked differences in concentration, however, were found between trees of different species. Thus, the concentration of (+)-catechin and (+)-gallocatechin appeared consistently lower in black than in green wattle barks. In the barks of black x green wattle hybrids, the concentration of these two catechins approximated to those in green wattle. This finding appeared to be of diagnostic value in connection with an investigation of bark constituents of uncultivated wattle trees grown in the Albany district (S.A.). These trees, which are classified as black wattle, show considerable differences in the concentration of their bark constituents when compared with authentic black wattle trees grown in plantations in Natal. The concentration of individual catechins is generally higher in the bark of wattle trees grown in Grahamstown (Albany district) than in black wattle trees grown in Natal (S.A.). Thus, (+)-catechin is present in an average concentration of 1.0 - 1.2%, compared with 0.51 - 0.57%; and (+)-gallocatechin within an average concentration of 0.76 - 1.1%, compared with 0.55 - 0.62%, for trees grown in Grahamstown and in Natal respectively. The higher concentration of (+)-catechin and (+)-gallocatechin in the bark of black wattle trees grown in Grahamstown, may indicate some hybridization with green wattle as the latter has a higher average concentration of these two constituents (1.22% and 0.81%

respectively) when compared with authentic black wattle (0.51 - 0.57% and 0.55 - 0.62% respectively). Authentic black x green wattle hybrids show almost identical concentrations of (+)-catechin and (+)-gallocatechin when compared with black wattle trees grown in Grahamstown.

Considering the four wattle species, a correlation appears to exist between the distribution of their catechin constituents in the bark, and the order of their closeness to black wattle in botanical character. The concentration of (-)-robinetinidol, which appears to be the characteristic compound of these Acacia species (Roux and Maihs, 57), progressively decreases in the sequence A. mearnsii, A. mearnsii x A. decurrens hybrids, A. decurrens, A. dealbata and A. pycnantha (0.23% - 0.18% - 0.11% - 0.08% - 0.05% respectively). At the same time the number of catechin constituents of the "phloroglucinol series" increases in the same sequence of species. Thus, A. decurrens, which is closest to A. mearnsii, has the identical composition of known catechins in the bark, but with those of the "phloroglucinol series" [(+)-catechin and (+)-gallocatechin] present in higher concentration. A. dealbata and A. pycnantha have two additional catechins of the "phloroglucinol series" [(-)-epicatechin and (-)-epigallocatechin], A. dealbata having the lower average concentration of these two cate-

chins when compared with A. pycnantha. A. pycnantha, in addition, contains the 3-galloyl esters of the latter catechins. Hence,the concentration and distribution of bark components in these Acacia species agree with the accepted systematics.

It has been shown previously by Erdtman (182) that the subgenera of coniferous plants may be differentiated by examination of the wood components. It now appears, that a differentiation between species may be possible by the examination of their bark components.

Under the conditions of the "anthocyanidin reaction" (Pigman et al., 87), extracts of black-, green-, silver- and golden wattle barks generate a number of anthocyanidins. The anthocyanidins were separated on one dimensional paper chromatograms, and their relative concentrations estimated by visual comparison. Three anthocyanidins, robinetinidin, fisetinidin and delphinidin, were generated from the extracts of all four species. The concentration of robinetinidin generated from A. mearnsii, A. decurrens and A. pycnantha was much higher than from A. dealbata. The concentration of fisetinidin from the four Acacia species was generally lower than that of robinetinidin and delphinidin and showed little variation, while the concentration of delphinidin generated increased in

the sequence black, golden, green, and silver wattle. The extracts of green and silver wattle generated an additional anthocyanidin, cyanidin, in high concentration. Cyanidin appeared to be absent from the reaction products of black and golden wattle extracts. Under the conditions of the anthocyanidin reaction, the extracts of all four wattle species generated an unidentified orange pigment. This pigment was found to be identical with an orange pigment from a complex leuco-robinetinidin tannin, which had been isolated from the barks of A. mearnsii and A. pycnantha. The concentration of the orange pigment appears to parallel that of robinetinidin chloride in the reaction products of various wattle extracts, and of the complex leuco-robinetinidin tannin. A correlation between the distribution of leuco-anthocyanidins in the bark of the four wattle species, and the botanical character, as in the case of catechin constituents, does not apparently exist.

The isolation of a complex leuco-anthocyanidin tannin, constituent D, from the bark of Acacia mearnsii is of interest, as it represents the first isolation of a flavonoid tannin from commercial vegetable tannin sources.

Constituent D was isolated from black wattle bark extract as[a] chromatographically pure, cream-coloured, amorphous substance. The substance is optically active, $[\alpha]_D$ -269°.

The tannin shows similarity to (+)-leuco-robinetinidin in C, H and O content, in colour reactions and ultraviolet and infrared absorption spectra, but also marked differences. From molecular weight estimations, the tannin appears to be dimeric. On heating with dilute mineral acid three pigments, robinetinidin, an orange pigment and a trace of delphinidin, was generated. The orange pigment was not an anthocyanidin. Enzymic degradation of the tannin with tannase yields the orange pigment as the only product. The pigment crystallised in brown red crystals, which do not melt below 330°C. The orange substance was found to have acidic properties. It gave resorcinol and gallic acid as main degradation products on alkali fusion. On fuming with ammonia or on the addition of sodium ethoxide, the pigment, in common with anthocyanidins, showed a big colour shift towards longer wavelength. A similar colour shift, on addition of ethanolic aluminium chloride, reflected the presence of vicinal orthodihydroxy- or orthotrihydroxy-groups, or a carbonyl group so placed with respect to a phenolic hydroxyl group as to allow the formation of metal complexes. Infrared absorption curves showed the presence of strong carbonyl absorption at 1680 $cm^{-1}$, possibly due to aryl-ketone, aryl-carboxylic or quinoid C=O groups. The presence of a conjugated carbonyl group was also indicated

by ultraviolet absorption maxima at 282 and 342 mμ. Absorption, due to carbonyl groups, is absent from the spectra of the parent compound D. The orange pigment is therefore a transformation product and not a simple hydrolytic fraction of D.

On the basis of a $C_{15}$ structure ($C_{15}H_{14}O_6$), acetylation of the orange pigment affords an acetyl derivative containing the exact equivalent of four acetyl groups. Diazomethane reacts to the equivalent of four methoxyl groups, corresponding to the methylation of four acidic, possibly phenolic hydroxyl, groups. Of the remaining two oxygen atoms per $C_{15}H_{14}O_6$, one was shown to belong to a carbonyl group, while the other is presumably involved in an aryl-alkyl-ether link. This leaves two alternative structures for the orange pigment. The first would correspond to a molecule with a total of four phenolic OH groups in the A and B nuclei, and a carbonyl group in the heterocyclic ring. The second alterantive corresponds to a structure with a quinoid carbonyl group in one of the two phenolic rings, and an acidic hydroxyl group in the heterocyclic ring.

Alkali microfusion of the tannin (D) gave resorcinol, β-resorcylic acid and gallic acid as main, and phloroglucinol as minor, degradation products. Selective spray

reagents did not reflect the presence of phloroglucinol , only of resorcinol and pyrogallol nuclei in the parent substance. Acidic breakdown under the conditions of the leuco-anthocyanidin reaction, furnished β-resorcylic acid, resorcinol, gallic acid and the two pigments as the main reaction products. Furthermore, these pigments, robinetinidin and the orange body, contain only resorcinol and pyrogallol nuclei and do not furnish phloroglucinol on alkali fusion. The presence of phloroglucinol among the reaction products of alkaline degradation of the tannin (constituent D), is therefore surmised to originate from a trace of an associated leuco-delphinidin.

Calculated to a $C_{15}$ basis, constituent D methylates to the equivalent of four methoxyl groups, corresponding to the methylation of all phenolic hydroxyl groups in the suggested 7:3':4':5'-tetrahydroxyflavan-3:4-diol. Direct acetylation afforded an acetyl derivative, containing the equivalent of five acetyls per $C_{15}$ unit, and presumably representing the substitution of the equivalent of four phenolic and one aliphatic hydroxyl group. On the assumption that D is a dimer of 7:3':4':5'-tetrahydroxyflavan-3:4-diol ($C_{15}H_{14}O_7$), it is apparent that one of the two remaining oxygen atoms per $C_{15}$ unit, which neither methylates nor acetylates, belong to the aryl-alkyl-ether of the pyrone

ring. The remaining equivalent of one aliphatic hydroxyl group per $C_{15}$ unit is, presumably, involved in direct condensation, or has undergone conversion into tertiary alcohol as a result of condensation, or, alternatively, has become sterically hindered or hydrogen bonded following condensation. An exact parallel has been found in the leucofisetinidin tannins by the isolation of a trimeric tannin from the heartwood of A. mearnsii by Roux (23).

On the basis of available evidence, it appears that constituent D is a dimer of 7:3':4':5'-tetrahydroxyflavan-3:4-diol, condensed, possibly, through an ether link between the heterocyclic ring of the one $C_{15}$ unit with the C-2 position of the second molecule.

A second tannin obtained from the bark of black wattle, "constituent B", consists of two overlapping substances, which are difficult to separate. At the best, very partial separation on paper chromatograms could be achieved, showing the presence of pyrogallol and catechol nuclei with the ferric alum reagent.

The tannin B was found to have an average molecular weight of 676, and it is considered likely that both substances may be dimolecular flavonoids. The presence of resorcinol, catechol and pyrogallol nuclei, was established by alkali microdegradation. On heating with dilute mineral

acid, three pigments formed, two of which were identified as fisetinidin and robinetinidin chlorides. The third was found identical to the orange pigment generated from constituent D and other complex leuco-robinetinidin tannins with dilute mineral acid, or enzymic degradation of the above with tannase.

"Constituent B" therefore consists of a mixture of complex leuco-robinetinidin and leuco-fisetinidin tannins.

From paper chromatographic comparison it is surmised, that the two bark tannins D and B are also present in A. decurrens, A. dealbata and A. pycnantha. D was also isolated from the bark of A. pycnantha and found identical to the corresponding substance from A. mearnsii.

# EXPERIMENTAL PART

## CHAPTER 3

## GENERAL ANALYTICAL AND PREPARATIVE TECHNIQUES.

Determination of melting points.

All melting points are uncorrected. Mixed melting points were made on the crystalline residue remaining after dissolving equal weights of both compounds in a suitable solvent and removing the solvent under vacuum.

Determination of optical rotations.

Optical rotations were measured on a micro scale using a Bellingham & Stanley Model A polarimeter with glass circle and micrometer drum, reading to $0.01^{o}$. The material (10-70 mg) was dissolved in 2.5 ml of a suitable solvent in which complete optical clarity could be obtained and the rotation measured in a 2 dm Zeiss polarimeter tube. The specific rotations and standard deviations were calculated from a minimum of ten readings.

Determination of U.V. and visible range spectra.

The U.V. and visible range spectra were measured with a Beckmann Model DU Quarz spectrophotometer with photomultiplier. The sample was diluted (ethanol) to have a maximum optical density of less than 1.0, measurements being made against a blank containing the solvent alone. In the far U.V. range (Photomultiplier) of 200-240 mμ, water had to be used as solvent, because ethanol itself shows an absorption in that range.

In the case of the unknown orange pigment generated from leuco-robinetinidin tannins,the absorption spectra of the substance in ethanolic aluminium chloride (Geissman, Jörgensen and Harborne, 192; Swain, 193; Harborne, 193) and in ethanolic 0.002 M sodium ethoxide (Mansfield, Swain and Nordström, 194; Nordström and Swain, 184, Harborne, 195) were also determined.

Determination of infrared spectra.

Infrared spectra were recorded by Dr. J.R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. The spectra (2.5-15 μ) were determined from KBr discs (13 mm, concentrations 0.5-1.0 mg/250 mg KBr) using a Perkin Elmer Infracord spectrophotometer.

Determination of molecular weights.

The molecular weights of methylated or acetylated polyphenolic constituents were determined by the ebulliometric method (acetone) of Evelyn (164, 165).

Microanalysis.

Analysis of C, H, methoxyl and acetyl (transesterification, Matchett & Levine, 156) were by Weiler & Strauss, Oxford, or by Dr. J.R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria.

Tannin test.

The gelatine test in which tannins produce a

heavy precipitate with gelatine-salt solution was carried out as described in "Procter's Leather Chemists Pocket Book", E & F. N. Spon, London, 1937, p.136.

Tannin analysis.

Tannin analyses are by the ultraviolet photometric method for black wattle tannins (Roux 196, 197).

Microdegradation technique, KOH fusion.

Information regarding the A and B nuclei of a $C_{15}$ compound may be obtained from alkali fusion of the constituent according to the improved microdegradation technique introduced by Roux (169).

2 to 4 mg of the substance were weighed into a 15 x 1 cm hard glass tube and fused for 90 seconds with molten KOH. The soluble degradation products were separated by the bicarbonate technique into phenolic-and phenolic-carboxylic-degradation products. The degradation products were identified on paper chromatograms by comparison with resorcinol, phloroglucinol, $\beta$ -resorcylic acid, gallic acid and protocatechuic acid as reference components.

Leuco-anthocyanidin reaction.

The reaction was carried out according to Pigman et al. (87). 1 to 6 mg of the substance were weighed into 8 ml pressure tubes and 5 ml of the mixture isopropyl-alcohol : 3N HCl (4:1) were added. The tube was heated on

a boiling water bath for one hour. The formation of deep orange, red or purple pigments (anthocyanidin chlorides) reflects the presence of leuco-anthocyanidins in the examined tannin. The generated anthocyanidins can be identified by paper chromatographic comparison with synthetic reference compounds (Table I). The reaction mixture is heavily streaked onto the starting line of a one-dimensional chromatogram (Whatman no. 1) and developed by the descending method with 90% formic acid : 3 N HCl = 1:1 (Roux 51, 52, 55). Additional evidence is supplied by the formation of the aluminium complexes of anthocyanidins having orthodihydroxy or orthotrihydroxy groups (Geissman 192, 205; Roux 52; Harborne 206). The reaction is carried out by applying one to three drops of 5% (w/v) ethanolic aluminium chloride onto the anthocyanidin spot. The spot is then cut out and the light absorption measured.

TABLE I.

Anthocyanidin chlorides.

| | $R_F$ * | $\lambda_{max.}$ * | $\lambda_{max.}$ of aluminium complex * | Colour in visible light. | Colour in U.V. light |
|---|---|---|---|---|---|
| 3:7:4'-trihydroxy-flavylium chloride | 0.60 | 485 mμ | no complex | orange yellow | yellow |
| fisetinidin chloride | 0.43 | 525 " | 545 mμ | pink | bright orange |

| | $R_F$ * | $\lambda_{max.}$ * | $\lambda_{max.}$ of aluminium complex * | Colour in visible light. | Colour in U.V. light. |
|---|---|---|---|---|---|
| robinetinidin chloride | 0.26 | 532 mμ | 570 mμ | pink purple | deep pink |
| pelargonidin chloride | 0.33 | 530 " | no complex | red | orange red |
| cyanidin chloride | 0.22 | 545 " | 560 " | magenta | pink |
| delphinidin chloride | 0.11 | 555 " | 580 " | purple | mauve |
| 3:7:8:3':4'-pentahydroxyflavylium chloride | 0.30 | 540 " | 590 " | magenta | |

* values by Roux (52).

Acetylation of flavonoid compounds.

Two methods were used in this work. Acetylation with acetic anhydride and sodium acetate was carried out as described by Roux and Evelyn (56). The proportions are: 500 mg substance, 5 ml acetic anhydride and 1 g sodium acetate. The mixture was kept boiling for 2 to 3 minutes, cooled and reboiled for another 2 to 3 minutes. After final cooling the reaction mixture was poured into 25 ml water and the acetic anhydride allowed to hydrolyse at room temperature over night. For acetylation with acetic anhydride in pyridine as described by Roux and Paulus (60) 500 mg of the substance were dissolved in 2 ml of pyridine, and 3 ml of acetic anhydride were added. The mixture was allowed

to stand at room temperature for five hours. The product was poured into 25 to 50 ml of water, and the acetic anhydride allowed to hydrolyse over-night. Both methods allow the acetylation of as little as 100 mg of a flavonoid substance.

Methylation of flavonoid compounds.

Isolated wattle constituents were methylated with an excess of diazomethane by the method described for (-)-leuco-fisetinidin by Roux and Evelyn (56). 500 mg of the substance was dissolved in 50 ml methanol (at -5°C) and 45 ml of ethereal diazomethane (generated from 10 g of nitrosomethylurea) added. The reaction was allowed to continue (at 5°C) for at least 24 hours. The product was finally concentrated under vacuum to a volume of 5 ml and poured into 50 ml of cold water. The white flocculent precipitate formed was allowed to stand over-night. This method allows to methylate as little as 100 mg of a flavonoid substance.

Preparation of Tannase.

The enzyme was prepared according to Maihs (240). To gain a high esterase activity the fungus, Aspergillus niger, was grown on a substrate of tannic acid, ammonium sulphate and secondary potassium phosphate only. The mycelium (200 mg) was collected before it turned brown. Where the mycelium had turned brown due to an excess of spores, it was discarded.

The wet mycelium was carefully washed 6 times with distilled water and then pressed, to remove excess water and frozen to break the cell walls. The mycelium was extracted for 3 days at room temperature with daily renewal of the solvent (water). The combined extracts were freeze-dried and the activity of the enzyme tested with methyl gallate (gallic acid monomethylester).

Substances to be hydrolysed were dissolved in water (10mg substance in 2 ml water), and one ml of a 1% enzyme solution was added. One drop of toluene was added to prevent fungal growth and the mixture kept at 40°C for 24 to 48 hours. The progress of the enzymic degradation was followed by two-way paper chromatography.

Paper chromatography of flavonoid compounds.

Bate-Smith and Westall (129) investigated the relationship between constitution and partition coefficient of natural and synthetic $C_{15}$ compounds in the solvent system-n.-butanol:acetic acid:water = 4:1:5. They demonstrated that in this solvent, weight of hydroxylation dominates all other factors in its effects of $R_F$ value. The addition of one hydroxyl group shows almost the same decrease of $R_F$ value to whatever class the substance belongs. Another

factor influencing the migration of flavonoid substances on paper chromatograms however, is the stereochemical configuration of the substances (84).

Roberts and Wood (84) demonstrated the separation of the optical antipodes of catechin and gallocatechin on paper chromatograms with water as the irrigant and showed that the (-)-forms have the lower $R_F$ in this solvent. In the epicatechin series this effect is reversed, (+)-epicatechin and (+)-epigallocatechin having the lower $R_F$ in water.

More recently Roux et al. (183) showed that the enantiomers of flavan-3-ols, flavan-3:4-diols and 2:3-dihydroflavonols may be separated in water, sometimes with the sequence of migration of the (+)- and (-)-forms reversed. Amongst the flavan-3-ols, those of 2:3-trans configuration of substituent groups always have an appreciably higher $R_F$ than the related epimer of 2:3-cis configuration. The presence of a carbonyl group in the 4-position (2:3-dihydroflavonols) introduces a pronounced reduction in $R_F$ compared with the corresponding flavan-3-ol. The introduction of a hydroxyl group in the 4-position produces a small increase of $R_F$, whereas the introduction of hydroxyl groups in positions 5 and 5' reduces the $R_F$ values. The introduction of a galloyl group in the 3-position slightly

reduces the $R_F$ of the corresponding 2:3-cis-flavan-3-ol.

Solvents.

The following solvents have been used in this work:

| | | Abbrev.: |
|---|---|---|
| 1.) | n-butanol:acetic acid:water = 6:1:2 | n.-B.A.W. |
| 2.) | sec.-butanol:acetic acid:water + 14:1:5 | sec.-B.A.W. |
| 3.) | n-butanol water saturated | n-butanol |
| 4.) | sec.-butanol water saturated | sec.-butanol |
| 5.) | 2% acetic acid in water | |
| 6.) | 15% acetic acid in water | |
| 7.) | 90% formic acid : 3N HCl 1:1 | |
| 8.) | 90% formic acid : 3N HCl 1:2 | |

The addition of acetic acid to the solvents is of dual purpose. Acetic acid prevents the weaker organic acids from trailing by depressing their ionisation. Acetic acid, keeps the pH down thus reducing atmospheric oxidation of the otherwise sensitive phenolic bodies. The stabilising effect of acetic acid is of importance in the "preparative method" (Roux 55).

The solvents 6, 7 and 8 have been used in the separation of anthocyanidins and will be discussed in that section.

The solvents used for counter current distribution

in the Craig machine will be discussed in a separate chapter and so will column chromatography.

Two dimensional chromatograms were developed in the first direction with the solvents 1 to 4 (partitioning) and in the second direction with 2% aqueous acetic (adsorption). To eliminate repetition the following abbreviations will be used: system n-B.A.W./water , instead of: first direction n-B.A.W. and second direction water ; or, system sec.-butanol/water , instead of: first direction sec.-butanol and second direction water.

Apparatus.

For runs in n-butanol:acetic acid:water and sec.-butanol:acetic acid: water (solvents n-B.A.W. and sec.-B.A.W.) on Whatman No. 1 paper, Shandon all glass boxes with ground glass lid and of the following dimensions have been used: height 20 in. width 20 in. and depth 8.5 in. Six chromatograms of 16 by 16 inches were run simultaneously in one box. A second Shandon box of the same type was used for the water direction (solvent 5).

The chromatograms were developed by the ascending method in both solvents. In the partitioning direction (solvents n-B.A.W. and sec.-B.A.W.) the chromatograms were developed for 20 to 24 hours, while in the water direction the solvent reached the top of the chromatograms after 5 hours.

Alternatively boxes made of heavily waxed wood were used with solvents 3 and 4. These boxes were fitted with front windows and glass lids. Their dimensions were governed by the size of the chromatographic papers which were 18¼ by 11¼ in. (=one half of the standard size sheet of 18¼ by 22½ in.). Chromatography boxes for the partitioning direction were 20 in. wide, 13 in. high and 13 in. deep, while the boxes for the water direction were 20 in. high, 13 in. deep and 15 in. wide.

Up to eight chromatograms were suspended on stainless steel frames which were inserted into stainless steel trays containing the solvents. After irrigation by the ascending method the chromatograms were dried on the frames, in a current of air.

Preparative paper chromatography.

For the "preparative method", as outlined by Nordström and Swain (184) and other authors (Roux 55,169; Brownell et al. 185), Whatman no. 3 chromatographic paper (18¼ by 22½ in.) has been used throughout this work. For chromatography in 2% aqueous acetic acid the paper was used without prior washing, because minor impurities migrated with the solvent front and did not interfere with the phenolic substances. For preparative runs in organic solvents, however, the paper sheets had to be washed for 12 hours with distilled, deionized water.

For adsorption chromatography (water direction) boxes 32 in. high, 30 in. wide and 11 in. deep were used. The boxes were of wooden structure with a front window and a glass lid. Each box held 5 sheets ($18\frac{1}{4}$ x $22\frac{1}{4}$ in.) suspended on a stainless steel frame. The sheets dipped into a stainless steel tray containing the solvent (2% acetic acid/water). The sheets were developed by the ascending method.

For partitioning chromatography (solvents 1-4) by the descending method, boxes of the same dimensions were used with the difference that here a glass tray was suspended in the top part of the boxes to hold the solvent. Each sheet (2 per box) was clamped between two stainless steel rods and inserted into the solvent tray. The sheets were held down in the solvent tray with a heavy glass rod.

All chromatographic operations were carried out in a constant temperature room at 20°C.

Selective spray reagents for the identification and estimation of flavonoid compounds associated with tannins.

Ammoniacal silver nitrate spray reagent.

This reagent due to Partridge (189) is used extensively in all fields of chromatography, mainly for the detection of strong reducing organic components such as carbohydrates, aldehydes and phenols. The reagent was introduced into the flavonoid field by Bate-Smith (129,190). Vicinal phenolic hydroxyl groups such as orthodihydroxy

and orthotrihydroxy groups (catechol and pyrogallol groups) reduce silver nitrate instantly in the cold and show up as brown, black (catechol) or metallic black spots (pyrogallol ).

In higher concentrations catechol and pyrogallol groups both show up as deep black spots. The shade of colouration developed, namely black, grey and brown depends mainly on the concentration of the hydroxyphenol but also on the nature of the reducing group. Mono- and meta-hydroxy-phenols reduce silver nitrate very slowly and, therefore, give either weak reaction or no reaction with this reagent. The silver nitrate spray reagent is made up in approx. 10% strength by dissolving 14 g of silver nitrate in 100 ml distilled chlorine free water and adding 6N ammonium hydroxide until the silver oxide formed just dissolves. The chromatograms are sprayed in a fume cabinet. Even spraying is obtained with an all-glass spray gun (Kopp, laboratory Supplies Inc., New York) and compressed air system. The use of diluted reagent must be avoided as the full intensity of the spot is not developed merely by heavy spraying. Spraying with weak reagents also results in spreading of the spots. For quantitative estimations (chapter 5 ) the chromatograms must be sprayed on both sides of the paper. Chromatograms intended to be

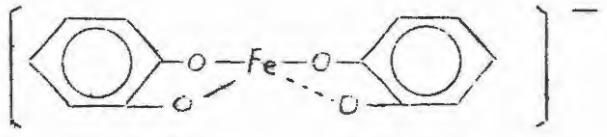
kept as records or for quantitative estimations, must be washed after spraying. Chromatograms may be washed three times with distilled water in a large 29 x 24 inch stainless steel dish and then fixed with 0.1% sodium thiosulphate for about 3 to 4 minutes.

A final wash of 20 minutes with tap water is necessary to remove traces of thiosulphate and to prevent staining of the paper chromatograms on ageing.

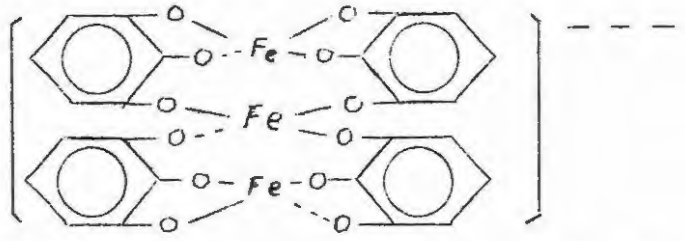
The chromatograms were then allowed to dry in the washing dish to avoid tearing of the wet filter paper.

The ferric alum spray reagent.

Ferric salts are known to give green or blue colorations with condensed tannins. Green colorations are due to a ferric complex with orthodihydroxyphenols and blue colorations to a ferric complex with orthotrihydroxyphenols.



green complex



blue complex

For paper chromatography a 3% solution of ferric alum (ferric ammonium sulphate) (186) is superior to an

aqueous ferric chloride solution.

The reagent is not as sensitive as the ammoniacal silver nitrate reagent, requiring approximately 100µg of a catechol- or pyrogallol- containing substance to produce a visible spot on the chromatogram. By comparison, ammoniacal silver nitrate produces visible colorations with 10-20µg of substances containing the same hydroxylation pattern. Ferric alum also coordinates weakly with mono- and meta-hydroxy-phenols present in high concentrations to give weak purple colorations (for example with β -resorcyclic acid).

The bis-diazotised benzidine spray reagent.

This reagent due to Lindstedt (191) is a very selective spray reagent for phenolic bodies. Flavans containing a phloroglucinol nucleus give an amber colour, whereas those of the "resorcinol series" give a yellow. Details of colour reactions are cited by Roux and Maihs (186).

The shade of colour produced varies slightly with the concentration of the phenolic substance on the chromatogram. The optimum concentration for a $C_{15}$ unit is 30 to 40µg, while $C_6$ units (resorcinol, phloroglucinol) give deep coloured spots with 1 to 2µg of the phenolic substance. Visible spots are developed with as little as 5µg of a phloroglucinol- or resorcinol- containing flavonoid com-

pound ,while phloroglucinol and resorcinol may be detected at concentrations as low as 0.5 to 1.0µg.

The benzidine reagent consists of two solutions mixed immediately before use in the proportions 3 part A with 2 parts B.

Solution A : Benzidine (5g) or benzidine hydrochloride (6g) are stirred with concentrated HCl (14 ml) and the suspension dissolved in 980 ml water.

Solution B : 10% aqueous sodium nitrite. The single solutions will keep several months if not exposed to direct sunlight. When mixed they should be used immediately.

If it is desired to keep a chromatogram for record purposes or for quantitative estimations it should be washed in a flat tray for at least 20 minutes with running tap water to avoid a yellow to brown background.

After spraying, the chromatogram should be allowed 1 to 3 minutes for full colour development before washing.

The toluene-p-sulphonic acid reagent.

This spray reagent developed by Roux (52) is exceptionally suitable for the identification of some monomeric and polymeric leuco-anthocyanidins. Leuco-anthocyanidins not hydroxylated in the 5 position (leuco-robinetinidin, leuco-fisetinidin, and melacacidin) give scarlet

colorations, while leuco-anthocyanidins hydroxylated in the 5-positions (leuco-cyanidin etc.) show up as yellow red spots after prolonged heating. The reagent is a 3% solution of toluene-p-sulphonic acid in absolute ethanol. The chromatograms are sprayed lightly and then baked in an oven for 5 to 10 minutes at 80 to 100°C. Prolonged heating should be avoided.

The Vanillin-toluene-p-sulphonic acid spray reagent.

This reagent, due to Roberts, Cartwright and Wood (187), is suitable for the detection of substances containing phloroglucinol nuclei. The reagent consists of a freshly prepared solution of 2 g vanillin and 1 g toluene-p-sulphonic acid in 100 ml absolute ethanol. The sprayed chromatogram is heated for 5 to 10 minutes at 80 to 100°C. A strong violet red color is developed with flavonoid substances containing phloroglucinol nuclei (eg. catechin and gallocatechin). Flavonoid bodies containing a resorcinol nuclei (eg. robinetinidol, leuco-fisetinidin) give weak pink colours after more prolonged heating.

Identification of wattle constituents on paper chromatograms under U.V. light.

Flavones and flavonols show a yellow fluorescence under U.V. light, but 5-deoxyflavonols (fisetin, robinetin) give an exceptionally brilliant greenish yellow

fluorescence (129).

The coumarins and chlorogenic acid exhibit a light blue fluorescence and phloroglucinol a brilliant blue when the spot is fumed with ammonia vapour.

The aglycons of anthocyanins give typical bright yellow red or purple fluorescence under U.V. light. Thus 3:7:4'-trihydroxyflavylium chloride shows a yellow, fisetinidin a bright orange, robinetinidin a deep pink, pelargonidin an orange red, cyanidin a light pink and delphinidin a mauve colour under U.V. light.

Determination of $R_F$ values.

The $R_F$ values of the parent coumpounds were determined as described by Bate-Smith and Westall (129). (+)-Catechin and robinetinidin chloride were used as markers for paper chromatograms of the catechins and anthocyanidins respectively.

# CHAPTER 4.

## THE EXTRACTION AND FRACTIONATION OF TANNINS FROM BLACK WATTLE BARK.

### Choice of starting material, sampling.

Comparison of commercial and of fresh black wattle bark extract by paper chromatography (Figures 1 & 2), shows clearly that the concentration of components, which show up as discrete spots on a two dimensional chromatogram, is considerably reduced in the commercial extract. The reduced concentration of these constituents in the commercial extract is, according to Roux (154, p.110 and personal communication), possibly due to two main factors; 1. oxidation of the bark on air drying after stripping; 2. hot aqueous leaching of the dry bark at the extract factories. Freshly stripped bark of light cream colour turns pink and darkens within hours in exposure to air. Under $SO_2$ atmosphere the bark retains its original colour due to the lack of oxygen and the inactivation of enzymes present. In order to prevent freshly stripped bark from becoming oxidised it may be covered with paraffin wax, or may best be extracted with organic solvents immediately after collection.

Fresh bark samples were collected from healthy trees (free of gummosis), varying in age (6-10 years), growing in the Grahamstown district. The best time for

stripping was one or two weeks after heavy rainfalls, when the bark peeled off with ease after cutting with a stainless steel knife. The bark was collected in strips of 2 to 3 inches width and of 10 inches length. According to the age of the tree the bark was about 5 to 10 mm thick. The bark was cut across the grain with a stainless steel knife to yield slivers of 1 mm thickness of a pale yellow colour. It is essential to use a stainless steel knife in order to prevent Fe ions from contaminating the phenolic bark constituents. The slivers were immediately covered with the organic solvent to reduce atmospheric oxidation. Extraction at room temperature proceeded over 5 days with daily renewal of the solvent. Extraction was carried out in 1 L. flat bottom flasks, the slivers filling 2/3 of each flask. The extracts were evaporated under vacuum in a rotary evaporator. The evaporation was heated in a water bath below 70°C.

Extraction of fresh black wattle bark with different solvents.

Three organic solvents have been tried for the extraction of the bark. These solvents had to be volatile, they had to be good solvents for polyphenols and poor solvents for lignins, gums and carbohydrates.

Methanol extraction of fresh black wattle bark, at room temperature, gave an extract of light cream colour. A two-way chromatogram, sprayed with ammoniacal silver nitrate, shows the presence of discrete spots standing out prominently against an overall dark background material in the lower $R_F$ area of the chromatogram. A methanol-extract will, in addition to the polyphenolic material, contain almost quantitatively all present sugars and some of the gums.

Ethanol extracts of fresh, moist black wattle bark are similar to methanol extracts. On a two dimensional chromatogram, sprayed with silver nitrate, the proportion of the higher molecular weight (lower $R_F$) tannins seem to be reduced, the discrete spots in the higher $R_F$ area stand out more prominently. An ethanol extract will still contain most of the sugars, but the gums will be absent due to their insolubility in ethanol.

Ethyl acetate extracts of fresh, moist black wattle bark contain typical black wattle constituents judging from two way chromatograms, sprayed with silver nitrate. The proportion of the highly polymerised background trail is, however, remarkably low compared with methanol or ethanol extracts. These findings are in agreement with those of Evelyn (209), who found the ethyl

acetate-soluble fraction of black wattle bark of lower average molecular weight (M.W. 921), compared with that of the whole fresh bark extract (M.W.1275). Ethyl acetate, therefore, has a fractionating effect. The proportion of sugars in the ethyl acetate extract is reduced and gums are absent. Ethyl acetate, on the other hand, is a good solvent for natural waxes, and ethyl acetate extracts will, therefore, have to be freed of wax prior to preparative fractionation of the polyphenolic constituents.

Table II compares the rate of extraction of damp freshly stripped black wattle bark using three different solvents. The figures not only reflect the total yield of extract, but also show the tannin content of these extracts, and the extent to which tannins have been extracted from the bark.

The moisture content of the solid extract was estimated by drying duplicate samples at 100°C in a vacuum oven. The percentage of tannins extracted from the bark was calculated on the basis of a tannin content of 18% for a moist ("green"), freshly stripped bark (197, 198). In the commercial language the damp freshly stripped bark is called "green" bark. This term will be avoided in this thesis to avoid confusion with green wattle (A. decurrens) bark.

Table II

Extraction of fresh black wattle bark with different solvents.

| | 200 g. samples extracted with:- | | |
|---|---|---|---|
| | Ethyl acetate | Methanol | Ethanol |
| 1st day | 16.2 g | 35.0 g | 34.0 g |
| 2nd. day | 8.8 g | 10.6 g | 12.3 g |
| 3rd. day | 1.1 g | 4.3 g | 4.2 g |
| 4th day | 1.0 g | 1.8 g | 1.8 g |
| 5th day | 0.7 g | 1.2 g | 1.0 g |
| Total extractives | 27.8 g | 52.9 g | 53.3 g |
| Moisture content of extractives | 12.5% | 6.0% | |
| Tannin content of vacuum dried extract. | 92.5% | 70.0% | |
| % tannins extracted from bark | 62.0% | 96.5% | |

The figures in Table II show that ethyl acetate gives an extract rich in polyphenolic material and is, therefore, the most suitable solvent.

Enrichment of the lower molecular weight constituents of black wattle tannins.

The ethyl acetate extract with an average molecular weight of 921 (Evelyn 209) was shown to contain highly polymerised tannins by means of paper chromatography (Fig. 2). Further enrichment of the low molecular weight fraction is essential to enable the separation of low molecular weight constituents such as catechins and leuco-anthocyanidins. Two methods have been tried for the removal of the high molecular weight fraction:-

1. Precipitation of higher molecular weight tannins with chloroform.
2. Retention of these tannins by column chromatography due to their affinity to cellulose.

1. Fractional precipitation of high molecular weight tannins with chloroform (Roux 169).

Samples of 100g ethyl acetate extractives were dissolved in 1 litre ethyl acetate (10% solution), and placed in ten 250 ml Erlenmeyer-flasks (100 ml per flask). Chloroform (100 ml)was added to each flask, in small quantities, with vigorous shaking and the precipitate allowed to settle for about 5 minutes. It is advisable to stopper the flasks to prevent moisture from contaminating the mixture. The presence of moisture will result in

a sticky precipitate, difficult to filter. When working under extremely humid conditions, it is advisable to do all filtering operations in big desiccators. While the precipitates were allowed to settle, 10 big funnels (4") were fitted with filters (Whatman no. 541) folded for rapid filtration. Each precipitate was rapidly filtered. The filtration of the white to cream precipitate was promoted by carefully stirring the precipitate with a spatula, without damaging the filter paper. The white precipitate turned brown on its surface, after a short period if not dried immediately. To free the precipitate completely from the filtrate, it may be centrifuged for 5 minutes at 2,500 R.P.M. in a centrifuge holding four 250 ml centrifuge tubes. The combined filtrates were evaporated to dryness with a rotary evaporator under vacuum on a water bath below 50°C. The filtrates of 50 precipitations, comprising 500 g starting material, gave 45 g of a low molecular weight tannin enriched fraction.

The rate of precipitation may be adjusted by altering the proportions ethyl acetate : chloroform.

| Chloroform : ethyl acetate | Yield of low molecular weight fraction. |
|---|---|
| 1 : 1 | 9 % |
| 1 : 1.5 | 13 % |
| 1 : 2 | 16 % |

When a lower proportion of chloroform is used for the preci-

pitation, the amount of polyphenols staying in solution is increased. A similar effect may be achieved by the addition of methanol to the ethyl acetate tannin solution, prior to the precipitation of the tannins with chloroform. Unfortunately both methods increase not only the proportion of low molecular weight tannins staying in solution, but also result in a similar increase in the proportion of higher molecular weight tannins.

The result of the fractional precipitation of highly polymerised tannins with chloroform was examined by two-way paper chromatography. Thus Fig. 2 shows an ethyl acetate extract of fresh black wattle bark, Fig. 3 the low molecular weight enriched filtrate, and Fig. 4 the chloroform precipitate. It may be noticed that the concentration of constituent D is decreased in the low molecular weight enriched fraction when compared with the ethyl acetate extract.

2. Retention of the high molecular weight tannins by column chromatography on cellulose.

Roux (199) has shown that components of black wattle extract are separated on cellulose and collagen (hide powder) columns. Solka Floc cellulose (Brown Co., Berlin, New Hampshire, U.S.A.) was used for all column chromatographic operations in the present work. Solka Floc cellulose is not a chemically pure product. Traces of Fe-salts have to be removed, before the cellulose

may be used for the separation of polyphenols. Failure to purify the cellulose, results in a bluish band of the well known Fe-tannin complex migrating with the front of the tannins. In some instances this may be a useful marker.

A chromatographic tube of 4 cm diameter and 55 cm length was closed at the bottom end with a porcelain disc, and the cellulose powder (150 g in 1 litre distilled water) introduced in a slurry. The final height of the cellulose was 40 cm. The column was freed of Fe-ions by washing with both 10% aqueous acetic acid (8 hours) and with distilled deionized water (8 hours).

Ethyl acetate extracted black wattle tannins (20 g) were dissolved in 125 ml warm water. A filter paper disc was placed on top of the cellulose column to prevent stirring up, and the tannin solution was slowly introduced to the column. After the tannin solution completely penetrated into the cellulose, and the surface of the tannin solution reached the filter paper disc, the space above the cellulose (15 cm) was filled with water and the column connected to an automatic feeding system to ensure constant water supply and pressure. $FeCl_3$ in solution as indicator, was introduced into the receiving flask (vacuum flask). After 5 to 6 hours the colour of the solution in the receiving flask changed from yellow to blue, thus indicating the presence of tannins. The following fractions (25 ml each) were collected. The collection of fractions continued until a sharp decrease in the tannin concentration of the collected

fractions was noticed, using the $FeCl_3$ test. A total of 625 ml had been collected. The high molecular weight tannins remained as a wide band on top of the column, while tannins of lower molecular weight and most of the individual polyphenolic constituents had been collected. Each fraction was examined by two-way paper chromatography. The fractions showed a gradual increase in the proportion of higher molecular weight constituents with time of development. The fractions were evaporated in a rotary evaporator under vacuum, with the temperature of the water bath kept at 50°C. The first 6 to 7 g of collected material (evaporated column fractions) represented the low molecular weight fraction, as reflected by paper chromatography.

Enrichment of low molecular weight tannins by chromatography on cellulose columns proved to have some advantages, but also marked disadvantages when compared with the fractional precipitation method. Column fractionation has the advantage that it yields much sharper fractions. These fractions, on the other hand, are in aqueous solution and, therefore, care must be taken on evaporation to prevent oxidation of the very sensitive phenolic compounds.

The fractional precipitation method was finally chosen for further investigations mainly because the enriched low molecular weight fraction is dissolved in a volatile solvent (ethyl acetate/chloroform) and may therefore be concentrated to dryness without the danger of oxidation of the tannins.

Bark Waxes.

Ethyl acetate, ethanol or methanol extractives of wattle barks were found to contain small amounts of waxes. If these waxes are not removed prior to paper chromatographic separations, they deposit on the surface of the cellulose and retard the development of the chromatograms considerably when water is used as irrigant. On preparative sheets, for example, the development which normally requires 16 hours, often requires 20 hours or longer when traces of wax are present.

Chloroform was found to be the best solvent for the removal of the waxes (Soxhlet) from bark extractives. Waxes extracted with this solvent showed molecular weights of 592-632 (ebulliometric method in benzene). The waxes from black, green, silver and golden wattle bark appeared to be identical.

The separation of flavonoid compounds from complex polyphenolic mixtures.

Bradfield and Penney (82) fractionated tea polyphenols on silica columns with wet ether as irrigant. The fractions of the first column were again fractionated with different solvents on subsequent columns. Vuataz et al.

(200) used the gradient elution technique of partition column chromatography, in which wet cellulose powder was used as stationary phase, and a series of solvents of increasing polarity as mobile phase. Vuataz isolated small quantities of tea polyphenols with this method. Nordström and Swain (184) separated flavonoid compounds from "Dandy", a blue garden variety of Dahlia variabilis, by "preparative paper chromatography", a technique also applied by Roux (179) for the separation of (+)-catechin and (+)-gallocatechin from Casuarina equisitifolia. Roux and Paulus (61) isolated (-)-fisetinidol from black wattle heartwood by means of Craig countercurrent distribution and preparative paper chromatographic techniques. Roux (169) attempted preliminary fractionation of black wattle bark tannins with a Craig machine, using both phases of the mixture amyl alcohol:acetic acid:water = 4:1:5. He also showed that Craig countercurrent distribution shows a similar separation effect as partitioning chromatography on cellulose sheets, and pointed out that Craig countercurrent distribution was found to be more effective in dealing with selected higher $R_F$ fractions of the extract than with the tannin mixture as a whole.

Fractionation of an enriched low molecular weight tannin mixture by Craig countercurrent distribution.

The Craig machine was a fully automatic model with 160 tubes built by "Glasapparatebau Göttingen, Helmut Rettberg". Each tube holds 50 ml upper- and under- phase.

First run.

10 g of enriched low molecular weight fraction (see previous chapter and Fig. 3) were dissolved in 400 ml of the under phase of sec-butanol:benzine:water = 1:1:2, and introduced into the first eight tubes of the apparatus. The remaining 152 tubes were filled with under phase and the automatic feeding flask filled with upper phase. The operation was started with a 3 minute shaking period followed by 5 minutes rest period in which the two phases were allowed to separate. These two operations were followed by a transfer operation, transferring the upper phase of each tube into the following tube. After 130 transfers, the operation was discontinued and the upper and under phase of every tenth tube was examined by two dimensional paper chromatography.

Second run.

100 g methanol extract of black wattle bark was dissolved in one litre distilled water and introduced into a liquid/liquid extractor. The extractor was then filled

with ether and the aqueous tannin solution was extracted with this solvent for four days. On evaporation the ether solution gave 5 g enriched low molecular weight tannins. This fraction was dissolved in 200 ml. of the under phase of sec.-butanol:benzine:water=1:1:2 and introduced into the first 4 tubes of the Craig machine. The operation was discontinued after 130 transfers and the upper- and under-phase of every tenth tube was examined by two dimensional paper chromatography (direction one: sec.-butanol:acetic acid:water; direction two: water.).

The results of Craig countercurrent distribution of black wattle polyphenols are reflected in Table IV. The chromatograms of the corresponding upper and under phase did not show differences in the composition but only in concentration. The concentration of phenolic bodies in the under phase was higher than in the upper phase. Run I and Run II showed very little difference in concentration and composition of wattle constituents for corresponding tubes. The front fractions (tube 130-110) of both runs contained yellow bark waxes only. Tubes 100-90 contained phenolic bodies of unknown structure in very low concentration. Tubes 80-30 contained the phenolic bodies A, $A_1$, F, G, H. The rest of the mobile phenolic black wattle bark constituents B, $A_1$, E, C, remained in tubes 1-20 due to low solubility in the solvent mixture.

Table IV.

Craig countercurrent distribution of black wattle bark polyphenols.

| | Relative concentration: | Remarks: | Total yield of 10 tubes: |
|---|---|---|---|
| 10 | unresolved starting | Presence of low $R_F$ background | |
| 20 | material | | |
| 30 | F (++); A (+++) | | 500 mg |
| 40 | F (+++); $A_1$(+);G(+) | | 400 mg |
| 50 | G (+++); $A_1$(+) | | 200 mg |
| 60 | G (+++); H(+++) | highest concentration of single bodies. | 200 mg |
| 70 | G (+++); H(+++) | Presence of 2 unknown bodies of high $R_F$ in n.-B.A.W. | 100 mg |
| 80 | G (+) | | 50 mg |
| 90 | unknown phenolic bodies | | traces |
| 100 | unknown phenolic bodies | | traces |
| 110 | " | | not estimated |
| 120 | yellow waxes | | " |
| 130 | " | | " |

Tubes 55 to 65 contained over 200 mg chromatographically pure (+)-catechin and (-)-robinetinidol in about equal concentration.

Fractionation of enriched low molecular weight polyphenols by "preparative" paper chromatography.

Preliminary experiments were carried out to find the optimum concentration for the preparative paper chromatography of polyphenolic mixtures on thick paper sheets (Whatman no. 3) in the three solvents, 2% aqueous acetic acid, sec. - B.A.W. and n. - B.A.W. Concentrations which were too high resulted in trailing of the substances and the bands on such chromatograms do not have straight fronts. At too low a concentration, on the other hand, atmospheric oxidation of the very sensitive phenolic constituents may result. Due to a higher solubility of the polyphenolic substances in sec.-butanol than in n.-butanol, sec.-B.A.W. allows a higher concentration of polyphenolic substances to be separated per sheet compared with n.-B.A.W. Up to 400 mg polyphenols per sheet may be chromatographed on Whatman no. 3 paper in water if the mixture consists of fairly equal proportions of lower and higher molecular weight constituents.

Table V lists the optimum concentrations for different phenolic black wattle fractions on Whatman no. 3 paper (weight 50 g of cellulose) for the 3 different solvents cited above.

Table V.

Optimum concentrations of different phenolic black wattle fractions per sheet (Whatman no. 3).

| Material to be chromatographed: | optimum concentration per sheet 2% acetic acid: | sec.-B.A.W.: | n-B.A.W.: |
|---|---|---|---|
| | upward migration | downward migration | |
| ethyl acetate extract of black wattle bark | 400 mg | 250 mg | 250 mg |
| enriched low mol. weight fraction | 300 mg | 200-250 mg | 200 mg |
| single band (material from previous preparative chromatographic separations) | 200 mg | 150-200 mg | 150 mg |
| two single constituents | 150-200 mg | 100-150 mg | 100 mg |
| single impure constituent. | 100 mg | 50 mg | 50 mg |

The "preparative" paper chromatography of mixtures of polyphenols, as outlined by Nordström and Swain (184) and by Roux (169), was finally chosen for future investigations, mainly for the following reasons:

1) The expected fractionation of a mixture can be exactly forecast from the results of two dimensional paper chromatograms in the same solvent system which will be used for the "preparative" operations.

2) The fractions are much sharper than in the case of cellulose column chromatography or Craig countercurrent distribution.

3) The fractions can be easily detected either under U.V. light, or by cutting a narrow strip from one side of the preparative chromatogram and spraying it with a selective spray reagent.

4) The preparative thick paper sheets (Whatman no. 3) dry easily in a current of air, and after marking and cutting, the fractions may be eluted with a minimum of a volatile solvent (e.g. ethanol/water = 7:3).

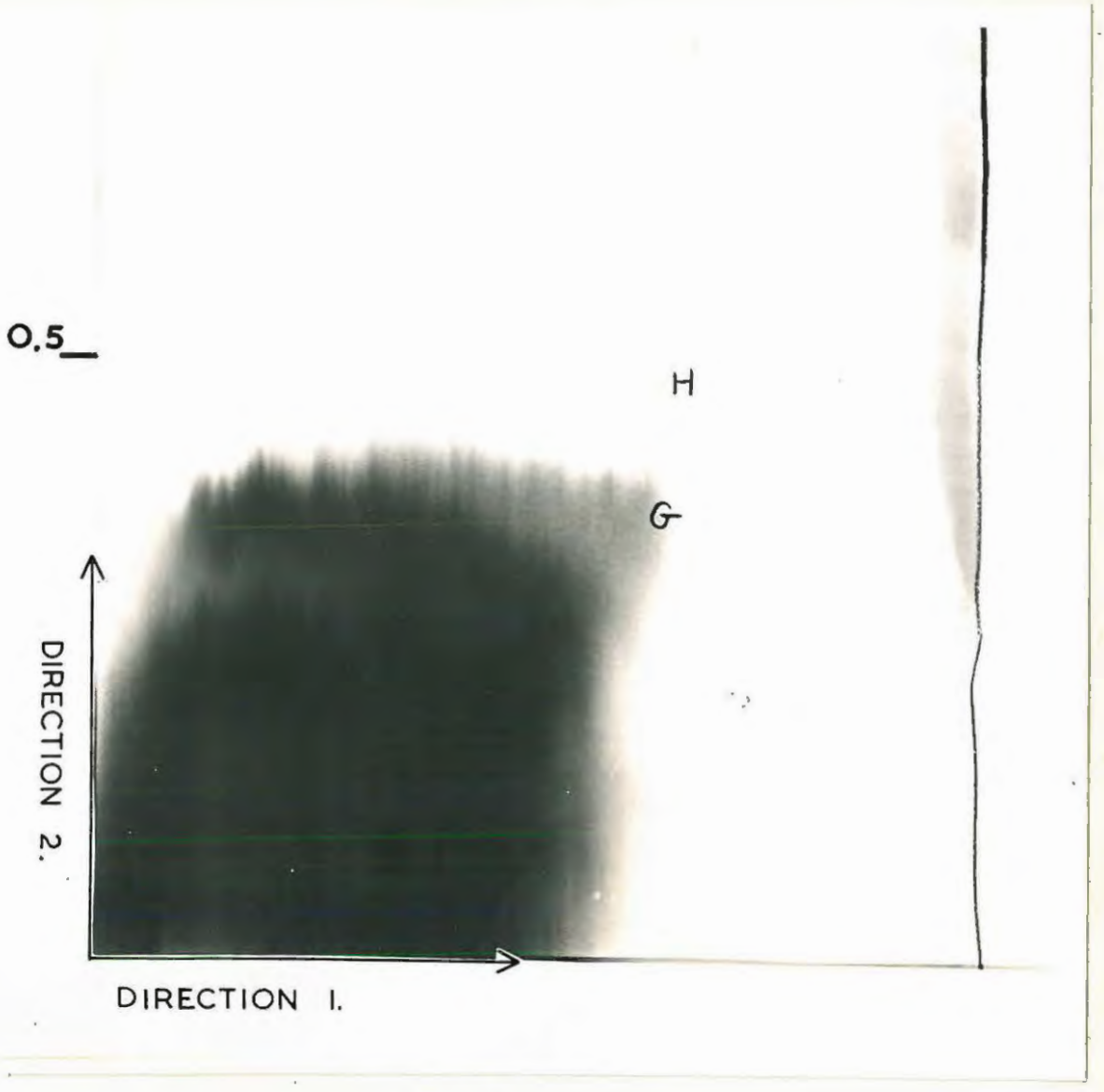



Fig. 1.

Commercial black wattle extract, concentration 4 mg.
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with ammoniacal silver nitrate.
H = (-)-robinetinidol
G = (+)-catechin

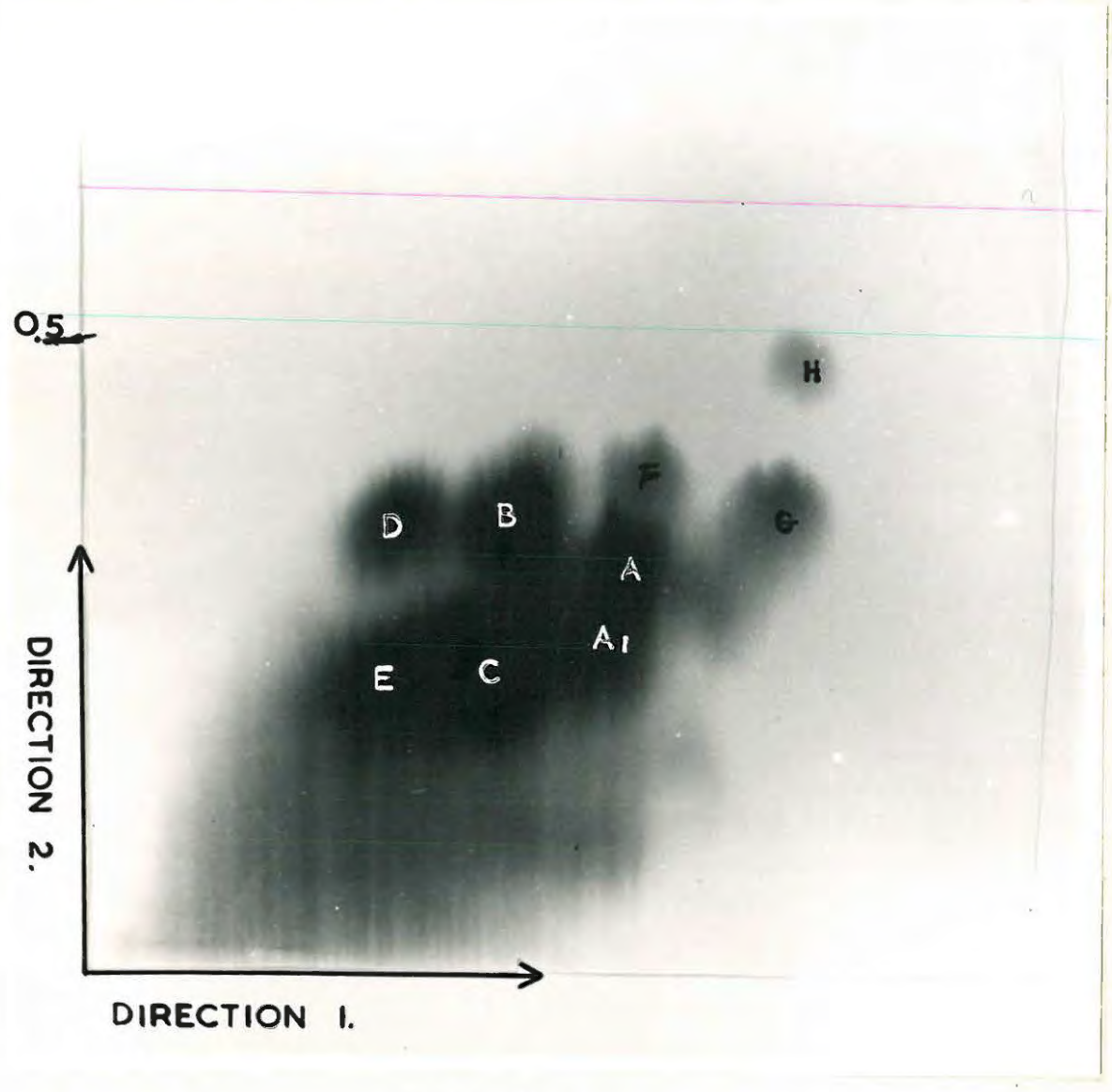



Fig. 2.

Ethyl acetate extract of fresh black wattle bark. (3 mg)
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with ammoniacal silver nitrate.
H = (-)-robinetinidol G = (+)-catechin
A= (+)-gallocatechin D = dimeric leuco-robinetinidin

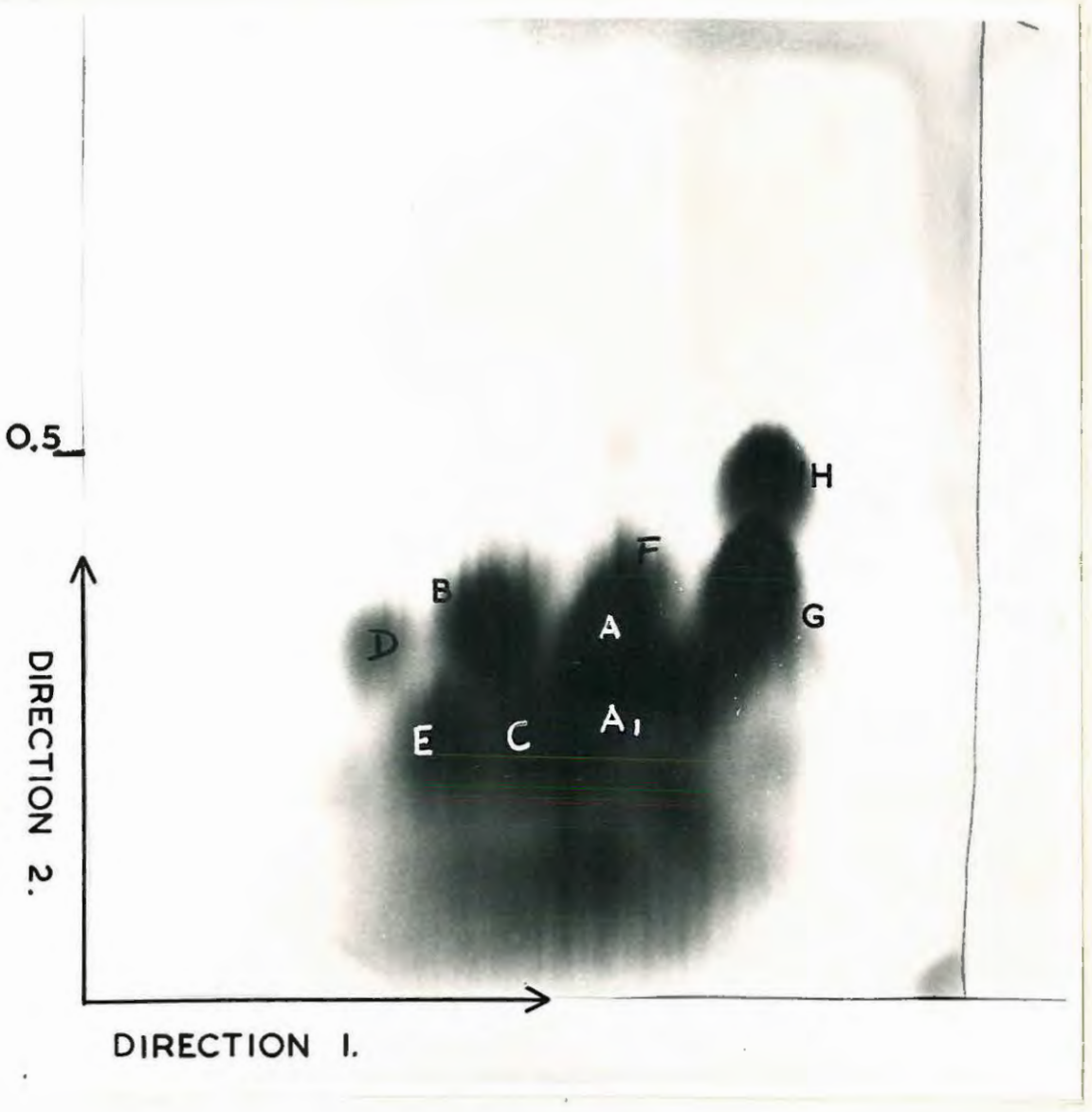



Fig. 3.

Enriched low molecular weight fraction of fresh black wattle bark ethyl acetate extract. (2 mg)
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with ammoniacal silver nitrate.
H = (-)-robinetinidol G = (+)-catechin
A = (+)-gallocatechin D = dimeric leuco-robinetinidin

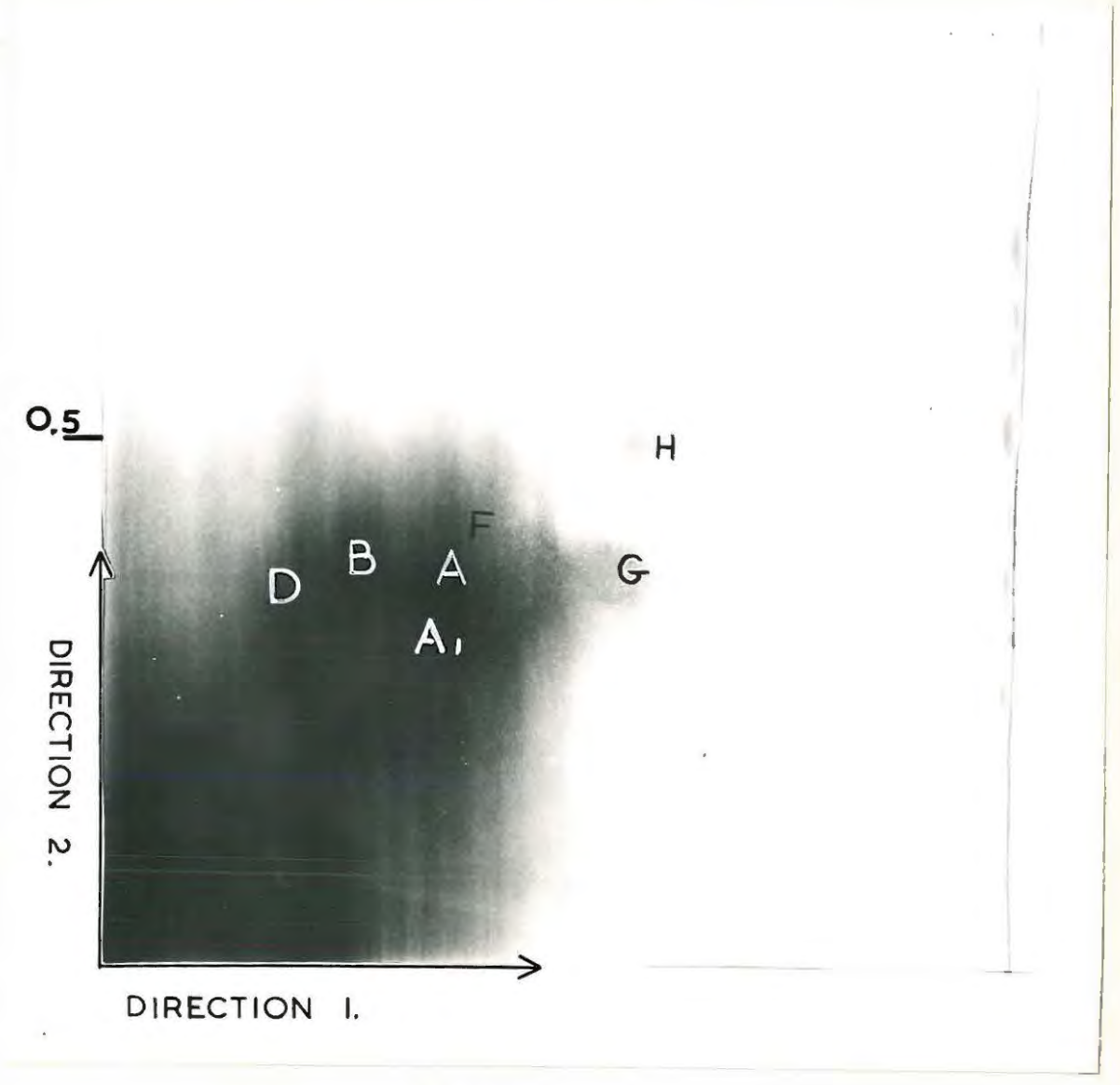



Fig. 4.

Black wattle bark tannins precipitated with chloroform.
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with ammoniacal silver nitrate.
H = (-)-robinetinidol G = (+)-catechin
A = (+)-gallocatechin D = dimeric leuco-robinetinidin

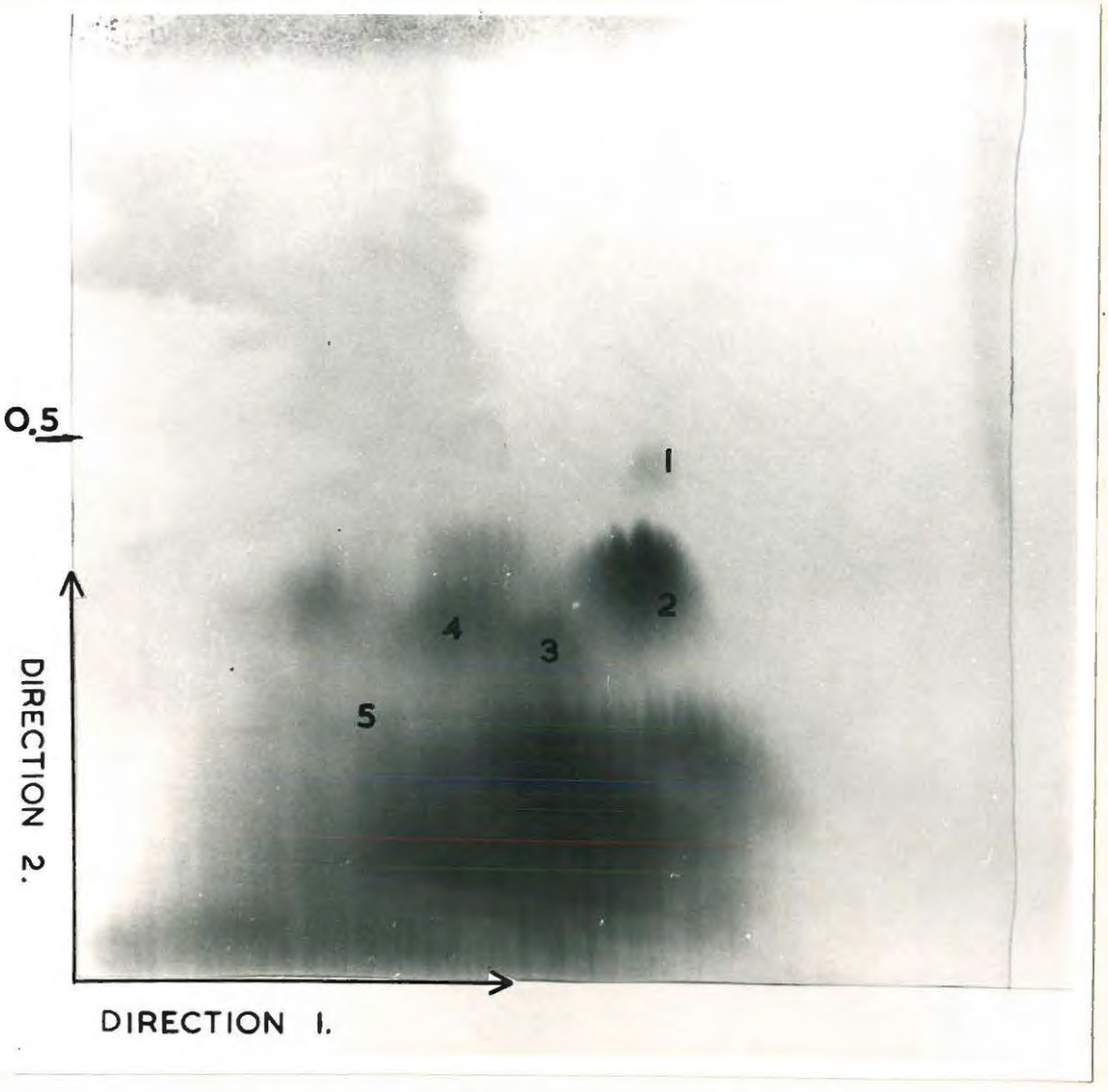



Fig. 5.

Ethyl acetate extract of dry silver wattle bark. (3 mg)
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with ammoniacal silver nitrate.
1 = (-)-robinetinidol 2 = (+)-catechin
3 = (-)-epicatechin 4 = (+)-gallocatechin
5 = (-)-epigallocatechin

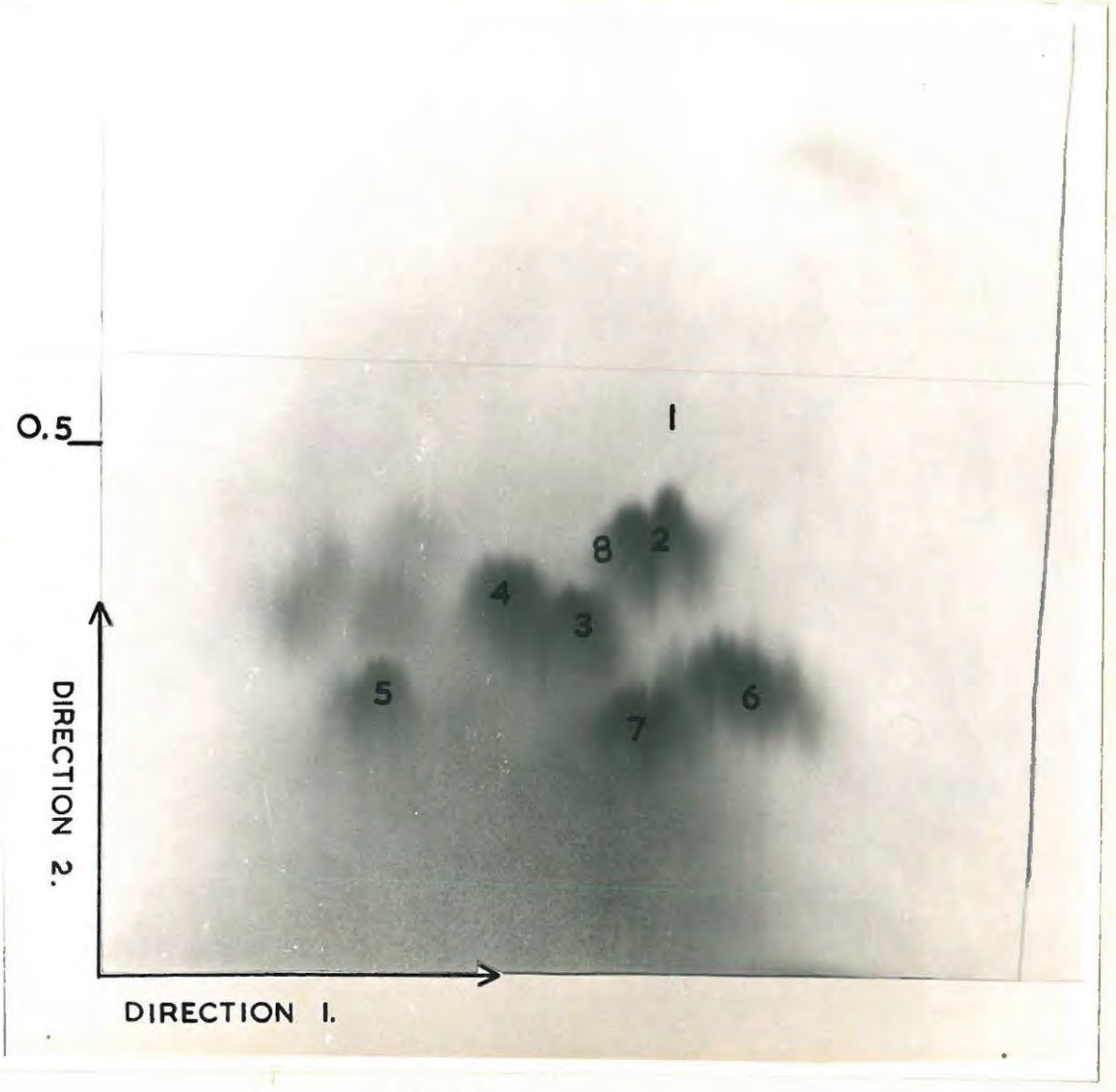



Fig. 6 .

Ethyl acetate extract of dry golden wattle bark. (3 mg)
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with the **bis**diazotised benzidine reagent.

1 = (-)-robinetinidol
2 = (+)-catechin
3 = (-)-epicatechin
4 = (+)-gallocatechin
5 = (-)-epigallocatechin
6 = (-)-epicatechin gallate
7 = (-)-epigallocatechin gallate
8 = gallic acid

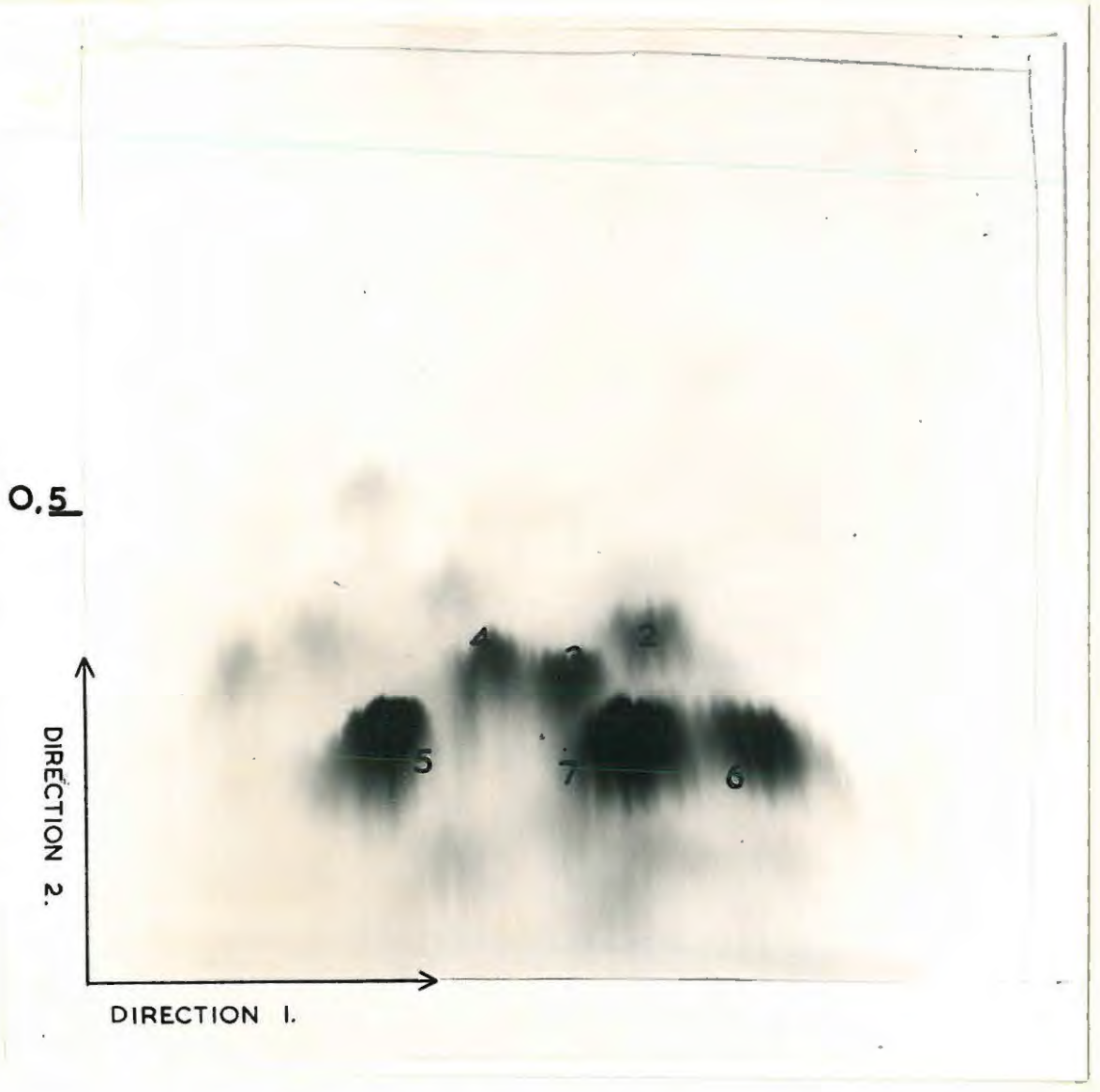



Fig. 7 .

Methanol extract of dry green tea leaves. (1 mg)
Direction 1: n.-butanol:acetic acid:water (6:1:2)
Direction 2: 2% aqueous acetic acid.
The chromatogram was sprayed with the bisdiazotised benzidine reagent.

1 = (-)-robinetinidol
2 = (+)-catechin
3 = (-)-epicatechin
4 = (+)-gallocatechin
5 = (-)-epigallocatechin
6 = (-)-epicatechin gallate
7 = (-)-epigallocatechin gallate

# CHAPTER 5

## COMPARATIVE STUDY OF CATECHIN COMPONENTS OF BLACK, GREEN, SILVER AND GOLDEN WATTLE BARKS.

### SECTION A.

### The isolation and identification of catechins from fresh black wattle bark.

Initial investigations showed that the attempt to isolate polyphenolic constituents from a source as complex as black wattle bark will only be successful if advantage is taken of preliminary enrichment procedures. Thus a scheme, starting with fresh black wattle bark, enriching the low molecular weight constituents and finally fractionating these constituents by both, adsorption and partition chromatography was chosen.

Table VI.

Fractionation scheme for black wattle tannins.

Fresh damp black wattle bark

↓ extraction with commercial ethyl acetate

720 g ethyl acetate extractives

↓ precipitation of low $R_F$ constituents with chloroform

68 g enriched low molecular weight fraction

↓ L.M.W.F. (Soxhlet extraction of waxes with chloroform).

64 g wax-free extract.

Further fractionation by preparative paper chromatography with water as the solvent, using 400 mg substance per sheet

| Fractions: | Average $R_F$ | | | |
|---|---|---|---|---|
| | 0.43 | E Band * | yield: | 8 g |
| | 0.48 | C Band | yield: | 9-10 g |
| | 0.56 | R Band | yield: | 2.4 g |

Further fractionation of the C band * by preparative paper chromatography with n.-B.A.W. or with sec.-B.A.W. as solvents; 200 mg substance per sheet.

| Fraction | $R_F$ n.-B.A.W.: | $\Delta R_F$: | $R_F$ sec.-B.A.W.: | $\Delta R_F$: | constituents: | yield: |
|---|---|---|---|---|---|---|
| C-7 | 0.24 | 0.06 | 0.34 | 0.09 | D | 0.5 g |
| C-6 | 0.30 | 0.04 | 0.43 | 0.07 | B,D | 0.6 g |
| C-5 | 0.34 | 0.14 | 0.50 | 0.05 | B | 1.6 g |
| C-4 | 0.48 | 0.05 | 0,55 | 0.07 | B,A,F | 2.1 g |
| C-3 | 0.53 | 0.11 | 0.62 | 0.06 | A,F | 2.0 g |
| C-2 | 0.64 | 0.06 | 0.68 | 0.06 | G,A,F | 0.5 g |
| C-1 | 0.70 | | 0.74 | | G | 1.6 g |

* The bands are named after the constituent, which according to its $R_F$, is expected to be present in highest concentration in the particular band. Thus, R band stands for robinetinidol, C band for catechin and E band for constituent E.

The enriched low molecular weight fraction of black wattle bark polyphenols (68 g) was freed of wax as described in a previous chapter and the wax-free product (64 g) dissolved in methanol to give an 8% solution. 5 ml solution (400 mg substance) was streaked evenly along a line 3 inches from the narrower end of an $18\frac{1}{4}$" by $22\frac{1}{2}$" Whatman no. 3 sheet. Development for 12 hours was permitted using the ascending method. After that time the solvent front had reached the top of the sheets and the development was discontinued.

The air-dried sheets were examined under ultraviolet light in a dark room. Fluorescent bands were observed at $R_F$ values 0.43 (dark blue fluorescent) E band: 0.48 (dark blue) C band: 0.56 (dark blue) R band: 0.64 (light blue) and 0.73 (dark mauve). The bands were marked under U.V. light with a soft pencil on each sheet. Bands C and R were separated by a narrow bright blue fluorescent band which serves as an excellent marker and enables these bands to be demarcated accurately. A strip of 2 cm width was cut from the wider end of one of these sheets and sprayed with bis-diazotized benzidine, giving a bright yellow with the R band, and an intense ochre with the C band. With the same spray reagent the E band gave an ochre, while the whole lower $R_F$ region of the chromatogram appeared yellow orange.

Strips corresponding to the C and the R bands were cut and eluted with 70% ethanol (Nördstrom and Swain 184) in air-tight boxes for 24 to 48 hours. The combined eluates of each band were evaporated at 50°C in a rotary evaporator under vacuum until the ethanol and most of the water had been removed. Final drying was carried out under vacuum, in a desiccator over dry $CaCl_2$.

Following the removal of ethanol from the eluates of the R band, white microcrystals separated out on standing. After standing for 2 days at 1°C the white crystalline material was filtered and washed with a minimum of iced water. After drying over $CaCl_2$ in vacuum the white crystals had a total weight of 2.4 g. A two dimensional chromatogram of the crystals showed the presence of body H contaminated with a trace of body G. A two dimensional chromatogram of the mother liquor showed the presence of the constituents H, G and traces of F.

Fractionation of the C band.

Evaporation of the eluates of band C yielded 9 g of a light yellow powder. A two-way paper chromatogram thereof reflected the presence of at least 6-7 individual reducing, probably phenolic, constituents, H,G,A,$A_1$,B,F and D (Fig. 3).

For further fractionation, the dried eluates of the C band were dissolved in 250 ml methanol and streaked onto washed sheets of Whatman no. 3 chromatographic paper.

5 ml of the solution was applied with a pipette, in a band 2 inches wide, along a line 4 inches from the narrower end of the sheet. The solvent front reached the bottom of the sheet after 11 hrs. and development was continued for another hour as it was not intended to collect front fractions.

When the dried sheets were examined under U.V. light, two dark mauve bands C-1 ($R_F$ 0.70 in n-B.A.W. or $R_F$ 0.74 sec.-B.A.W.) and C-3 ($R_F$ 0.53 n-B.A.W. or $R_F$ 0.62 sec.-B.A.W.)were visible, and were separated by a light blue band C-2 ($R_F$ 0.64 n-B.A.W. or $R_F$ 0.68 sec.-B.A.W.). A strip of 2 cm. width was cut from the wider end of a sheet and sprayed with bis-diazotised benzidine. C-1 and C-3 appeared as deep ochre, C-5 and C-7 as orange and yellow bands respectively. Band C-2 appeared as a narrow weak pink band. To ensure accurate cutting every 4th chromatographic sheet was sprayed with benzidine, as above. The bands were marked with a soft pencil and cut and eluted as described.

On evaporation, white crystals separated from the eluates of C-1. A two dimensional paper chromatogram showed that these were identical with constituent G. The substance was found to be impure by paper chromatography and accordingly, recrystallised from water (1.6 g in 5 ml water). The final yield of pure substance, after two recrystallisations, was 0.83 g.

Following removal of the ethanol from the eluates

of band C-3, the aqueous solution was evaporated in a desiccator over $CaCl_2$, under vacuum. A two dimensional paper chromatogram showed the presence of components A, F and a trace of B. The whole C-3 fraction was, therefore, re-run in both water and n-butanol solvent systems and special care was taken to cut the bands as accurately as possible. The final eluate was dried, under vacuum, in a desiccator Following the first signs of crystallisation, the flask was stoppered and placed in the refrigerator. After several days more white crystals separated, and were filtered off. The yield of the pure white crystalline material, which was identical with constituent A, was 30 mg.

Identification of constituent H.

The white crystals from the R-band (2.4 g) were recrystallized in a minimum of water (2.1 g). The crystalline substance was run on two dimensional chromatograms having $R_F$ values of $R_F$ 0.76 in n-B.A.W. and $R_F$ 0.47 in 2% aqueous acetic acid. The chromatograms were sprayed with selective spray reagents. The ferric reagent gave a sharp blue spot, indicating the presence of a pyrogallol group. Silver nitrate spray reagent gave a metallic black spot, indicating strongly reducing groups (ortho-dihydroxy or ortho-trihydroxy). The vanillin-toluene-p-sulphonic acid reagent on prolonged heating gave a weak pink spot, a reaction

typical of flavonoid bodies containing a resorcinol nucleus (Roux and Maihs 186). The bis-diazotised benzidine reagent produced an intense yellow pigment, thus, confirming the presence of a resorcinol A nucleus (reaction = weak yellow) and also indicating the presence of a pyrogallol B nucleus (reaction = intense yellow). The toluene-p-sulphonic acid reagent gave no immediate colour reaction on heating (5 minutes). On prolonged heating, a very weak brown spot developed, a reaction observed with catechins (Roux & Maihs 186). The negative leuco-anthocyanidin reaction with this reagent was confirmed when no pigment developed on heating the substance with dilute mineral acid in alcoholic solution, under pressure, according to Pigman et al. (87). Under U.V. light the substance exhibited no fluorescense. A comparison of the rates of migration of constituent H with (+)-catechin in partitioning mixtures, (n-B.A.W.; n-butanol and sec.-butanol, both water saturated), showed similar $R_F$ values in each instance. Craig countercurrent distribution showed that both substances [constituent H and (+)-catechin] have identical rates of migration, and, therefore, the same partition coefficients. This suggests a similar total number of hydroxyl groups per $C_{15}$ unit for both substances.

Alkali microfusion of the crystalline substance confirmed the presence of resorcinol (A) and pyrogallol (B) nuclei in constituent H, giving resorcinol, $\beta$ -resorcylic acid and gallic acid as main degradation products.

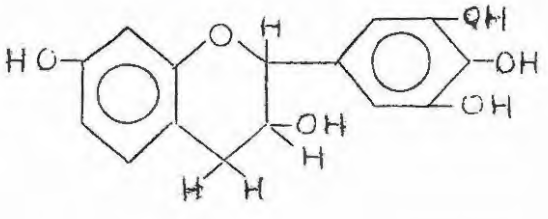
Component H crystallized from water as a white hydrate with a melting point (uncorrected) of 207°C. $\lambda_{max.}$ 282 mμ; $\varepsilon_{max.}$ 3636. Found: C, 56.2; H, 5.8: loss at 100°C, 8.8%. Calculated for $C_{15}H_{14}O_6 \cdot 1.5\ H_2O$: C, 56.8; H, 5.4, $H_2O$ 8.5%. The crystals, dried over $P_2O_5$ under vacuum in a drying pistol at the temperature of boiling ethanol (78°C) for 4 hours, retained one molecule of water. Found: C, 58.6; H, 5.4. Calculated for $C_{15}H_{14}O_6, H_2O$: C, 58.4; H, 5.2%. On vigorous drying at 110°C for two hours the crystals lost water of crystallization. Found: C, 61.7; H, 5.1. Calculated for $C_{15}H_{14}O_6$: C, 62.1 and H, 4.9%. $[\alpha]_D^{20}$ -10.7 $\pm$ 0.3° in acetone:water = 1:1 (c, 1.42).

To gain further evidence for the pattern of hydroxylation the substance was methylated and acetylated. The recrystallized constituent H (500 mg) was methylated with excess diazomethane by the method described by Roux and Evelyn (56), giving 0.4 g crystalline (ethanol) tetramethoxy derivative. The melting point, 141-142°C increased to 143-144 after two further recrystallizations. Found : C, 65.9; H, 6.7: OMe, 35.4. Calculated for $C_{19}H_{22}O_6$: C, 65.9;

H, 6.4 and OMe, 35.8%. $[\alpha]_D^{20}$ -21.6$^{\pm}$ 0.4 in $C_2Cl_4H_2$ (c,2.3).

Acetylation of the recrystallized constituent H (490 mg) with acetic anhydride and sodium acetate (Roux & Evelyn, 56) gave a crystalline penta-acetyl derivative (600 mg) with a melting point of 132 - 133°C, after re-crystallization from ethanol. Found: C, 60.0; H, 4.9; $COCH_3$, 42.0. Calculated for $C_{25}H_{24}O_{11}$: C, 60.0; H, 4.8 and $COCH_3$ 43.0%. $[\alpha]_D^{20}$+4.6 $\pm$ 0.3° in $C_2Cl_4H_2$; (c, 2.15).

The above findings indicate that constituent H has a total of 5 hydroxyl groups, four of which are phenolic. It was further shown that constituent H has a resorcinol A and a pyrogallol B nucleus on the assumption that it is a flavonoid body. The optical rotation of constituent H and its derivatives reflect the presence of at least one asymmetric carbon atom. This and the fact that constituent H is colourless eliminates the possibility of a flavone, flavonol or chalkone structure, the latter having an intrinsic yellow colour. A flavan-3:4-diol structure is being ruled out by negative leuco-anthocyanidin reactions. A catechin (flavan-3-ol) structure was therefore proposed for this constituent.




Constituent H
(-)-robinetinidol

Dr. Weinges of Heidelberg University kindly supplied a sample of synthetic (-)-robinetinidol, (-)-7:3':4':5'-tetrahydroxyflavan-3-ol, for reference. The synthetic product exhibits the same colour reactions as body H when sprayed with different spray reagents, and has an identical rate of migration in 5 different solvent systems (sec. B.A.W.; n-B.A.W. sec.-butanol; n-butanol acid; and water). A molecular mixture of the two substances (mixed in equal quantities, dissolved and recrystallized) showed no depression (m.p. 206-207°C). Infrared absorption curves of constituent H and the synthetic (-)-robinetinidol, and those of their derivatives, were superimposable over the range 2.5 - 15 μ.

A comparison of the physical constants of both the natural and synthetic (-)-7:3':4':5'-tetrahydroxyflavan-3-ol is summarized below:

| | natural (-)-robinetinidol | synthetic (-)-robinetinidol (Weinges 115) |
|---|---|---|
| M.P. | 207°C. | 207 - 209°C |
| rotation (acetone:water = 1:1) | $[\alpha]_D^{20}$ -10.7°; c,1.42 | $[\alpha]_D^{25}$ -11.5°; c.2.0 |
| $\lambda_{max.}$ $\varepsilon_{max.}$ | 282 mμ 3636 | not reported. |
| acetyl derivatives M.P. | 132 - 133°C | 131 - 133°C |

| | | |
|---|---|---|
| acetyl derivatives | natural (-)-robinetinidol | synthetic (-)-robinetinidol (Weinges 115) |
| rotation (tetrachloroethane) | $[\alpha]_D^{20}+4.6^\circ$; c,2.15 | $[\alpha]_D^{25}+4.9^\circ$;c,2.0 |
| methyl derivatives | | |
| M.P. | 143 - 144°C | 140 - 144°C |
| rotation (tetrachloroethane) | $[\alpha]_D^{20}-21.6^\circ$; c,2.3 | $[\alpha]_D-22.3^\circ$; c,2.0 |

Identification of constituent G.

The recrystallized chromatographically pure constituent G (830 mg) was spotted on several chromatograms at a concentration of 20-40µg and run in the solvent systems n-B.A.W. ($R_F$ 0.75) or sec.-butanol ($R_F$ 0.77) for the first, and in 2% aqueous acetic acid ($R_F$ 0.35) for the second direction. Constituent G gave the following colour reactions when sprayed with selective spray reagents: silver nitrate reagent - black to brown; benzidine reagent - claret maroon; ferric alum reagent - green; vanillin -toluene-p-sulphonic acid reagent - violet red; toluene-p-sulphonic acid reagent - no reaction. The substance was colourless in ordinary light and did not fluoresce under U.V. light. The colour reactions indicate the presence of phloroglucinol (A) and catechol (B) nuclei. The absence of a flavan-3:4-diol structure, as indicated by the negative reaction with toluene-p-sulphonic

acid, was confirmed by a negative leuco-anthocyanidin reaction on heating the substance with dilute mineral acid in alcoholic solution, under pressure. The absence of fluorescence under U.V. light, rules out the possibility of a flavonol structure.

Alkaline microdegradation (KOH) of the crystalline substance gave phloroglucinol, and protocatechuic acid as main degradation products, thus confirming the presence of phloroglucinol (A) and catechol (B) nuclei in the molecule.

The melting point of the crystalline substance (water) was 172 - 174°C, $[\alpha]_D^{21}$ +17.0° in acetone:water = 1:1 (c, 1.1). Found: C, 56.8; H, 5.7. Calculated for $C_{15}H_{14}O_6$,1.5 $H_2O$: C, 56.8; H, 5.4%.

The recrystallized substance G (100 mg) was acetylated with acetic anhydride in pyridine, yielding 124 mg crystalline penta-acetyl derivative, m.p. 131°C; $[\alpha]_D^{20}$ +40.1° in $C_2H_2Cl_4$ (c, 1.3), and $[\alpha]_D^{23}$ +29.4±0.4° in acetone (c, 0.9).

A mixed m.p. of G and of (+)-catechin from Uncaria gambir, showed no depression. Similarly, a mixed m.p. of acetylated G and penta-acetyl-(+)-catechin, showed no depression, thus confirming the identify of G with (+)-catechin. The infrared absorption curves of (+)-catechin and component G were superimposable over the range 2.5 - 15μ.

The ultraviolet absorption curve of constituent G (6.12 mg substance in 100 ml ethanol) showed a $\lambda_{max.}$ 280 mμ and $\varepsilon_{max.}$ 3830, which are in agreement with values reported by Roux (79) for (+)-catechin from Uncaria gambir.

Listed below are the physical constants for constituent G and (+)-catechin from U. gambir.

| | G | (+)-catechin |
|---|---|---|
| M.P. | 172-174°C | 172°C (Roux 79) |
| opt. rotation acetone:water 1:1 | $[\alpha]_D^{20}$ +17.0° | $[\alpha]_D$ + 17.1° (Freudenberg 28) |
| U.V. absorption | $\lambda_{max.}$ 280 mμ<br>$\varepsilon_{max.}$ 3830 | $\lambda_{max.}$ 280 mμ (Roux 79)<br>$\varepsilon_{max.}$ 3986 |
| penta-acetyl derivative M.P. | 131 - 132°C | 131°C |
| rotation $C_2H_2Cl_4$ | $[\alpha]_D^{20}$ +40.1° | $[\alpha]_D$ +40.6° (Freudenberg 28) |
| acetone | $[\alpha]_D^{23}$ +29.4° | $[\alpha]_D^{20}$ +31.9° (Roux 79) |

The isolation and identification of constituent G as (+)-catechin, (+)-5:7:3':4'-tetrahydroxyflavan-3-ol, confirms Roux's (50) chromatographic identification of this component in black wattle bark extracts.

Identification of constituent A.

The chromatographically pure microcrystals (30 mg) from fraction C-3 were examined by two-dimensional paper chromatography, $R_F$ 0.57 in n-B.A.W.; $R_F$ 0.59 in sec.-B.A.W. and $R_F$ 0.32 in 2% aqueous acetic acid. On paper chromatograms constituent A gave the following colour reactions with selective spray reagents: silver nitrate reagent - metallic black; ferric alum reagent - blue; benzidine reagent - claret-maroon; vanillin -toluene-p-sulphonic acid reagent - red; toluene-p-sulphonic acid reagent - no reaction. The substance was colourless in visible light and did not fluoresce under U.V. light. The negative reaction with toluene-p-sulphonic acid was confirmed by a negative leucoanthocyanidin reaction when the substance was heated with dilute mineral acid in alcoholic solution under pressure.

Alkali microfusion (KOH) of constituent A gave phloroglucinol and gallic acid as the only breakdown products, thus confirming the presence of phloroglucinol (A) and pyrogallol (B) nuclei as already indicated by the above colour reactions.

The melting point of the crystalline substance (crystallized from water) was 184°C. After drying at 110°C for two hours, Found: C, 55.8; H, 5.4. Calculated for $C_{15}H_{14}O_7$, $H_2O$: C, 55.6; and H, 5.0%. $\lambda_{max.}$ 271 mμ in

ethanol. $[\alpha]_D^{20}$+10.7 in acetone:water = 1:1 (c, 0.5).

Colour reactions with selective spray reagents and the results of alkali microdegradation indicate that constituent A contains a phloroglucinol A and a pyrogallol B nucleus, assuming the substance is a flavonoid body. The optical activity of the substance rules out the possibility of a flavone, flavonol or chalkone structure. Negative leuco-anthocyanidin reactions indicate that constituent A has no flavan-3:4-diol structure. A mixed melting point of the substance (m.p. 184°C) with (+)-gallocatechin from Casuarina equisitifolia (m.p. 183°C kindly supplied by Dr. Roux), showed no depression. Infrared absorption curves of component A and an authentic sample of (+)-gallocatechin were identical over the range 2.5 - 15 μ. Listed below are the physical constants of constituent A and of (+)-gallocatechin from the bark of Quercus sessiliflora, (Mayer and Bauni, 208):

| | Constituent A; | (+)-gallocatechin: |
|---|---|---|
| M.P. | 184°C | 185°C |
| opt. rotation (acetone:water= 1:1) | $[\alpha]_D^{21}$+10.7° | $[\alpha]_D$+ 14.7° |
| U.V. absorption | $\lambda_{max.}$ 271 mμ | $\lambda_{max.}$ 271 mμ |

The apparent identity of the substance, isolated from bark extracts of the black wattle tree, with (+)-gallocatechin confirms the tentative identification of (+)-gallocatechin in black wattle extracts by Roux (50).

## SECTION B

### The catechin constituents of green wattle bark.

Samples of damp, green wattle bark (covered with paraffin wax) from trees grown in Natal (S.A.) were kindly supplied by the Wattle Research Institute, Pietermaritzburg. Ethyl acetate extracts of these samples were run on two-way paper chromatograms with n.-butanol:acetic acid: water (6:1:2) for the first, and 2% aqueous acetic acid for the second direction. After spraying with selective spray reagents, these chromatograms showed the same pattern of constituents as chromatograms of black wattle bark extracts. Two dimensional paper chromatograms of both green- and black-wattle bark, when developed for the same time in the same solvent systems, were superimposable. To gain further proof for the identity of black- and green-wattle bark constituents, (+)-catechin, (-)-robinetinidol and (+)-gallocatechin were spotted together with green wattle bark extract onto the starting point of a two-way chromatogram. The areas of the suspected three catechins on the chromatogram of green wattle extract appeared to be greatly increased and intensified. From the above, it was assumed that the main catechin constituents in green wattle bark are (+)-catechin, (-)-robinetinidol and (+)-gallocatechin.

The similarity of bark constituents of the two

closely related wattle species, which, until recently, were regarded by some authorities (Williams 176) as varietal forms of the same species (*Acacia decurrens* Willd., var. *mollis*) is of interest.

SECTION C.

## The isolation and identification of catechins from silver wattle bark.

Two dimensional paper chromatograms of ethyl acetate extract of fresh or dry silver wattle bark (from trees grown in Natal and kindly supplied by the Wattle Research Institute, Pietermaritzburg, S.A.) show a similar pattern of constituents to black wattle bark extracts (Fig. 3 and 9). It was therefore surmised, that the mobile polyphenolic constituents of silver wattle bark are identical to those isolated from black wattle bark extracts, i.e. (-)-robinetinidol, (+)-catechin, (+)-gallocatechin and the constituents F, B and D. In addition to the above mentioned constituents, silver wattle bark contains a new constituent of $R_F$ 0.64 in n.-B.A.W. and $R_F$ 0.29 in water. This body shows identical colour reactions, and has identical $R_F$ values to (-)-epicatechin (from cacao beans), a sample of which had been kindly supplied by Dr. Forsyth of the Colonial Microbiological Research Laboratory, Trinidad.

Another constituent, present in very low concentration and which had not been identified in the barks of black- and green-wattle trees, had $R_F$ values of 0.39 in n.-B.A.W. and 0.24 in water. This body gave identical colour reactions and had identical $R_F$ values to (-)-epigallocatechin from golden wattle.

2.1 kg of dry silver wattle bark was ground in a Wiley mill, and the powdered material extracted in 5 litre flat-bottom flasks with commercial ethyl acetate for 5 days. The solvent was renewed every day and the combined extracts concentrated in a rotary evaporator under vacuum. The temperature of the water bath was kept below 70°C.

The weight of the combined dry extracts was 560 g. The extract was redissolved in 5 L ethyl acetate, containing 5% of methanol. Some material remained undissolved and was separated (90 g). The 470 g extract in solution were precipitated in batches of 100 ml with 100 ml chloroform, as described for black wattle. The united filtrates were concentrated under vacuum to give 56 g enriched low molecular weight fraction. The low molecular weight fraction was dissolved in methanol (700 ml) and the solution allowed to stand in the refrigerator for 2 hours. The bark waxes separated as light green precipitate. After filtration, the solution was streaked onto 140 thick paper sheets (Whatman no. 3) (400 mg extract per sheet) and the sheets developed in 2% aqueous acetic acid. The sheets were air dried, the bands marked as described for black wattle and the bands eluted with 70% ethanol. The united eluates of each band were evaporated under vacuum to give

the following quantities of dry extractives:

| | | |
|---|---|---|
| R band | $R_F$ 0.54 | 0.4 g |
| C band | $R_F$ 0.48 | 5.0 g |
| E band | $R_F$ 0.44 | 2.2 g |

Each band consisted of several constituents:

| | Main constituents: | Impurities: |
|---|---|---|
| R band | (-)-robinetinidol | (+)-catechin, F. |
| C band | (+)-catechin, (+)-gallocatechin, F,B,D. | (-)-robinetinidol. |
| | | (-)-epicatechin. |
| E band | (-)-epicatechin | (+)-catechin B,D. |
| | (+)-gallocatechin | and background trail. |

The material from each band was subjected to further fractionation in a partitioning solvent (n.-B,A.W.). The substances were dissolved in methanol and streaked onto washed Whatman no. 3 sheets at a concentration of 200 mg (5 ml) per sheet. After developing by the descending method for 12 hours, the sheets were dried in a current of air and the bands marked, cut and stripped, as described for black wattle.

40 mg pure (-)-robinetinidol crystallised from the eluates of the R band after most of the solvent had been evaporated.

The C band was divided into 7 fractions; C-1 to C-7:

| Fraction. | $R_F$ n.B.A.W. | Main component. | Impurities. |
|---|---|---|---|
| C-1 | 0.70 | (+)-catechin | (-)-robinetinidol |
| C-2 | 0.55 | (-)-epicatechin | (+)-catechin, (+)-gallocatechin |
| C-3 | 0.45 | (+)-gallocatechin | (-)-epicatechin, F,B |
| C-4 | 0.40 | (+)-gallocatechin | |
| C-5 | 0.34 | B | D, background trail |
| C-6 | 0.30 | B, D | " |
| C-7 | 0.24 | D | " |

The E band in a similar way was divided into 7 fractions: E-1 to E-7:

| | | | |
|---|---|---|---|
| E-1 | $R_F$ 0.7 | (+)-catechin | (-)-epicatechin |
| E-2 | 0.55 | (-)-epicatechin | (+)-catechin, (+)-gallocatechin |
| E-3 | 0.45 | (+)-gallocatechin | F,B, (-)-epicatechin |
| E-4 | 0.40 | (+)-gallocatechin | background trail |
| E-5 | 0.34 | B | " |
| E-6 | 0.30 | B,D | D, " |
| E-7 | 0.24 | D | " |

Fraction C-1 was re-run in 2% aqueous acetic acid. 800 mg of (+)-catechin crystallized from the eluates of the corresponding fraction after the ethanol had been removed under vacuum. The mother liquor contained traces of (-)-robineti-

nidol and (-)-epicatechin. Another 200 mg (+)-catechin crystallized on evaporation of fraction E-1.

Fraction C-2, comprising (-)-epicatechin, was contaminated with (+)-catechin and (+)-gallocatechin. The fraction was united with fraction E-2. The combined fractions C-2 and E-2 were rerun in both the water and partitioning direction, and the bands marked and stripped as before. The eluates were concentrated in a desiccator under vacuum over $CaCl_2$. After the ethanol and most of the water had been evaporated the remaining aqueous solution was seeded with (-)-epicatechin crystals from cacao beans, kindly supplied by Dr. Forsyth of the Colonial Microbiological Research Laboratory, Trinidad. On standing at room temperature and slow evaporation, microcrystals separated after 4 days. The flask was put in the refrigerator for 3 more days. After that period more crystals separated. They were filtered off, washed with a minimum of ice water and dried, yield 200 mg (-)-epicatechin.

The concentration of (+)-gallocatechin in fraction C-3 was low. The united fractions C-3 and E-3 were re-run in both the partitioning and water direction for further purification. The eluates of the nearly pure (+)-gallocatechin bands were concentrated in a desiccator under vacuum over $CaCl_2$ as described before. After the ethanol and most of

the water had been removed, the aqueous solution was seeded with (+)-gallocatechin from golden wattle bark. The solution was allowed to stand at room temperature in a desiccator over silica gel without vacuum. After 3 days microcrystals formed and the conical flask with the solution was stoppered and put in the refrigerator. After one week the crystallization was complete. The crystals were filtered, washed with a minimum of ice water and dried, yield 18 mg pure (+)-gallocatechin.

Identification of crystallized silver wattle bark constituents. (-)-Robinetinidol, (-)-7:3':4':5'-tetrahydroxyflavan-3-ol.

The crystalline material exhibited the same colour reactions and had identical rates of migration in different solvents on 2-way paper chromatograms, as authentic (-)-robinetinidol from black wattle. For example, when both substances were run on one chromatogram in two different solvent systems, they migrated as one discrete spot. The substance did not give a colour reaction with toluene-p-sulphonic acid, nor did it develop any pigment when heated with 3 N HCl and isopropylol (anthocyanidin reaction), thus eliminating the possibility of flavan-3:4-diol structure. The substance did not exhibit fluorescence under ultraviolet light. Alkali microfusion of the crys-

talline substance showed the presence of resorcinol, β-resorcylic acid and gallic acid as main degradation products.

The melting point of the chromatographically pure substance from silver wattle was 202°C. A mixed melting point (equal quantities of substances, crystallized together), with an authentic sample from black wattle, (m.p. 205°C) showed no depression; mixed m.p. 202°C. Found : C,56.72; H,5.47%; calculated for $C_{15}H_{14}O_6$, 1.5 $H_2O$: C,56.78; H,5.40%.

The U.V. absorption of (-)-robinetinidol from silver wattle was measured over the range 200 mμ - 300 mμ; $\lambda_{max.}$ 282.5mμ, $\varepsilon_{max.}$ 3,992; c = 3.7 mg substance in 100 ml ethanol. A second maxima exists at $\lambda_{max.}$ 202.3 mμ (solvent, water). The infrared absorption curves of (-)-robinetinidol from black and from silver wattle were identical over the range 2.5 - 15 μ. The optical rotation of the crystalline silver wattle constituent was $[\alpha]_D^{23}$ -10.9±0.5° in acetone:water = 1:1 (c, 1.4). From the above findings it can be deducted, that (-)-robinetinidol, crystallized from silver wattle, is identical with the substance from black wattle (compare p.102 )

(+)-Catechin, (+)-5:7:3':4'-tetrahydroxyflavan-3-ol.

The substance crystallized from bands C-1 and E-1 (1.0 g) was not pure and had to be recrystallized from a minimum of water. The recrystallized material (850 mg) had a melting point of 176°C and was shown pure by paper chromatography. A mixed melting point, with an authentic sample (black wattle (+)-catechin), showed no depression (m.p. 176°C).

(+)-Catechin from black- and silver-wattle barks, migrated as one discrete spot when run together on a two dimensional chromatogram, $R_F$ 0.75, n-B.A.W.; $R_F$ 0.67, sec-butanol and $R_F$ 0.35 in 2% aqueous acetic acid. With selective spray reagents (see p. 65 ) the substance from silver wattle shows identical colour reactions with (+)-catechin from black wattle.

Alkali fusion gave phloroglucinol and protocatechuic acid as main degradation products. No colour developed when the substance (4 mg) was heated with 3 N HCl in isopropylol (anthocyanidin reaction). The substance did not fluoresce under U.V. light. Found: C,59.18, H,5.58%; Calculated for $C_{15}H_{14}O_6$, $H_2O$: C,58.44, H,5.23%. $[\alpha]_D^{23}$ +16.97 ±0.1° in acetone:water = 1:1; (c = 2.28). $\lambda_{max.}$ 281 mμ (ethanol), $\varepsilon_{max.}$ 3838 and a second peak at $\lambda$ 207.5 mμ (water). Infrared absorption curves of silver wattle (+)-catechin and an authentic sample from black wattle bark were identical.

(+)-Catechin (500 mg) was acetylated with acetic anhydride in pyridine, giving 580 mg of the penta-acetyl derivative (m.p.131°). A mixed m.p., with an authentic sample from black wattle, (m.p.131°) showed no depression.

(-)-Epicatechin, (-)-epi-5:7:3':4'-tetrahydroxyflavan-3-ol.

The crystalline product from silver wattle (200 mg) and (-)-epicatechin from cacao beans had identical $R_F$ values ( 0.64 in n.-B.A.W.; 0.30 in water), and showed identical colour reactions on two-way paper chromatograms.

Alkali microfusion gave phloroglucinol and protocatechuic acid. A mixed m.p. of the substance from silver wattle (m.p.235°) and (-)-epicatechin from cacao beans (m.p.236°), gave a m.p. 235°. Found: C,58.87; H,5.08%. Calculated for $C_{15}H_{14}O_6$ $H_2O$: C,58.44; H,5.1 %. $[\alpha]_D^{23}$ -57.1±0.7° in acetone:water=1:1 (c,0.61), $\lambda_{max.}$ 281 mμ, $\varepsilon_{max.}$ 3886 (6.4 mg in 100 ml ethanol), $\lambda_{max.}$ 207.5 mμ (water). Infrared absorption curves of the component from silver wattle and (-)-epicatechin from cacao beans, were identical. For comparison of the physical constants see Tables VIII and XI.

(+)-Gallocatechin, (+)-5:7:3':4':5'-pentahydroxyflavan-3-ol.

(+)-Gallocatechin was crystallized in extremely low yield (18 mg) from silver wattle bark extracts. This may be due to the fact that the fractions rich in this compound (C-3 and E-3), are heavily contaminated with

neighbouring constituents, mainly body F, body B and (-)-epicatechin. Silver wattle appears to be rich in constituent F, which has only slightly different $R_F$ values to (+)-gallocatechin.

| | | | | | |
|---|---|---|---|---|---|
| (+)-gallocatechin | $R_F$ | 0.55 | n.-B.A.W. | $R_F$ | 0.32 in water |
| Constituent F | " | 0.57 | " | " | 0.35 " |
| (+)-catechin | " | 0.75 | " | " | 0.35 " |
| (-)-epicatechin | " | 0.64 | " | " | 0.30 " |

Crystalline (+)-gallocatechin from silver wattle, when run on two dimensional chromatograms and sprayed with various selective spray reagents, shows the same colour reactions as an authentic sample from Casuarina equisitifolia (Roux 79) and from black wattle. When run on a two dimensional chromatogram, the three substances migrate as one discrete spot. No colour developed when the substance from silver wattle (2 mg) was heated with 3 N HCl (1 ml) in isopropylol (4 ml) for one hour under pressure. The absence of a flavan-3:4-diol structure was further proved by a negative reaction with the toluene-p-sulphonic acid reagent. The substance did not fluorescence under U.V. light. The substance had a melting point of m.p. 185°C, a mixed melting point, with an authentic sample, showed no depression. $\lambda_{max.}$ 270.5 mμ; $\varepsilon_{max.}$ 1.760; c = 6.4 mg/100 ml ethanol. The optical rotation of the sub-

stance had to be measured immediately after crystallization due to rapid darkening on ageing; $[\alpha]_D^{23}$ +10.1±0.4°. (c, 1.31) in acetone:water = 1:1. The small quantity of gallocatechin available from silver wattle bark precluded the preparation of derivatives. The physical constants of the substance isolated from silver wattle bark and of authentic (+)-gallocatechin are listed in Table VIII.

TABLE VIII

Physical constants of catechin constituents from silver wattle bark.

| | m.p.(°C) | $\lambda_{max.}$ | $\varepsilon_{max.}$ | rotation |
|---|---|---|---|---|
| S.W. (-)-robinetinidol | 202 | 282 mμ | 3992 | $[\alpha]_D^{23}$ -10.9° acetone:water |
| B.W. " | 207 | 282 mμ | 3636 | $[\alpha]_D^{20}$ -10.7° acetone:water |
| S.W. (+)-catechin | 176 | 281 mμ | 3830 | $[\alpha]_D^{23}$ +16.97° acetone:water |
| B.W. " | 172-174 | 280 mμ | 3830 | $[\alpha]_D^{21}$ +17.0° acetone:water |
| S.W. (+)-catechin, penta-acetate | 131 | | | $[\alpha]_D^{23}$ 29.4° acetone |
| B.W. (+)-catechin, penta-acetate | 131-132 | | | $[\alpha]_D^{20}$ 28.4° acetone |
| S.W. (-)-epicatechin | 235 | 281 mμ | 3886 | $[\alpha]_D^{23}$ -57.1° acetone:water |
| Cacao bean " | 237 | | | $[\alpha]_D^{20}$ -58.0° acetone:water |
| S.W. (+)-gallo-catechin | 185 | 270.5 mμ | 1760 | $[\alpha]_D^{23}$ +10.1° acetone:water |
| Casuaria-equistifolia (+)-gallocatechin | 183 | 271.0 mμ | 1734 | $[\alpha]_D^{21}$ +13.1° acetone:water |

## SECTION D

## The isolation and identification of catechins and catechin gallates from golden wattle.

Two dimensional chromatograms of an ethyl acetate extract of dry golden wattle bark show a number of mobile constituents migrating as discrete spots (Figure 6). Many of these are absent from two dimensional chromatograms of black wattle bark extract. The similarity of the distribution of golden wattle components as well as their colour reactions, suggested their identity with green tea catechins (Figure 7).

Green tea catechins had previously been isolated by Bradfield and Penny (81, 82) in 1948 and very recently by Vuataz, Brandenberger and Egli (200), but were not available from these authors. The catechins were, accordingly, separated from green tea (Part II) to enable comparison with those from golden wattle (Part I).

### Part I.

700 g dry golden wattle bark from the Government Plantations at Eerste Rivier, Cape, and kindly supplied by the Department of Forestry, was powdered in a Wiley mill No. 1 and extracted 3 times with commercial ethyl acetate, at room temperature, in a 10 L flat bottomed flask. The solvent was renewed, and the extract evaporated under vacuum daily. The weight of the combined dry extractives was 230 g. The extract had a smell typical of wattle wax and had to be

freed of wax by extraction with chloroform in a Soxhlet. The wax-free extract was redissolved in an ethyl acetate: methanol (20:1) mixture to give a 10% tannin solution. The high molecular weight fraction was precipitated with chloroform (v/v = 1/1), as described for black wattle. The combined filtrates were evaporated to dryness, under vacuum, to give 32 g of low molecular weight fraction (yield 14%).

The dry low molecular weight fraction was redissolved in 400 ml. methanol and the solution allowed to stand in the refrigerator for 1 hour. A small quantity of light green waxy material separated and was filtered off. The solution was streaked onto 80 sheets of Whatman no. 3 (400 mg extract per sheet) and the sheets developed with 2% aqueous acetic acid, for 12 hours, by the ascending method. The bands were located under U.V. light and by spraying with the benzidine reagent. Some bands were not sharply demarcated, but the R and the C bands were clearly separated. (Table IXa). The bands were cut and eluted as described for black wattle. The eluates of each band were dried under vacuum in a rotary evaporator at 60°C.

TABLE IXa.

Colour reactions, $R_F$ values and yields of golden wattle bark fractions.

| Band: | $R_F$: | colour under U.V. light: | colour developed with benzidine: | weight: |
|---|---|---|---|---|
| R band | 0.56 | light blue | yellow | 0.2 g |
| | 0.54 | dark | | |
| C band | 0.50 | dark | ochre | 2.0 g |
| | 0.46 | light blue | pink | |
| E band | 0.42 | dark | ochre | 2.2 g |
| | 0.39 | light blue | | |
| G band I | 0.35 | dark | ochre | 2.8 g |
| G band II | 0.29 | dark | pink | 4.3 g |
| | 0.24 | light blue | orange | |

The bands are named after the constituent predominantly present in the band: R band for robinetinidol; C band for catechin; E band for epicatechin and G band for epigallocatechin gallate. The constituents present in each band are listed in Table IXb.

TABLE IXb.

Constituents present in golden wattle bark fractions.

| | Main constituents: | Minor constituents: |
|---|---|---|
| R band | (-)-robinetinidol | (+)-catechin, unknown polyphenols. |
| C band | (+)-catechin, (+)-gallocatechin, gallic acid. | (-)-epicatechin |
| E band | (-)-epicatechin, (+)-gallocatechin. | (-)-epicatechin gallate, (+)-catechin, (-)-epigallocatechin. |
| G band I | (-)-epicatechin gallate, (-)-epigallocatechin | (-)-epicatechin (-)-epigallocatechin gallate. (-)-epicatechin |
| G band II | (-)-epigallocatechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin. | |

The eluates of the 5 bands (Table IXb) were further fractionated in a partitioning mixture (n.-B.A.W.), at a concentration of 200 mg per Whatman no. 3 sheet (Table IXc).

TABLE IXc.

Fractionation of golden wattle bands.

R band

| | | | |
|---|---|---|---|
| FRACTION: | R-1 | (-)-robinetinidol | $R_F$ 0.70 (n.-B.A.W. Whatman no. 3), unknown phenolic bodies of $R_F$ 0.85, 0.54, 0.39. |

C band

| | | | |
|---|---|---|---|
| FRACTION: | C-1 | (+)-catechin, gallic acid. | $R_F$ 0.70 (n.-B.A.W.) |
| | C-2 | (-)-epicatechin | $R_F$ 0.55 |
| | C-3 | (+)-gallocatechin | $R_F$ 0.45 |
| | C-4 | (-)-epigallocatechin | $R_F$ 0.40 |

E band

| | | | |
|---|---|---|---|
| FRACTION: | E-0 | (-)-epicatechin gallate | $R_F$ 0.75 (n.-B.A.W.) |
| | E-1 | (+)-catechin | $R_F$ 0.70 |
| | E-2 | (-)-epicatechin | $R_F$ 0.55 |
| | E-3 | (+)-gallocatechin | $R_F$ 0.45 |
| | E-4 | (-)-epigallocatechin | $R_F$ 0.40 |

G band I

| | | | |
|---|---|---|---|
| FRACTION: | I-0 | (-)-epicatechin gallate | $R_F$ 0.75 |
| | I-1 | (-)-epigallocatechin gallate | $R_F$ 0.62 |
| | I-2 | (-)-epicatechin | $R_F$ 0.55 |
| | I-3 | | $R_F$ 0.45 |
| | I-4 | (-)-epigallocatechin | $R_F$ 0.40 |

G band II

| | | | |
|---|---|---|---|
| FRACTION: | II-0 | (-)-epicatechin gallate | $R_F$ 0.75. (n.-B.A.W.) |
| | II-1 | (-)-epigallocatechin gallate | $R_F$ 0.62 |
| | II-4 | (-)-epigallocatechin | $R_F$ 0.40 |

The combined eluates of each fraction were evaporated under vacuum at not more than 50°C. Two dimensional chromatograms of all fractions showed the presence of minor impurities. The eluates of each constituent, for example, (-)-epicatechin, E-2 and C-2; or (-)-epicatechin gallate, E-0 and I-0; had to be re-run in both the partitioning and the water direction. The repurified constituents crystallized on standing at room temperature, after the ethanol and most of the water had been evaporated.

The eluates of fraction C-1 crystallized on evaporation to give 850 mg of a white crystalline product. The crystals were sucked off and washed with a minimum of ice water. When these were run on a two dimensional paper chromatogram (n.-B.A.W. / 2% aqueous acetic acid), two spots separated and were identified as (+)-catechin and gallic acid. After re-running in the partitioning mixture on Whatman no. 3. (n.-B.A.W.), the corresponding bands were located under U.V.-light and with the benzidine reagent. The bands were cut and stripped with 70% ethanol. The eluates were evaporated under vacuum in a desiccator over $CaCl_2$ to

give 150 mg pure crystalline gallic acid and 600 mg pure, crystalline (+)-catechin. More (+)-catechin crystallized from the E 1 fraction (100 mg). The following table lists the yields and melting points of each constituent isolated and crystallized from golden wattle bark.

TABLE X.

Yields of crystallized golden wattle constituents.

| | Yield (mg): | m.p.; |
|---|---|---|
| (-)-robinetinidol | 32 | 204°C. |
| (+)-catechin | 700 | 177°C. |
| gallic acid | 150 | 253°C, decomp. |
| (-)-epicatechin | 80 | 236°C. |
| (+)-gallocatechin | 410 | 182-183°C. |
| (-)-epigallocatechin | 55 | 215°C. |
| (-)-epicatechin gallate | 135 | 258-255°C. |
| (-)-epigallocatechin gallate | 150 | 219-220°C. |

Identification of golden wattle constituents.

(-)-Robinetinidol, (-)-7:3':4':5'-tetrahydroxyflavan-3-ol.

The substance crystallized from water, m.p. 204°C, had an identical rate of migration on two-way paper chromatograms to (-)-robinetinidol from black wattle bark. Colour reactions with various selective spray reagents were identical with those given by (-)-robinetinidol, thus indicating a resorcinol A and a pyrogallol B nucleus. Micro-fusion with KOH yields resorcinol, β-resorcylic acid and gallic acid as main degradation products. The substance does not fluoresce under ultra-violet light. The absence of a flavan-3:4-diol grouping was established by a negative reaction with the toluene-p-sulphonic acid reagent, and a negative anthocyanidin reaction, on heating the substance with 3N HCl. A mixed melting point of the crystalline substance (m.p. 204°) with an authentic sample from black wattle (m.p. 207°), gave a m.p. of 204°C . The ultraviolet spectrum of the substance from golden wattle ( $\lambda_{max.}$ 282 mμ) was identical to that of (-)-robinetinidol from black wattle. The limited quantity of the substance isolated from golden wattle bark precluded the formation of its derivatives.

From the above findings, it can be assumed that the substance crystallized from golden wattle bark is

identical with (-)-robinetinidol from black- and silver wattle bark.

(+)-Catechin, (+)-5:7:3':4'-tetrahydroxyflavan-3-ol.

The substance crystallized from water with ease, 700 mg from fractions C-1 and E-1 (Tables IXc and X) m.p. 177°C . The substance had an identical rate of migration to (+)-catechin from Uncaria gambir on a two dimensional chromatogram (Whatman No. 1 paper, solvent system n.-B.A.W./ water). Colour reactions with selective spray reagents reflected the presence of a phloroglucinol A and a catechol B nucleus; see black wattle (+)-catechin . Micro-degradation with KOH yields phloroglucinol and protocatechuic acid as the main degradation products. The absence of a flavan-3:4-diol structure was proved by a negative reaction with toluene-p-sulphonic acid and a negative "leuco-anthocyanidin reaction". A mixed melting point with an authentic sample of (+)-catechin, showed no depression. Found: C,58.25; H,5.38%; Calculated for $C_{15}H_{14}O_6H_2O$: C,58.44; H,5.23%. The ultraviolet absorption curve was measured in ethanol, $\lambda_{max.}$ 281 mμ; $\varepsilon_{max.}$ 4080; c, 6.15/100 ml. . The infrared-absorption curve of the crystalline substance was identical to that of an authentic sample. $[\alpha]_D^{23}$ +16.8 ±0.4° in acetone: water = 1:1; (c, 2.29%).

Penta-acetyl-(+)-catechin.

120 mg. of the substance were acetylated with acetic anhydride in pyridine, as described for black wattle. Yield: 142.6 mg crystalline (from ethanol) penta-acetyl-(+)-catechin M.P. 131. $[\alpha]_D^{23}$ +28.4 ± 0.4° acetone, (c, 0.82%). Mixed melting points with authentic penta-acetyl-(+)-catechin from silver wattle and from black wattle, showed no depression.

Gallic acid, 3:4:5-trihydroxybenzoic acid.

The pure, pale-cream substance, recrystallized from water (150 mg), decomposed at 253°. A mixed melting point with an authentic sample of gallic acid, showed similar decomposition at 253°. The substance had $R_F$ values of $R_F$ 0.35 in 2% aqueous acetic acid and $R_F$ 0.67 in n.-B.A.W. on Whatman No. 1 paper. The colour reactions of the substance with selective spray reagents were identical to those of an authentic sample of gallic acid: Benzidine reagent - deep yellow; ferric reagent - blue; silver nitrate reagent - metallic black; toluene-p-sulphonic acid reagent - no reaction.

(-)-Epicatechin, (-)-epi-5:7:3':4'-tetrahydroxyflavan-3-ol.

As in silver wattle bark, (-)-epicatechin was only present in low concentration in golden wattle bark, and was difficult to separate from neighbouring (+)-catechin and

(+)-gallocatechin. After repeated chromatographic purification procedures (as previously described), pure white crystals (80 mg) separated from water, m.p. 236°. A mixed melting point with an authentic sample of (-)-epicatechin (from cacao beans) (m.p. 236°), showed no depression (m.p. 236°). (-)-Epicatechin from cacao beans and the crystalline material from golden wattle, migrated as one discrete spot when run together on a two dimensional chromatogram, using the solvent system n.-B.A.W./water. Colour reactions with selective spray reagents and microfusion with KOH confirmed the presence of a phloroglucinol A and a catechol B nucleus. Negative leuco-anthocyanidin tests showed the absence of a flavan-3:4-diol structure. The substance did not fluoresce under U.V. light, migrated in water, and was optically active, thus eliminating the possibility of a flavonol structure. The ultraviolet absorption curve reflected $\lambda_{max}$ 280 mμ, $\varepsilon_{max}$ 4008 (c, 6.73 mg in 100 ml ethanol). Infrared-absorption curves of the substance crystallized from golden wattle and of an authentic sample of (-)-epicatechin, showed similarity over the range 2.5 - 15 μ. Found: C, 56.53; H, 5.19%. Calculated for $C_{15}H_{14}O_6$ 1.5 $H_2O$: C, 56.78, H, 5.40%. $[\alpha]_D^{23}$ -63.2±0.5° in ethanol (c, 0.44) and $[\alpha]_D^{23}$ -57.4±0.5° in acetone:water= 1:1; (c, 0.61). For comparison, the physical constants of (-)-epicatechin from three different sources are listed in Table XI.

TABLE XI.

Physical constants of (-)-epicatechin isolated from:

| | cacao beans (Forsyth 216): | silver wattle bark: | golden wattle bark: |
|---|---|---|---|
| m.p. | 237°C | 235°C | 235°C |
| rotation (acetone: water 1:1) | $[\alpha]_D$ -58° (c, 2.00) | $[\alpha]_D^{23}$ -57.1° (c, 0.61) | $[\alpha]_D^{23}$ -57.4 (c, 0.61) |
| $\lambda_{max}$ | * 280 mμ ethanol | 281 mμ ethanol | 280 mμ ethanol |
| $\varepsilon_{max}$ | * 3.580 | 3.886 | 4.008 |

* values reported by Vuataz (200) for (-)-epicatechin from green tea.

(+)-gallocatechin (+)-5:7:3':4':5'-pentahydroxyflavan-3-ol.

The pure substance (410 mg) crystallized with relative ease after preparative paper chromatographic separations. The substance gave identical colour reactions to (+)-gallocatechin from black wattle bark and from Casuarina equisitifolia (Roux 79). Microdegradation reflected the presence of phloroglucinol and gallic acid, thus confirming the presence of a phloroglucinol A and a pyrogallol B nucleus. The substance from golden wattle bark had identical rates of migration on two dimensional paper chromatograms (solvent system n.-B.A.W./water, Whatman No. 1 paper) to (+)-gallocatechin from black wattle bark, and an authentic sample from Casuarina equisitifolia, which was kindly supplied by Dr. Roux. The ultraviolet absorption curve had a $\lambda_{max.}$ 270.5 mu and $\varepsilon_{max.}$ 1.820 (3.9 mg in 25 ml ethanol).

Infrared absorption curves of both (+)-gallocatechin from black wattle bark and the substance from golden wattle bark, were identical over the range 2.5 - 15 μ. Found: C,52.19; H, 4.80%. Calculated for $C_{15}H_{14}O_7$ 2 $H_2O$: C, 52.63; H, 5.30%. $[\alpha]_D^{23}$ +10.1±0.3° in acetone;water = 1:1; (c, 1.33%).

Hexa-acetyl-(+)-gallocatechin.

The crystalline hexa-acetate (116 mg from ethanol), m.p. 141, was prepared by acetylation of 120 mg substance with acetic anhydride (1 ml) in pyridine (0.7 ml). $[\alpha]_D^{23}$ +28.6 ±0.4° in acetone (c, 0.61). For comparison, the physical constants of (+)-gallocatechin from three different sources are listed in Table XII.

TABLE XII.

Physical constants of (+)-gallocatechin from:

| | Casuarina equisitifolia (Roux 79) | oak and sweet chestnut (Mayer 208) | golden wattle |
|---|---|---|---|
| M.P. | 183°C | 185-188°C | 182-183°C |
| rotation (acetone: water) (1:1) | $[\alpha]_D^{20}$ +13.1° | $[\alpha]_D$ +14.7° | $[\alpha]_D^{23}$ +10.1° |
| $\lambda_{max}$ | 271 mμ | - | 270.5 mμ |
| $\epsilon_{max}$ | 1.734 | - | 1.820 |
| Hexa-acetyl-(+)-gallocatechin. | | | |
| M.P. | 143°C | 142-143°C | 141°C |
| rotation (acetone) | $[\alpha]_D^{20}$ + 30.0° | $[\alpha]_D$ +31.0° | $[\alpha]_D^{23}$ +28.6° |

(-)-Epigallocatechin, (-)-epi-5:7:3':4':5'-pentahydroxy-flavan-3-ol.

This substance has not been isolated from other wattle species. The golden wattle constituent and (-)-epigallocatechin from green tea had identical rates of migration on two-dimensional paper chromatograms ($R_F$ 0.39 in n.-B.A.W. and $R_F$ 0.24 in water) and gave identical colour reactions when sprayed with selective spray reagents: Ferric alum reagent - blue; benzidine reagent - claret-maroon; silver nitrate reagent - deep black; vanillin - toluene-p-sulphonic acid reagent - violet red; toluene-p-sulphonic acid reagent - no reaction. On microdegradation, (KOH fusion) both substances gave the same breakdown products, namely, gallic acid and phloroglucinol. These findings suggested the presence of phloroglucinol (A) and pyrogallol (B) nuclei. The negative leuco-anthocyanidin reaction with toluene-p-sulphonic acid was confirmed by a negative reaction on heating the substance with dilute mineral acid in alcoholic solution, under pressure. The substance did not fluoresce under U.V. light and showed optical activity (see below) and was therefore no flavonol or flavon.

The melting point of the pure substance (55 mg) was 215°C. A mixed melting point with an authentic sample

from green tea (m.p. 214 - 215°C), showed no depression (m.p. 215°C). The ultraviolet absorption curve had a $\lambda_{max.}$ 270.5 mμ and $\varepsilon_{max.}$ 1786 (concentration 3.3 mg/25 ml ethanol). Infrared absorption curves of the crystalline golden wattle constituent and of (-)-epigallocatechin from green tea, were identical over the range 2.5 - 15 μ, Found: C, 53.77; H, 4.99%; calculated for $C_{15}H_{14}O_7$, 1.5 $H_2O$; C, 54.05; H, 5.14%. $[\alpha]_D^{23}$ -58.7±0.5° in ethanol (c, 0.48%) and $[\alpha]_D^{23}$ -65±1.4° in acetone:water (1:1).

Hexa-acetyl-(-)-epigallocatechin.

The hexaacetate of the substance [acetylation as described for (+)-gallocatechin] crystallized from ethanol, m.p. 195°C. $[\alpha]_D^{23}$ -16.4±0.3° in acetone (c, 0.72).

Table XIII compares the physical constants of (-)-epigallocatechin reported in the literature (Bradfield et al. 82, 83, Vuataz et al. 200) with values found for the golden wattle substance.

TABLE XIII.
Physical constants of (-)-epigallocatechin from:

| | green tea: | golden wattle bark: |
|---|---|---|
| M.P. | 212 - 215°C* 217°C‡ | 215°C |
| Rotation (ethanol) | $[\alpha]_D^{23}$ -59.5±2.0° | $[\alpha]_D^{23}$ -58.7±0.5° |
| $\lambda_{max.}$ | 271 mμ * | 270.5 mμ |
| $\varepsilon_{max.}$ | 1.340 * 1.450 | 1.786 |

* values reported by Bradfield et al. (82, 83)
‡ values reported by Vuataz et al. (200).

(-)-Epicatechin gallate, (-)-epi-5:7:3':4'-tetrahydroxyflavan-3-galloylester.

This substance was absent in other wattle species. After repeated preparative chromatographic separations, it crystallized from less than 2 ml water in white crystals, m.p. 257-258°C, (135 mg). A mixed melting point with (-)-epicatechin gallate from green tea (m.p. 254°C), showed almost no depression (m.p. 254 - 255°C). When run on two-dimensional chromatograms, the substance had identical $R_F$ values to (-)-epicatechin gallate from green tea; $R_F$ 0.84 in n-B.A.W. and $R_F$ 0.22 in 2% aqueous acetic acid. When sprayed with different spray reagents, the substance gave the following colour reactions (on paper chromatograms): benzidine reagent - claret maroon; ferric alum reagent - blue; silver nitrate reagent - brown black; vanillin-toluene-p-sulphonic acid reagent - red; toluene-p-sulphonic acid reagent - no reaction. The substance did not fluoresce under U.V. light.

Microfusion with KOH yielded phloroglucinol, protocatechuic acid and gallic acid in approximately equal concentrations as main degradation products. No colour developed when the substance was heated with 3 N HCl and iso-propylol for one hour under pressure.

The above findings indicated that the substance contains phloroglucinol, pyrogallol and catechol nuclei in

almost equal proportion. Identical colour reactions and the same rate of migration on two-dimensional chromatograms indicated the apparent identity of the substance and (-)-epicatechin gallate from green tea. Combustion analysis of the golden wattle substance; Found: C, 58.98, H, 4.32%; calculated for $C_{22}H_{18}O_{10}$ C, 58.54: H, 4.24%. $[\alpha]_D^{23}$ -181±0.1° in ethanol (c, 0.28%) and $[\alpha]_D^{23}$ -222.2±3.0° in acetone: water (1:1) (c, 0.61%). $\lambda_{max.}$ 280 mu; $\varepsilon_{max.}$ 13.570 (c, 6.34 mg in 500 ml ethanol).

The substance gave a heavy white precipitate with the gelatine salt reagent and thus proved to be a tannin.

Hepta-acetyl-(-)-epicatechin gallate.

The crystalline substance from golden wattle (123mg) was acetylated with acetic anhydride (0.7 ml) in pyridine (0.5 ml) as described for silver wattle (+)-catechin. The hepta-acetate crystallized from ethanol in white needles (130 mg m.p. 118.5°C). $[\alpha]_D^{23}$ -86.7±0.3° in acetone (c, 0.76%).

Table XIV compares the physical constants of (-)-epicatechin gallate reported in the literature. Bradfield et al. (82) and Vuataz et al. (200) with values found for the golden wattle substance.

TABLE XIV.

Physical constants of (-)-epicatechin gallate from:

| | green tea: | golden wattle bark: |
|---|---|---|
| M.P. | 253° ‡<br>252 -254° * | 257° |
| rotation (ethanol) | $[\alpha]_D$-190°, (c, 0.25) * | $[\alpha]_D^{23}$-181±0.1° (c, 0.28%) |
| $\lambda_{max.}$ (ethanol) | 280 mμ*<br>279 mμ ‡ | 280 mμ |
| $\epsilon_{max.}$ | 13 600 *<br>14 000 ‡ | 13 570 |
| heptaacetyl (-)-epicatechin gallate | | |
| m.p. | 119.5-120.5° * | 118.5° |
| rotation | $[\alpha]_D$-96°,(c, 0.56) * (in benzene) | $[\alpha]_D^{23}$-86.7±0.3° (c, 0.76%) (in acetone) |

* values by Bradfield et al. (82)

‡ values by Vuataz et al. (200)

Action of tannase on (-)-epicatechin gallate.

For hydrolysis, (-)-epicatechin gallate (10 mg) was dissolved in 2 ml of water, and one ml of the enzymic solution (10 mg enzyme in 1 ml water) was added. One drop of toluene was added and the mixture kept at 40°C for one day. The hydrolysate was investigated by two dimensional paper chromatography. Chromatograms were sprayed with selective spray reagents, showing the presence of 3 spots corresponding to (-)-epicatechin, gallic acid and the parent compound, (-)-epicatechin gallate.

When the hydrolysis is allowed to continue for two to three days (at 45°C) the spot corresponding to (-)-epicatechin gallate disappears completely while the concentration of the other two spots is increased.

(-)-Epigallocatechin gallate, (-)-epi-5:7:3':4':5'-pentahydroxyflavan-3-galloyl ester.

This substance could not be identified in other wattle species. Repeated chromatographic purification procedures of the combined fractions I-1 and II-1, afforded white crystals after the ethanol and most of the water had been removed from the eluates of the final purification step. The solution was allowed to stand 3 days at room temperature (with very slow evaporation of the aqueous solvent), and crystallization was continued for 4 days at 1°C in a refrigerator.

The crystals were sucked off, washed with a minimum of iced water and dried (140 mg, m.p. 220°C.) A mixed melting point with (-)-epigallocatechin gallate from green tea (m.p. 220°) showed no depression (m.p. 219-220°). The pure substance when dissolved in water (10 mg in 1 ml) precipitates gelatine and is therefore a tannin..

The substance from golden wattle had an identical rate of migration on two dimensional paper chromatograms ($R_F$ 0.71 in n-B.A.W.: $R_F$ 0.22 in 2% aqueous acetic acid), and gave identical colour reactions to (-)-epigallocatechin gallate from green tea: benzidine reagent - claret-maroon; silver nitrate reagent - metallic black; ferric alum reagent - blue; vanillin -toluene-p-sulphonic acid reagent - red. The negative reaction with the toluene-p-sulphonic acid reagent was confirmed by a negative leucoanthocyanidin reaction on heating the substance with dilute mineral acid in alcoholic solution under pressure. The substance did not fluoresce under U.V. light, migrated in water on paper chromatograms and was optically active (see below), thus indicating that it had not a flavonol or flavone structure. Alkali microfusion gave phloroglucinol and gallic acid as the main degradation products.

The ultraviolet absorption curve exhibits two maxima: $\lambda_{max.}$ 276 mμ, $\varepsilon_{max.}$ 9330 and $\lambda_{max.}$ 279.5 mμ

$\varepsilon_{max.}$ 9350 (concentration 4 mg/100 ml in ethanol). The double peak for (-)-epigallocatechin gallate was previously reported by Bradfield and Penny (118) for the substance crystallized from green tea $\lambda_{max}$ 279.5 mμ $\varepsilon_{max.}$ 9250; $\lambda_{max.}$ 275 mμ $\varepsilon_{max.}$ 9500. The galloyl catechins show much higher $\lambda_{max}$ values, a result to be expected from the introduction of the powerful galloyl chromophore. The infrared absorption curves of the substance isolated from golden wattle and of (-)-epigallocatechin gallate from green tea, show similarity over the range 2.5 - 15 μ. A mixed melting point of the two substances (both m.p. of 220°) showed no depression (decomposition at 221°).

The optical rotation of the substance from golden wattle was $[\alpha]_D^{23}$ -177 ± 0.7° in ethanol (c, 0.29) and $[\alpha]_D^{23}$ -220.5 ± 1.60° in acetone:water (1:1) (c, 0.27). Found: C, 55.16; H, 4.76 calculated for $C_{22}H_{18}O_{11}$, $H_2O$: C, 55.46; H, 4.23%.

Octaacetyl (-)-epigallocatechin gallate.

The substance from golden wattle bark (118 mg) was acetylated with acetic anhydride (0.6 ml) in pyridine, (0.5 ml) as described for silver wattle (+)-catechin. After two recrystallizations from ethanol, 95.5 mg octaacetate m.p. 119.5 - 120.5° were obtained. $[\alpha]_D^{23}$ -84.4 ± 0.3° in acetone (c, 0.84%).

Table XV compares the physical constants of (-)-epigallocatechin gallate reported in the literature (82, 200) with values found for the golden wattle substance.

TABLE XV.

Physical constants of (-)-epigallocatechin gallate from:

| | green tea | golden wattle bark |
|---|---|---|
| M.P. | 215 - 216° * <br> 218° ‡ | 220° |
| Rotation (in ethanol) | $[\alpha]_D$ -179° (c, 0.28) * | $[\alpha]_D^{23}$ -177° (c, 0.29%) |
| $\lambda_{max.}$ | 275 mμ * <br> 279.5 mμ* | 276 mμ <br> 279.5 mμ |
| $\varepsilon_{max.}$ | 9.500 * <br> 9.250 * | 9.330 <br> 9.350 |
| Octaacetyl (-)-epigallocatechin gallate | | |
| M.P. | not reported | 119.5 - 120.5° |
| Rotation ( in acetone) | not reported | $[\alpha]_D^{23}$ -84.4±0.3° (c, 0.84%) |

* values reported by Bradfield et al. (82)
‡ values reported by Vuataz et al. (200)

Action of tannase on (-)-epigallocatechin gallate.

10 mg of the golden wattle substance were hydrolysed with tannase as described for (-)-epicatechin gallate. The hydrolysate was examined on two-dimensional chromatograms and sprayed with different spray reagents. After hydrolysis for 24 hours, 3 main spots could be detected corresponding to

gallic acid, (-)-epigallocatechin and (-)-epigallocatechin gallate. After 3 days, gallic acid and (-)-epigallocatechin were the only products remaining.

The above confirms that the substance isolated from golden wattle bark is identical to (-)-epigallocatechin gallate from green tea.

PART II

The separation of catechins and catechin gallates from unfermented green tea.

400 g dry, green tea leaves from East Africa (Dr. T. Eden, Kericho) were extracted with 1 litre methanol for three days with daily renewal of the solvent. The liquid extract was dried in a rotary evaporator under vacuum, the temperature of the water bath being kept at 70°C. The combined methanol extractives (49g) contained high percentages of chlorophyll and waxes. The methanol extractives were extracted in a Soxhlet for 8 hours with chloroform to give 40 g wax- and chlorophyll-free polyphenols, and a mixture (9 g) of chlorophyll and wax.

The polyphenolic fraction when examined on a two-dimensional chromatogram (solvent system n.-B.A.W./water), reflected the presence of a number of constituents (Fig. 13). These could be recognised when compared with paper chromatograms published by Roberts and Wood (84, 215). The benzidine reagent showed the presence of six claret maroon spots, corresponding to (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate. The wax-free polyphenolic fraction was dissolved in 500 ml

methanol and streaked on 100 sheets Whatman no. 3 at a concentration of 400 mg (5 ml) per sheet. The preparative chromatograms were developed in 2% aqueous acetic acid for 12 hours by the ascending method.

The bands were marked and cut as described for golden wattle; the eluates were evaporated under vacuum.

Yields of eluted material per band:

| | | |
|---|---|---|
| C band | $R_F$ 0.47 | 2.55 g |
| E band | $R_F$ 0.42 | 5.10 g |
| Gallate band I | $R_F$ 0.35 | 5.80 g |
| Gallate band II | $R_F$ 0.29 | 6.30 g |
| | | 19.75 g |

49.5% of the starting material.

Only the E band, gallate band I and gallate band II were fractionated in order to isolate (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate, as (+)-catechin, (-)-epicatechin and (+)-gallocatechin were already available for reference. The solids eluted from these bands, were redissolved in methanol and streaked on washed sheets (Whatman no. 3), at concentrations of 900 mg for 8 sheets, for further fractionation in n.-B.A.W. by the descending method.

Yields of crude uncrystallized material were:

| | | |
|---|---|---|
| E band | (-)-epigallocatechin | 0.4 g |
| Gallate band I | (-)-epicatechin gallate | 1.1 g |
| | (-)-epigallocatechin gallate | 1.1 g |
| | (-)-epigallocatechin | 1.0 g |
| Gallate band II | (-)-epicatechin gallate | 1.1 g |
| | (-)-epigallocatechin gallate | 3.0 g |

The corresponding fractions were further purified by means of preparative paper chromatographic operations. The substances crystallized from the final eluates after the ethanol and most of the water had been removed under vacuum. The final yields after recrystallization from water were:

| | | |
|---|---|---|
| (-)-epigallocatechin | 225 mg | m.p. 214 - 215°C |
| (-)-epicatechin gallate | 115 mg | m.p. 254 - 255°C |
| (-)-epigallocatechin gallate | 120 mg | m.p. 220°C |

## SECTION E.

### The quantitative estimation of flavonoid compounds on paper chromatograms by the maximum colour density method.

The quantitative estimation of polyphenols which are resolved on one- or two- dimensional paper chromatograms has not yet been reported except for work published from this thesis (Roux and Maihs 27,57,59,62,186). For other organic substances, however, three estimation methods are known:

1) Quantitative estimation of substances by the area method of Fisher (217,218).

This method is based on the phenomenon that the spot area of a round or ovoid spot increases with the logarithm of the spot content. This relationship holds for a wide range of concentrations, but only approximate results can be obtained. Inaccuracies are introduced by the fact that the spot area is not only dependant on the concentration, but also varies with the developing time of the paper chromatogram. To obtain accurate results with the above method the spots must be clearly separated and have distinctive boundaries. The spots, which polyphenolic substances (in tannin mixtures) produce on two-way paper chromatograms, very seldom fulfil these requirements. The method was therefore found unsuitable for the estimation of constituents in wattle extracts.

2) Estimation of material eluted from spots.

This method is widely used for the estimation of carbohydrates (Flood 221, Fischer & Dörfel 231, Wallenfels 232) but is not suitable for polyphenols because the recovery of flavonoid substances from cellulose is never quantitative (Roux & Evelyn 55). Substantial losses apparently occur due to the residual affinity of flavonoid compounds for cellulose, and due to atmospheric oxidation of these very sensitive bodies on elution. The second difficulty is the fact that these substances are colourless, (except for the anthocyanidins), most of them do not fluoresce under ultraviolet light (except flavonols), and usually, when sprayed with reagents to form pigments, such pigments cannot be eluted.

3). The determination of spot intensity (colour density) of pigmented spots by means of visual comparison (Arden, 222), or photoelectric measurement (Wallenfels 223, Block 224,225, Grassman 226,227), was found to give rapid and accurate results also in the polyphenolic field.

Principles of photometric measurement of coloured spots.

The substances to be measured must either be a pigment, or form a pigment with relative ease. The amount of pigment formed must be proportional to the concentration

of the substance and must be relatively stable at room temperature. The increase of colour density should be proportional to the concentration of the substance over a relatively wide range of concentrations. Generally Lambert-Beer's law may be applied not only for solutions, but also for coloured foils, as was shown by Grassmann and Hannig (226,227) for transparent electropherograms of amino acids. If the paper chromatograms to be measured have not been made transparent prior to quantitative photometric estimations, the reduction of the incident light is not only due to absorption, but mainly due to scattering. This will lead to deviations from Lambert-Beer's law, (Block 228). A similar effect will take place if the spots on the chromatograms are measured by the reflectance method (King and White 229). It is practically impossible to fit these light scattering effects into a mathematical formula, or to correct them with a mathematical correction factor (226). For practical estimations agreement with de Beer's law is not necessary, if empirically found values are used for the preparation of calibration curves. The calibration curves should in no instance be extrapolated beyond actual experimental findings. If measured values, for unknown substances, are not within the standard range, new chromatograms of the substance to be estimated should be run at a higher or lower concentration respectively.

The light absorption of a spot may be measured in two ways. The chromatogram may be cut in strips and pulled

slowly across a beam of light (slit). The galvanometer deflection is read at mm intervals and plotted against the mm distance the filter paper had been moved.

The area on the graph is afterwards integrated with a planimeter and plotted against the concentration of each spot. This method is only applicable for one-dimensional chromatograms where all the spots are in one line. The above method is of less value for two-dimensional chromatograms, where the spots are scattered over the chromatogram.

For two-dimensional chromatograms it is preferable to measure the colour density of the whole spot. This can be done by fitting the densitometer with apertures of different sizes and diameters instead of a slit. The spots should obviously not be bigger, nor much smaller than the instrument aperture. Deviations in both directions will result in values which are too low. For accurate readings the ratio instrument aperture/spot size should be approximately 1 for reasons explained in detail by Grassmann and Hannig (226,227).

Chromatographic methods.

The effective resolution, of the flavonoid substances on two-dimensional chromatograms, is necessary for accurate quantitative estimations by the maximum colour density method.

Paper. Improved resolution was obtained by using the largest size of paper convenient for running in two directions without unnecessarily long irrigation times. Whatman no. 1 paper size 16 x 16 inches was used throughout. The substances were applied in spots of $\frac{1}{2}$ inch diameter, at a point in one corner, 1 inch from each edge.

Chromatography tanks. Six chromatograms were developed simultaneously in an 20 x 20 x $18\frac{1}{2}$ inches all glass tank as described before.

Solvents. The solvents used for quantitative operations were n-butanol: acetic acid: water = 6:1:2 for the first, and 2% acetic acid for the second direction. After each run the chromatograms were dried in a current of air at room temperature.

Time of development. The chromatograms were run approximately 20 hours in the first and 5 hours in the second direction.

Standard solutions. 10 mg flavonoid substance was dissolved in 10 ml pure absolute ethanol. 10 $\mu$l quantities were applied with a micropipette (10 $\mu$l capacity, graduated in 10 subdivisions; "Elphor", Bender and Hobein, Munich) until the desired concentration of the substance was obtained. Tannin solutions were made up ten times the concentration used for single flavonoids. Standard solutions of lower molecular phenols, phloroglucinol for example, which give stronger colour reactions than catechins (compare Fig.12), were made up one tenth of the strength of catechin solutions.

Spray reagents. Two spray reagents, ammoniacal silver nitrate and bisdiazotised benzidine, were found to give straight line relationships between log concentrations of the flavonoid compounds, and the galvanometer deflections, over a sufficiently wide range to allow accurate measurements. Constituents which are well separated on the paper chromatograms may be sprayed with either the silver nitrate reagent or the bisdiazotised benzidine reagent. Where, however, two constituents overlap (e.g. gallic acid and (+)-catechin) the silver nitrate reagent proved to be unsuitable. The interference of overlapping substances may be partially overcome using the more selective bisdiazotised benzidine reagent. This reagent produces claret-maroon with phloroglucinol-containing catechins, and yellow with resorcinol-containing flavonoid substances or with gallic acid. With the aid of a yellow green filter the colour density of the yellow substances may be depressed, and the density of the claret maroon sufficiently increased to allow an accurate estimation of the latter.

The densitometer. A densitometer (diagramatically illustrated in Figure 8) was built to measure the maximum colour density of spots on two dimensional chromatograms. A sliding mask with holes of different shapes and sizes, was used instead of a slit, which is the conventional form of aperture for densitometers (Extinktions - Schreiber II, Zeiss; Elphor densitometer, Bender and Hobein, Munich). The different

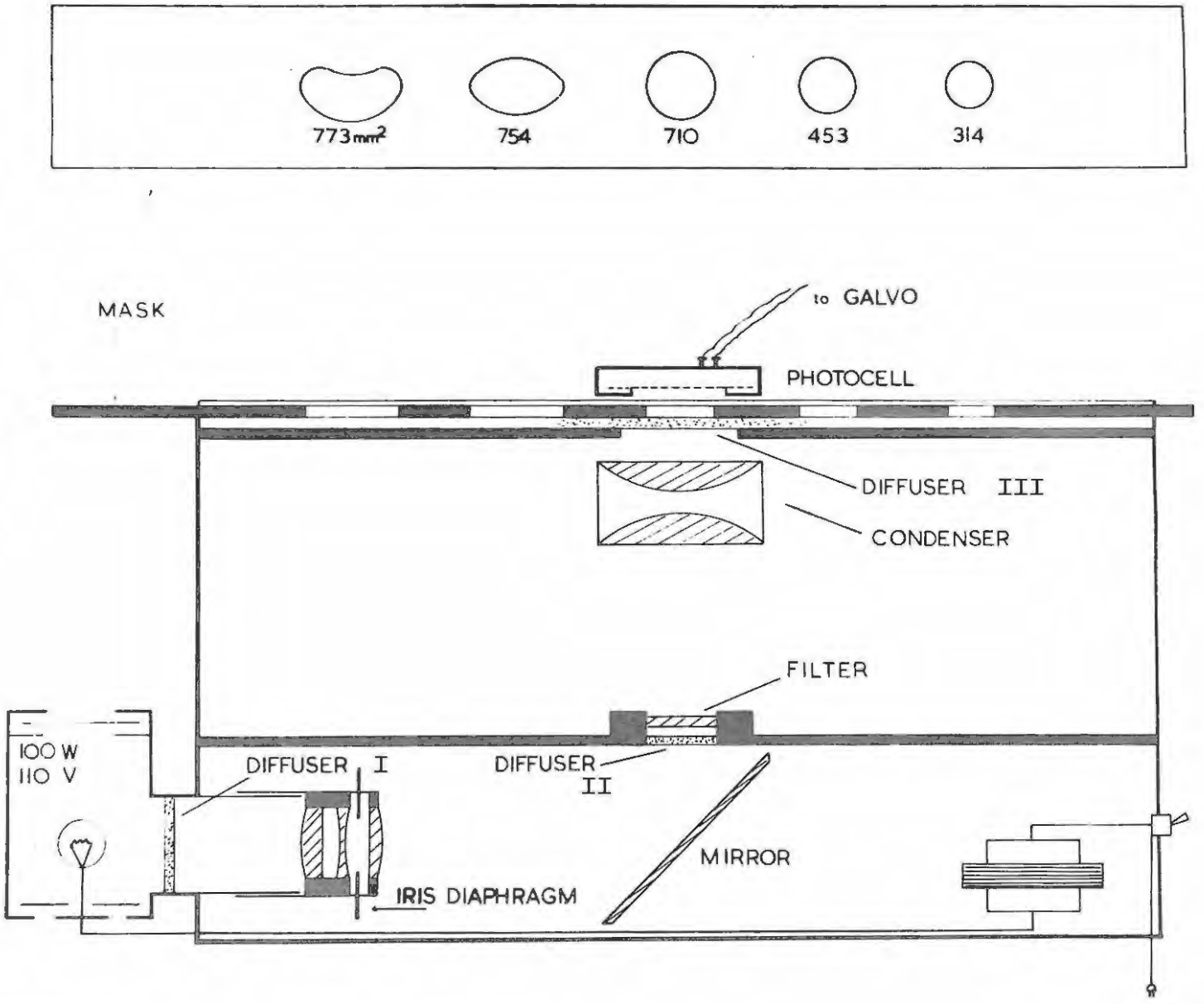
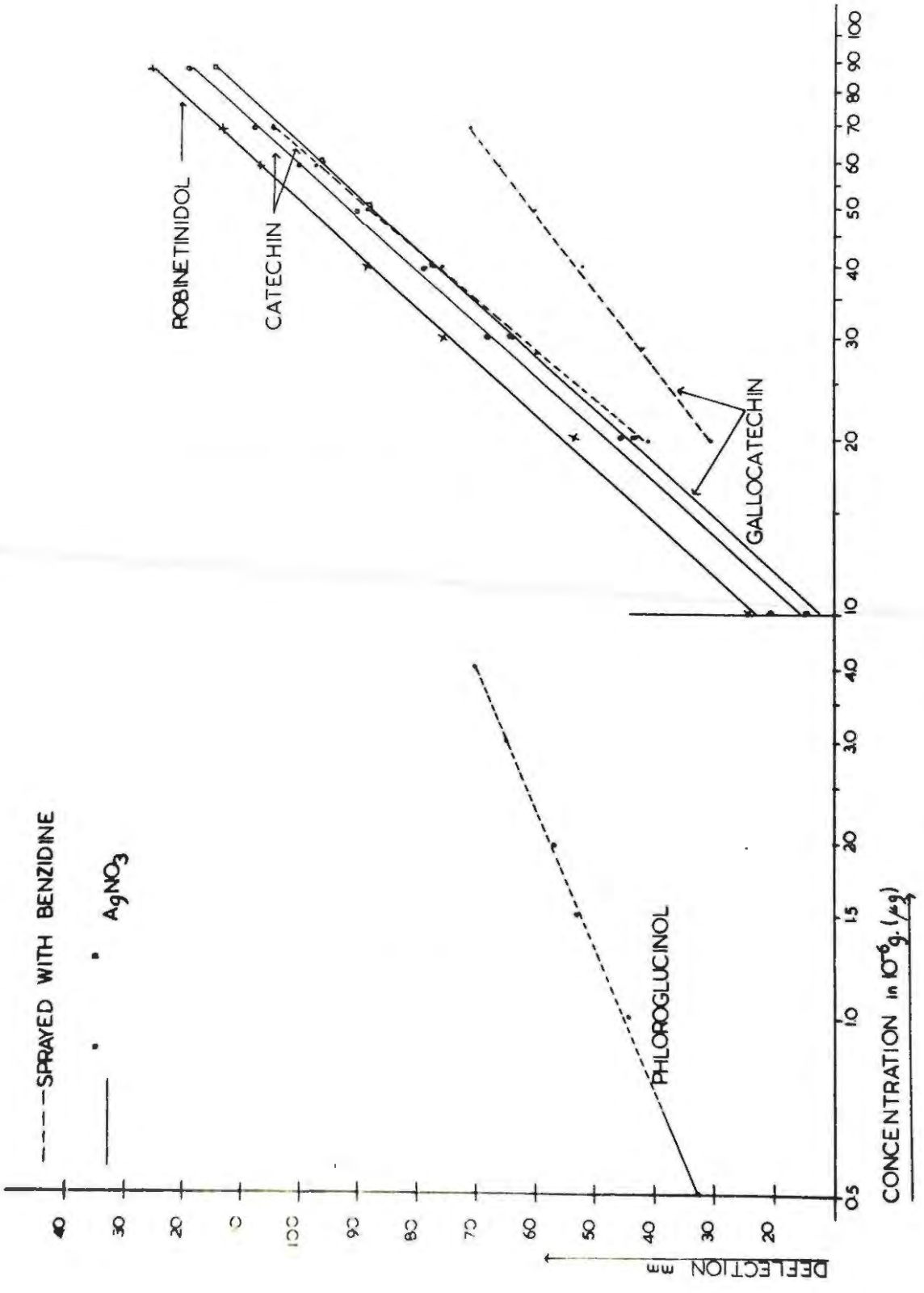



Fig. 8. DENSITOMETER

Fig. 9. CALIBRATION CURVES



sizes and shapes of the holes (=instrument aperture) allows for the measurement of spots of different shapes. The densitometer consists of a wooden box of the dimensions 16 x 9 x 9 inches, mounted on rubber feet and closed with a heavy glass lid at the top. The light source, a microscope illumination lamp (Leitz, Wetzler; 100 watt, 110 volt) is attached outside to allow effective loss of heat. The intensity of the light source is controlled with a rheostat, for course adjustment (setting the voltage to correct 110 volt), and with an iris diaphragm for fine adjustment. An optical system for the control of the light intensity was preferred to an electrical one, because it does not alter the spectrum of the light when the intensity is changed. This is important when coloured spots (benzidine reagent) are measured. The iris diaphragm is opened or shut with a handle on the right side of the instrument. The light, after passing the optical system, is reflected with a mirror on to a diffuser (II). A filter cupboard is fixed above the diffuser (II). A grey filter may be used for additional control of the light intensity, while coloured filters may be used to increase, or reduce, the colour density of pigmented spots. In focal distance of diffuser (II) two condenser lenses are attached to assure even illumination of another diffuser (III) below the aperture of the instrument. The light intensity is measured with a selenium

barrier layer photocell ("Eel" 37 x 50 mm) which is bigger than the biggest aperture of the sliding mask. The two terminals mounted at the rear of the photocell, are coupled directly with a sensitive galvanometer (Pye Scalamp galvanometer 7891/S). The photocell is mounted in a holder which is a lid of the size 5 x 7 inches. The holder is attached to the top of the instrument with hinges. The lid (photocell holder) holds the paper chromatogram in position, and prevents stray light reaching the photocell.

Preparation of calibration curves.

Two dimensional chromatograms, of increasing concentration were run of each substance to be estimated. The chromatograms were run in the system n-B.A.W./water and were sprayed with either silver nitrate,(-)-robinetinidol, (+)-catechin, (+)-gallocatechin , or the benzidine reagent, phloroglucinol, (+)-catechin, (+)-gallocatechin . The four substances, (-)-robinetinidol, (+)-catechin, (+)-gallocatechin and phloroglucinol, are well separated and may, therefore, be run simultaneously on one chromatogram. Chromatograms, five at each concentration, were run over concentrations ranging from 0.5 to 4.0 µg for phloroglucinol and from 10 to 80 µg for the catechins. The chromatograms were run, sprayed and washed under standardized conditions. One aperture was selected for the preparation of a calibration

curve and for subsequent estimations. Apertures of different shapes, but of the same total area may be used for one calibration curve. Other than shown in Fig. 12, the sliding mask should preferably have apertures of standard area, but of different shapes.

Measurement of colour density.

The maximum colour density of each spot was determined by placing a blank part of the paper chromatogram between the aperture and the photocell and to set the instrument to 100% transmission by opening or closing the iris diaphragm. The spot to be measured was placed on top of the aperture, resulting in a deflection (in mm) of the galvanometer. The average of replicate analyses at each concentration of flavonoid compound was plotted against log concentration on semi-logarithmic graph paper. Straight-line relationships between log concentrations and galvanometer readings were obtained over the range 20 to 80 µg for catechins and 0.5 to 4.0 µg for phloroglucinol (Fig. 9).

The following Tables XVI and XVII list the galvanometer deflections (in mm), obtained from paper chromatograms of flavonoid substances and of phloroglucinol sprayed with the ammoniacal silver nitrate and the bisdiazotised benzidine reagent respectively. The corresponding curves are shown in Fig. 9 (calibration curves).

TABLE XVI.

Galvanometer deflections in mm for different concentrations of flavanols sprayed with the silver nitrate reagent.

(-)robinetinidol

| substance in µg: | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 |
|---|---|---|---|---|---|---|---|---|
| deflections: | 24 | 55 | 80 | 89 | 100 | 110 | 112 | 116 |
| | 24 | 56 | 80 | 94 | 102 | 102 | 112 | 117 |
| | 27 | 53 | 76 | 91 | 98 | 105 | 118 | 118 |
| | 24 | 51 | 72 | 85 | 92 | 112 | 110.5 | 123 |
| | 25 | 50 | 71 | 87 | 98 | 108 | 118 | 126 |
| mean values : | 24.8 | 53 | 75.8 | 89 | 98 | 107.4 | 114.1 | 120 |

(+)-catechin

| substance in µg: | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 |
|---|---|---|---|---|---|---|---|---|
| deflections: | 23 | 44 | 67 | 77 | 90 | 103 | 108 | 119 |
| | 21 | 41 | 69 | 76 | 86 | 101 | 105 | 118 |
| | 21 | 46 | 73 | 81 | 86 | 103 | 113 | 123 |
| | 18 | 49 | 66 | 73 | 93 | 100 | 110 | 122.5 |
| | 17 | 45 | 66 | 77 | 96 | 98 | 109 | 120 |
| mean values : | 21 | 45.0 | 68.2 | 76.8 | 90.2 | 101 | 109 | 120.5 |

(+)-gallocatechin

| substance in µg: | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 |
|---|---|---|---|---|---|---|---|---|
| deflections: | 14 | 44 | 64 | 82 | 82 | 97 | 105 | 111 |
| | 14 | 41 | 65 | 94 | 94 | 96 | 109 | 114 |
| | 16 | 44 | 67 | 95 | 95 | 99 | 109 | 106 |
| | 17 | 45 | 60 | 83 | 83 | 101 | 100 | 108 |
| | 14 | 41 | 65 | 90 | 90 | 99 | 102 | 113.5 |
| mean values : | 15 | 43.0 | 64.2 | 79 | 88.8 | 98.4 | 105 | 110.5 |

TABLE XVII.

Galvanometer deflections in mm for different concentrations of two catechins and of phloroglucinol. Chromatograms were sprayed with the bisdiazotised benzidine reagent.

(+)-catechin

| substance in µg: | 20 | 30 | 40 | 50 | 60 | 70 |
|---|---|---|---|---|---|---|
| deflections: | 41.0 | 64.0 | 77.0 | 92.0 | 95.5 | 104.0 |
| | 40.0 | 62.0 | 79.0 | 91.0 | 96.5 | 105.0 |
| | 42.0 | 59.0 | 74.0 | 95.0 | 99.5 | 106.5 |
| | 43.0 | 58.5 | 74.0 | 82.0 | 96.0 | 108.0 |
| | 39.0 | 58.0 | 77.0 | 86.0 | 95.0 | 103.0 |
| mean values: | 41.0 | 60.3 | 76.2 | 89.2 | 96.5 | 105.1 |

(+)-gallocatechins.

| substance in µg: | 20 | 30 | 40 | 50 | 60 | 70 |
|---|---|---|---|---|---|---|
| deflections: | 32.0 | 43.0 | 51.0 | 51.0 | 67.0 | 71 |
| | 33.0 | 46.0 | 53.5 | 61.0 | 63.0 | 72 |
| | 31.0 | 41.5 | 51.0 | 63.0 | 66.5 | 70 |
| | 30.0 | 42.5 | 52.0 | 62.5 | 69.0 | 74 |
| | 30.0 | 39.5 | 53.0 | 55.0 | 67.0 | 70 |
| mean values: | 31.0 | 42.5 | 52.1 | 60.5 | 66.5 | 71.4 |

phloroglucinol

| substance in µg: | 0.5 | 1.0 | 1.5 | 2.0 | 3.0 | 4.0 |
|---|---|---|---|---|---|---|
| deflections: | 32.5 | 42.0 | 50.0 | 59.0 | 66.5 | 73.0 |
| | 34.0 | 43.0 | 50.0 | 57.0 | 67.0 | 72.0 |
| | 33.0 | 47.0 | 54.0 | 54.0 | 63.0 | 68.0 |
| | 33.0 | 45.0 | 59.0 | 58.0 | 64.0 | 69.0 |
| mean values: | 33.1 | 44.25 | 53.25 | 57.0 | 65.1 | 70.6 |

## SECTION F.

### A comparative study of the concentration of catechin constituents in the bark extracts of various wattle species.

Bark samples for this study were collected from trees grown in Grahamstown (black wattle), or were kindly supplied by the Wattle Research Institute, Pietermaritzburg (black wattle on shale, black wattle on dolorite, green wattle on shale, black x green hybrid, silver wattle) and by the Department of Forestry (golden wattle). The samples were exhaustively extracted with methanol (six extractions of powdered or chipped bark of 24 hr. each at room temperature).

Paper chromatograms for the quantitative estimation of flavonoid constituents were run in triplicate at concentrations ranging from 2 to 6 mg bark extractives per sheet. Additional standard curves were prepared for the estimation of (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate. The concentration of individual catechins and catechin gallates was estimated at different concentrations using the ammoniacal silver nitrate and the bisdiazotised benzidine reagent where possible. The concentration of (-)-robinetinidol was, however, exclusively determined on chromatograms sprayed with ammoniacal silver nitrate.

Values obtained from chromatograms sprayed with

the silver nitrate reagent were generally higher than those obtained from chromatograms sprayed with the bisdiazotised benzidine reagent (Tables XVIII and XIX). The differences were small for well separated constituents, (+)-catechin for example, but were high where constituents with similar reducing properties overlap, e.g. (+)-gallocatechin, constituent F and constituent $A_1$. Where components giving a pale yellow colour with the bisdiazotised benzidine reagent (gallic acid, constituents F and $A_1$ and others, at low concentration) are in close proximity or overlap the ochre colourations of catechins of the "phloroglucinol series", e.g. (+)-catechin, (+)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin and (-)-epicatechin gallate, the maximum colour density of the latter may be determined by using a yellow green filter (Leitz, yellow green, factor 5).

Visual comparison of the colour density of (+)-gallocatechin on the chromatograms used for the preparation of the calibration curves, showed that the estimated values for (+)-gallocatechin (Tables XVIII and XIV) were too high, even when estimated on chromatograms sprayed with the bis-diazotised benzidine reagent. It was therefore preferred to estimate this component by visual comparison rather than by the maximum colour density method.

The following tables list the concentrations of individual catechin constituents in various wattle barks.

TABLE XVII.

Concentration of catechins in the bark of black wattle trees grown in Grahamstown.

The values are expressed as percentage of dry methanol extractives. The chromatograms were sprayed with the silver nitrate reagent.

| | | (-)-robinetin-idol | (+)-catechin | (+)-gallocatechin |
|---|---|---|---|---|
| Sample: | 1 | 0.67 | 1.06 | 2.83 |
| | 2 | 0.72 | 1.02 | 2.81 |
| | 3 | 0.75 | 1.07 | 2.88 |
| | 4 | 0.82 | 1.10 | 2.85 |
| | mean | 0.72 | 1.06 | 2.84 |

TABLE XIX.

Concentration of catechins in the bark of black wattle trees grown in Grahamstown.

The values are expressed as percentage of dry methanol extractives. The chromatograms were sprayed with bisdiazotised benzidine.

| | | (-)-robinetin-idol | (+)-catechin | (+)-gallocatechin |
|---|---|---|---|---|
| Sample | 1 | not estimated | 1.21 | 1.83 |
| | 2 | " | 1.22 | 1.75 |
| | 3 | " | 1.18 | 1.77 |
| | 4 | " | 1.18 | 1.84 |
| | mean | | 1.20 | 1.79 |

by visual comparison 0.93

TABLE XX.
Concentration of catechins in the barks of black, black x green hybrids and green wattle trees grown in Natal.

Values are expressed as percetanges of dry methanol extract.

| Sample | No. | % (+)-catechin | % (-)-robinetinidol | % (+)-gallo-catechin |
|---|---|---|---|---|
| Black wattle on shale | 1 | 0.65 | 0.21 | 0.5 * |
| | 2 | 0.6 | 0.2 | 0.5 |
| | 3 | 0.6 | 0.25 | 0.55 |
| | 4 | 0.62 | 0.2 | 0.6 |
| | 5 | 0.6 | 0.3 | 0.55 |
| | 6 | 0.51 | 0.25 | 0.45 |
| | 7 | 0.5 | 0.21 | 0.45 |
| | 8 | 0.5 | 0.24 | 0.5 |
| | 9 | 0.6 | 0.21 | 0.6 |
| | 10 | 0.47 | 0.21 | 0.8 |
| | | 0.57 | 0.23 | 0.55 |
| Black wattle on dolorite | 21 | 0.6 | 0.12 | 0.5 * |
| | 22 | 0.5 | 0.10 | 0.7 |
| | 23 | 0.5 | 0.23 | 0.7 |
| | 24 | 0.45 | 0.20 | 0.6 |
| | 25 | 0.5 | 0.24 | 0.6 |
| | | 0.51 | 0.18 | 0.62 |
| Hybrid | 11 | 0.85 | 0.12 | 0.5 * |
| | 12 | 1.3 | 0.16 | 1.1 |
| | 13 | 1.25 | 0.17 | 1.05 |
| | 14 | 1.06 | 0.18 | 0.9 |
| | 15 | 1.3 | 0.25 | 1.2 |
| | | 1.15 | 0.18 | 0.93 |
| Green wattle on shale | 16 | 1.5 | 0.14 | 0.8 * |
| | 17 | 1.15 | 0.08 | 1.0 |
| | 18 | 1.2 | 0.11 | 0.8 |
| | 19 | 1.1 | 0.14 | 0.9 |
| | 20 | 1.15 | 0.10 | 0.9 |
| | | 1.22 | 0.11 | 0.81 |

* The values for (+)-gallocatechin are estimated by visual comparison of chromatograms sprayed with the benzidine reagent. The values for (+)-catechin are mean values obtained from chromatograms sprayed with the benzidine, and the silver nitrate reagent, respectively.

TABLE XXI.

Concentration of catechin constituents in the bark of silver wattle trees grown in Natal.

The values are expressed as percentage of dry methanol extractives.

Group A. Bark samples "rich" in (-)-epicatechin.

| | (-)-epicatechin | (+)-catechin | (-)-robinetinidol | (+)-gallocatechin |
|---|---|---|---|---|
| Sample: 1 | 0.60 | 0.60 | 0.07 | not estimated |
| 2 | 0.57 | 0.64 | 0.11 | " |
| 3 | 0.25 | 0.65 | 0.07 | " |
| 4 | 0.44 | 0.58 | 0.07 | " |
| 5 | 0.65 | 0.55 | 0.10 | " |
| mean | 0.50 | 0.60 | 0.08 | |

Group B. Bark samples "poor" in (-)-epicatechin.

| | | | | |
|---|---|---|---|---|
| Sample: 1 | 0.15 | 0.64 | not estimated | 0.06 |
| 2 | 0.11 | 0.60 | " | 0.04 |
| 3 | 0.09 | 0.66 | " | 0.06 |
| 4 | 0.14 | 0.61 | " | 0.09 |
| 5 | 0.13 | 0.50 | " | 0.05 |
| mean | 0.12 | 0.60 | | 0.06 |

Note: Values for (+)-gallocatechin are estimated by visual comparison of paper chromatograms sprayed with the bisdiazotised benzidine reagent.

TABLE XXII.

Concentration of catechins in the bark of golden wattle trees grown in the Cape Province.

The values are expressed as percentage of dry methanol extractives.

| | Sample: 1 | 2 | mean value |
|---|---|---|---|
| (+)-catechin | 0.71 | 0.59 | 0.65 |
| (-)-epicatechin | 0.49 | 0.41 | 0.45 |
| (-)-epicatechin gallate | 0.48 | 0.52 | 0.50 |
| (+)-gallocatechin | 0.42 | 0.38 | 0.40 |
| (-)-epigallocatechin | 0.20 | 0.21 | 0.20 |
| (-)-epigallocatechin gallate | 0.30 | 0.35 | 0.33 |
| (-)-robinetinidol | 0.05 | 0.05 | 0.05 |

Note: Values for (+)-gallocatechin are estimated by visual comparison of paper chromatograms sprayed with the bisdiazotised benzidine reagent. The concentration of (-)-robinetinidol in the methanol extract was too low to allow for its estimation. This component was therefore estimated in an enriched golden wattle fraction (see section D) and the value corrected accordingly. The value may be taken as an approximate figure.

TABLE XXIII.

Average concentrations of catechins in bark extracts of black, green, black x green hybrid, silver and golden wattles.

The values are expressed as percentage of dry methanol extractives. -, Absent.

| | (-)-robinetinidol | (+)-catechin | (-)-epicatechin | (-)-epicatechin gallate | (+)-gallocatechin * | (-)-epigallocatechin | (-)-epigallocatechin gallate |
|---|---|---|---|---|---|---|---|
| Species: | | | | | | | |
| Black wattle on shale, Natal | 0.23 | 0.57 | - | - | 0.55 | - | - |
| Black wattle on dolorite, Natal | 0.18 | 0.51 | - | - | 0.62 | - | - |
| Black wattle Grahamstown | 0.72 | 1.13 | - | - | 0.93 | - | - |
| Green wattle on shale, Natal | 0.11 | 1.22 | - | - | 0.81 | - | - |
| Black x green hybrids, Natal | 0.18 | 1.15 | - | - | 0.93 | - | - |
| Silver wattle** Natal | 0.08 | 0.60 | 0.50 | - | not estimated | trace | - |
| Silver wattle** | not estimated | 0.60 | 0.12 | - | 0.06 | trace | - |
| Golden wattle Cape Province | 0.05 | 0.65 | 0.45 | 0.50 | 0.40 | 0.20 | 0.33 |

* Values estimated by visual comparison. ** Silver wattles have barks both "rich" and "poor" in (-)-epicatechin.

Comparison of yields of isolated catechin constituents.

Individual catechin constituents in the bark extracts of the four Acacia species were usually found to constitute less than 1% each of the total extractives. In spite of this low concentration, in some instances, up to 46% of an individual constituent was recovered. In the following tables, the yields ( = isolated constituents expressed as percentages of dry ethyl acetate extractives ) and the recoveries ( = isolated constituents expressed as percentages of estimated constituents) are compared.

TABLE XXIV.

Yields of catechins isolated from the ethyl acetate extract of fresh black wattle bark from trees grown in Grahamstown.

Abbreviations: % est. catechin, % catechin estimated in dry methanol extractives; % yield, weight of isolated constituent expressed as percentage of dry ethyl acetate extractives; recovery, isolated constituent expressed as percentage of estimated constituent.

| | % est. catechin | weight of isolated catechin | % yield | % recovery |
|---|---|---|---|---|
| (-)-robinetinidol | 0.80 | 2400 mg | 0.33 | 41.0 |
| (+)-catechin | 1.40 | 830 mg | 0.12 | 8.6 |
| (+)-gallocatechin | 1.10 | 30 mg | 0.004 | 0.4 |
| Total individually | 3.02 | | | |

TABLE XXV.

Yields of catechins isolated from the ethyl acetate extract of dry silver wattle bark.

Abbreviations: see previous table.

| | % est. catechin | weight of isolated catechin | % yield | % recovery |
|---|---|---|---|---|
| (-)-robinetinidol | 0.08 | 30 mg | 0.007 | 9 |
| (+)-catechin | 0.60 | 1000 mg | 0.18 | 30 |
| (-)-epicatechin | 0.12 | 200 mg | 0.04 | 30 |
| (+)-gallocatechin | 0.05 | 18 mg | 0.004 | 8 |
| Total individually | 0.85 | | | |

TABLE XXVI

Yields of catechins isolated from the ethyl acetate extract of dry golden wattle bark.

Abbreviations: see TABLE XXIV.

| | % est. catechin | weight of isolated catechin | % yield | % recovery |
|---|---|---|---|---|
| (+)-catechin | 0.65 | 700 mg | 0.3 | 46 |
| (-)-epicatechin | 0.45 | 80 mg | 0.04 | 9 |
| (-)-epicatechin | 0.50 | 135 mg | 0.06 | 12 |
| (+)-gallocatechin | 0.40 | 410 mg | 0.18 | 45 |
| (-)-epigallocatechin | 0.20 | 55 mg | 0.025 | 12 |
| (-)-epigallocatechin gallate | 0.33 | 150 mg | 0.065 | 20 |
| (-)-robinetinidol | 0.05 | 32 mg | 0.02 | 40 |
| Total individually | 2.58 | | | |

Discussion of the distribution of catechin constituents in the barks of black, black x green hybrid, green, silver and golden wattle trees.

Quantitative estimation of polyphenolic substances on two-way paper chromatograms supplied for the first time, means for an accurate comparative study of the catechin constituents in the barks of the four wattle species.

The concentration of individual catechin constituents was generally found to show small tree to tree variations within species. In this respect silver wattle, the bark of which could be divided into two categories, those "rich" and those "poor" in (-)-epicatechin, was an exception.

Taxonomic significance may possibly be attached to an apparent correlation between the distribution of catechin constituents in the bark of the four wattle species, and the order of their closeness to black wattle in botanical character. Thus, the concentration of (-)-robinetinidol, which appears to be the characteristic component of these acacia barks progressively decreased in the sequence black, black x green hybrid, green, silver and golden wattle (0.23%, 0.18%, 0.11%, 0.08% and 0.05% respectively). This finding appears to parallel a correlation observed previously between the total number of catechins of the "phloroglucinol series" and accepted systematics. A. decurrens which is closest to A. mearnsii was found to have an identical com-

position of catechins to black wattle, with those of the "phloroglucinol series", (+)-catechin and (+)-gallocatechin, present in higher concentration. The bark of A. dealbata was found to contain two additional catechins of this series, (-)-epicatechin and (-)-epigallocatechin, while a total of six catechin constituents of the "phloroglucinol series" were isolated from the bark of A. pycnantha: (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (+)-gallocatechin (-)-epigallocatechin and (-)-epigallocatechin gallate.

It has been shown previously by Erdtman (182) that the subgenera of coniferous plants may be differentiated by the examination of the wood components. It now appears that a differentiation between species may be possible by the examination of their bark components.

Of special interest in view of the chemical taxonomy of plants is the bark of black wattle trees grown in Grahamstown. The bark of these trees show a generally higher concentration of individual catechins when compared with the bark of authentic black wattle trees grown in Natal (Table XXI). Thus (+)-catechin is present in an average concentration of 1.0-1.2% compared with 0.51 - 0.57%; (-)-robinetinidol in an average concentration of 0.33 - 0.70% compared with 0.18 and 0.23%; and (+)-gallocatechin in an average concentration of 0.76 - 1.1% compared with 0.55 -

0.62% for black wattle trees grown in Grahamstown and in Natal respectively. The average concentrations of (+)-catechin and (+)-gallocatechin in black wattle trees grown in Grahamstown are almost identical with the concentrations found in green wattle trees and green x black wattle hybrids: (+)-catechin, 1.22%, 1.15%, 1.1% and (+)-gallocatechin, 0.81%, 0.93% and 0.93% for green wattle, green x black hybrids and black wattle trees respectively. This apparent similarity may indicate hybridization of Grahamstown black wattle trees with green wattle. In those barks examined the higher concentration of (-)-robinetinidol in the barks of black wattle trees grown in Grahamstown can however, not be explained.

In spite of the low total concentration of catechins in the extracts of black, silver and golden wattle (2.3%, 0.85% and 2.58% respectively), some were recovered in remarkably high yields. Thus, more than 40% of the total (-)-robinetinidol present was isolated from the bark extracts of black and golden wattle trees in spite of the fact that this component represented only 0.8% and 0.05% of the extracts respectively. The recovery of (+)-catechin and (+)-gallocatechin from golden wattle was substantially higher than from black and silver wattles, probably due to less interference from neighbouring substances. Subsequently, the isolation of the above compounds from golden wattle was

much simpler. (-)-Epicatechin was isolated in good yield from silver wattle (200 mg = 30%) and in lower yield from golden wattle. (80 mg = 8%). From the extracts of golden wattle bark, more than 10% each of the total (-)-epicatechin gallate, (-)-gallocatechin and (-)-epigallocatechin gallate present, were isolated. In view of the strong interference from highly polymerised tannins, as reflected by two-way paper chromatograms, these yields are remarkably good.

## CHAPTER 6.

## THE ISOLATION AND ATTEMPTED IDENTIFICATION OF TWO LEUCO-ANTHOCYANIDIN TANNINS FROM THE BARK OF ACACIA MEARNSII.

### Isolation of constituents D and B from the extracts of fresh black wattle bark.

On spraying a two-way paper chromatogram of an enriched low molecular weight fraction of black wattle bark extract (Fig. 3) with toluene-p-sulphonic acid, a number of individual constituents show up as orange (constituents B and D) or orange red (constituents C and E) spots. It is therefore considered likely that these constituents are leuco-anthocyanidins.

Two of these constituents, D and B, were present in high concentration in black wattle fractions obtained previously (Chapter 4, Section A). The C band fractions C-4 to C-7 (9.9 g) were refractionated on cellulose sheets (Whatman no. 3) with sec.-B.A.W., at a concentration of 100 mg per sheet. After developing (12 h.) the sheets were dried, the bands marked under U.V. light and after spraying with the benzidine reagent cut and eluted as described before.

The following fractions were obtained.

| Fraction | $R_F$ | Predominent Constituents | Yield |
|---|---|---|---|
| C-3 | 0.62 | A, F | trace |
| C-5 | 0.50 | B | 1.6 g |
| C-7 | 0.34 | D | 1.1 g |

Fraction C-5 (1.6 g) when run on a two dimensional chromatogram occupied the same place as constituent B. When a small amount of substance B (60mg) was run together with 5 mg of C band on a two dimensional chromatogram the intensity of component B appeared considerably increased after spraying with ammoniacal silver nitrate. The crystallization of constituent B has not been achieved. The material (constituent D) eluted from band C-7 (1.1g) was still contaminated with traces of constituent B and had to be refractionated in sec.-B.A.W. at a concentration of 100 mg per sheet (Whatman no. 3). After air drying a dark bank ($R_F$ 0.3) serving as a marker, was visible under U.V. light. A strip (2 cm wide) was cut from the wider side of each sheet and sprayed with the silver nitrate reagent showing a deep black band at $R_F$ 0.34. The zone of highest concentration ($R_F$ 0.32 - 0.36) was carefully cut and eluted. The combined eluates were shown by two dimensional paper chromatography (solvent system sec.-B.A.W./water) to contain a small proportion of low $R_F$ impurities and were, therefore, rerun in 2% aqueous acetic acid. The combined eluates were dried in a desiccator under vacuum over $CaCl_2$ (700 mg cream coloured amorphous material). When examined by two dimensional paper chromatography the isolated material (constituent D) appeared pure.

Attempted identification of constituent D.

The amorphous, cream-coloured material (700 mg) from the fractions C-6 and C-7 did not crystallize from water, methanol or ethanol. When run on two way chromatograms, the chromatographically pure and homogeneous substance had $R_F$ values of 0.34 in sec.-B.A.W., 0.29 in n.-B.A.W. and 0.33 in 2% aqueous acetic acid (Whatman no. 1). The low $R_F$ of the substance, in partitioning solvents, indicatesthat the substance may be polymolecular by comparison with the monomolecular flavonoid bodies hitherto isolated. The substance developed the following colours when chromatograms were sprayed with selective spray reagents: silver nitrate reagent - black; ferric alum reagent - blue; benzidine reagent - deep yellow; vanillin-toluene-p-sulphonic acid reagent - deep yellow; toluene-p-sulphonic acid reagent - orange. These colour reactions suggest the presence of resorcinol (A) and pyrogallol (B) nuclei. The positive leuco-anthocyanidin reaction with toluene-p-sulphonic acid was confirmed by the formation of two pigments on heating the substance with mineral acid in alcoholic solution, under pressure.

Alkali fusion of chromatographically pure constituent D gave gallic acid, $\beta$-resorcylic acid and resorcinol as main, and phloroglucinol as minor degradation products. Under the conditions of the leuco- antho-

cyanidin reaction resorcinol, $\beta$-resorcylic acid, gallic acid, robinetinidin chloride and the orange pigment are the main degradation products. Constituent D (10 mg) was dissolved in 5 ml water, 2 mg tannase was added, and the reaction mixture kept at 40°C for two days in a stoppered flask. Two-way paper chromatography showed that the orange pigment was the only enzymatic breakdown product. * footnote.

The amorphous substance (D) was dried under vacuum at 110° for 2 hours. Found C, 58.22; H, 5.26%; $C_{15}H_{14}O_7$ requires C, 58.82; H, 4.61%. $[\alpha]_D^{23}$ -269±1.2° in methanol (c, 0.48%). The U.V.-absorption spectrum gave $\lambda_{max.}$ 280 mμ; $E_{1cm}^{1\%}$ 86 (5.08 mg in 100 ml ethanol). The infra-red-absorption spectrum of constituent D shows a general similarity with that of (+)-leuco-robinetinidin over the range of 2.5 to 15 μ (Fig. 5), having strong bands at 3450 (-OH), 1610, 1530, 1500 and 1450 (aromatic -C=C-), 1105 and a shoulder at 1120 ( aryl-alkyl-ether), and at 1020 $cm^{-1}$ (sec.-aliphatic OH). Constituent D precipitates gelatine-salt reagent and is therefore a tannin.

Methylated constituent D.

Pure constituent D (245mg) was methylated with diazomethane by the method described by Roux and Evelyn (56). Yield after two precipitations, 165 mg of light

* Under identical conditions, monomeric leuco-robinetinidin did not generate any orange pigment.

yellow amorphous product. The methyl derivative was dried under vacuum for 2 hours at 110°. Found: C, 63.9; H, 6.1; OCH, 35.0%. $C_{19}H_{22}O_7$ requires C, 63.0; H, 6.1; $OCH_3$, 34.3%. $[\alpha]_D^{23}$-103±0.7° in acetone (c, 0.32%). The molecular weight of the methylated constituent D was estimated by the ebulliometric method of Evelyn (164-166) and reflected a value of 795 (calculated for $OCH_3$ 34.3% the M.W. of constituent D is 670).

Acetylated D.

Pure constituent D (200 mg) was acetylated with acetic anhydride and pyridine, as described for (+)-catechin from black wattle. A yield of 210 mg white, acetylated constituent D was obtained after two precipitations. Attempts to crystallize the product from ethanol, methanol or acetic acid/water, failed. The derivative was dried under vacuum for 2 hours, at 110°C. Found: C, 58.22; H, 4.77; $COCH_3$, 42.78%. $C_{25}H_{24}O_{12}$ requires: C, 58.3; H, 4.67; $COCH_3$, 41.8%. $[\alpha]_D^{23}$-85.6±0.7° in acetone (c, 0.26). $[\alpha]_D^{23}$-68.4±0.5° in $C_2H_2Cl_4$ (c, 0.25). The molecular weight of the acetylated compound, estimated by the improved ebulliometric method (Evelyn 164-166), was found to be 1169. Recalculated for an acetyl content of 42.78%, the above estimated value reflects a M.W. of 692 for the parent substance.

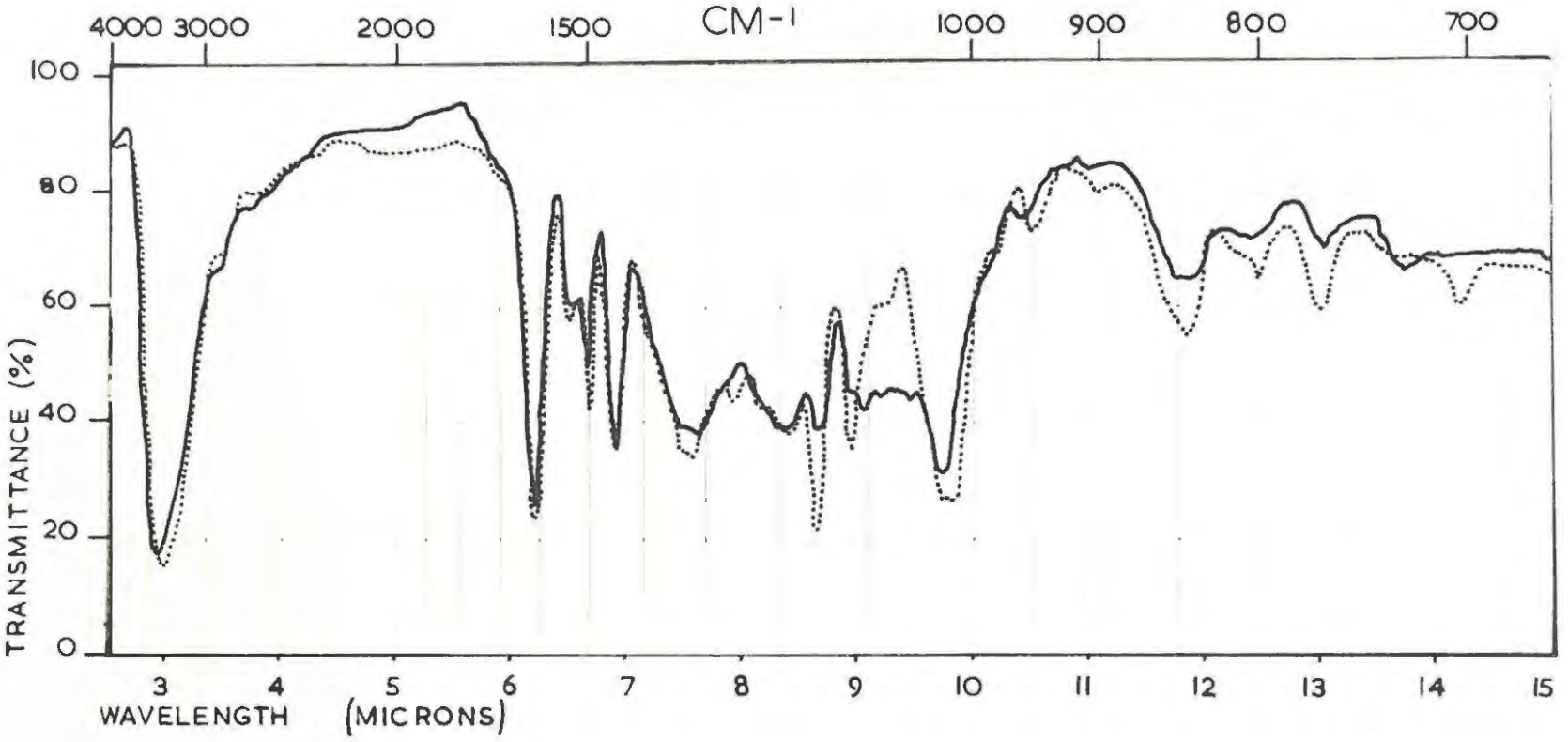



FIG. 10. INFRARED ABSORPTION CURVES OF CONSTITUENT D AND OF LEUCO-ROBINETINIDIN.

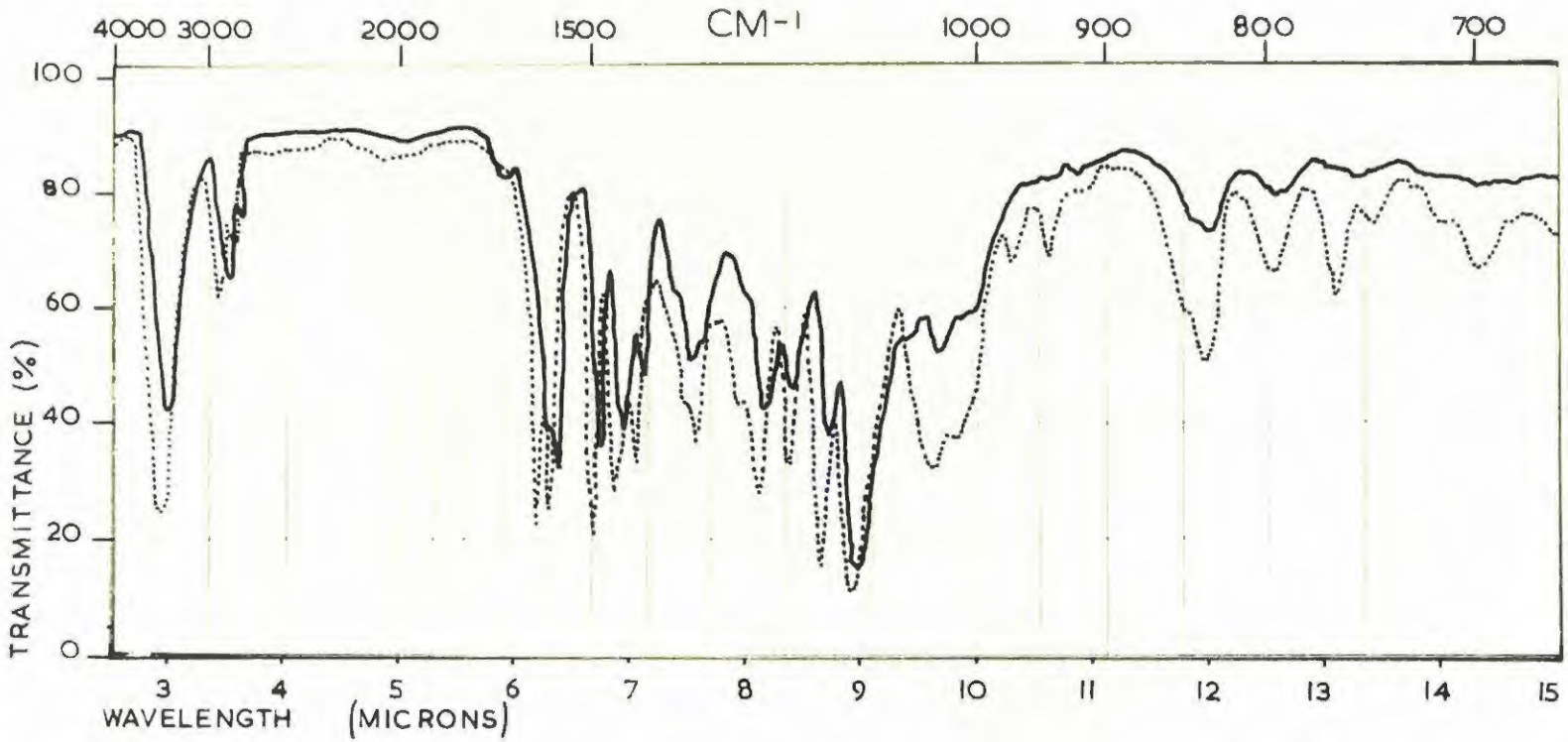



FIG. 11. INFRARED ABSORPTION CURVES OF METHYLATED CONSTITUENT D AND OF METHYLATED LEUCO-ROBINETINIDIN.

Identification of pigments generated from constituent D.

The pigments generated from constituent D, under the conditions of the leuco-anthocyanidin reaction, were separated on one dimensional paper chromatograms using 3N HCl : 90% formic acid = 1:1 as solvent. $R_F$ values of 0.26 and 0.56 for the purple-red and the orange pigment respectively, were found. Both pigments changed their colour when fumed with ammonia vapour; the purple-red pigment turning blue and the orange pigment claret-maroon. These colour changes indicated increased resonance of the molecules, due to ionisation of hydroxyl groups in unsaturated systems, and are typical for anthocyanidins, chalkones and aurones (Geissman, 205).

The purple-red pigment showed a deep pink fluorescense under U.V. light. The $\lambda_{max.}$ 532 mμ of the pigment was shifted to 570 mμ on formation of the aluminium complex (addition of two drops of 5% $AlCl_3$ in ethanol). When run simultaneously with synthetic robinetinidin chloride (synthesized, and kindly supplied by Dr. Roux, 96,100) on one-way paper chromatograms, both substances had identical $R_F$ values, identical colours in ordinary and U.V.-light, and identical absorption maxima and colour shifts (Table I). The purple-red pigment is therefore robinetinidin, thus establishing the presence of a flavan-3:4-diol

capable of conversion into robinetinidin in constituent D.

The second pigment of orange colour, ($R_F$ 0.56 in 3N HCl: 90% formic acid= 1:1) migrated in water ($R_F$ 0.26 in 2% aqueous acetic acid) and was therefore neither an anthocyanidin nor a chalkone; both classes of compounds having zero $R_F$ values in water due to their planar structures and low solubilities. (Roux, 199), (Roberts, 187, 211).

In order to make a detailed study of the orange pigment, it was necessary to isolate this compound. Pure constituent D (120 mg) was heated with 200 ml of the mixture, iso-propyl alcohol : 3N HCl=4:1, for one hour in 50 ml glass pressure tubes. The products generated were streaked onto 4 sheets of Whatman no. 3 paper and developed by the descending method for 8 hours, in 25% acetic acid/ water. Two bands of $R_F$ 0.35 (purple-red) and $R_F$ 0.58 (orange) were well separated. After drying, the orange band was cut and the substances eluted with 70% methanol. For further purification the combined eluates of this band were streaked onto two washed Whatman no. 3 sheets and developed with sec.-B.A.W. The orange pigment migrated as one sharp band of 1 inch width, $R_F$ 0.88. After elution with the mixture of 70% ethanol, 10%methanol, 20% water, the combined eluates were slowly evaporated in

a desiccator over $CaCl_2$ under light vacuum. After 4 days, dark red microcrystals had formed. These were allowed to grow for four more days. The crystalline material ( 6 mg) appeared chromatographically pure, having $R_F$ values of 0.87 in n.-B.A.W., 0.56 in 3N HCl:90% formic acid=1:1, and 0.25 in 2% aqueous acetic acid. The U.V.- and visible-range-absorption spectrum of the crystalline substance had three peaks at $\lambda_{max}$ 282, 342 and 468 mμ respectively.

The amount of crystalline material isolated from the reaction products of pure constituent D was small, (6mg), and the generation of the orange pigment from the ethyl acetate extract of fresh black wattle bark was therefore attempted. The concentration of 10 mg of wattle extract per 5 ml of the mixture of 3N HCl:iso-propanol=1:4, gave the highest total yields of orange pigment, under the conditions of the leuco-anthocyanidin reaction, in spite of the fact that, a concentration of 6 mg of extract per 5 ml of the reaction mixture, gave higher percentage yields. A concentration of 2.0 g wattle extract per litre reaction mixture was therefore chosen for large scale generation of the orange pigment.

For the preparative paper chromatographic separation of the pigment, the reaction mixture had to be concentrated. On concentration, the HCl concentration increased to such an extent that the amount of pigments progressively decreased due to polymerisation. Prior to concentration, the reaction mixture was therefore neutralised with bicarbonate solution (neutralisation of HCl), and then reacidified with acetic acid to reduce oxidation of the polyphenolic pigments. After the iso-propyl alcohol and some of the water had been evaporated (remaining volume about 350 ml), the orange pigment was extracted from the mixture by four successive extractions with ether. The combined ether extracts were concentrated under vacuum and applied to Whatman no. 3 sheets at a concentration corresponding to 200 mg starting material per sheet. The sheets were developed by the ascending method in 15% acetic acid/water for 16 hours. The bright orange bands ($R_F$ 0.45) of $1\frac{1}{2}$ inch width were cut and the pigment eluted with the mixture of 70% ethanol, 10% methanol, and 20% water. The eluates were examined by two-way paper chromatography. Only minor impurities ( $\beta$-resorcylic acid and a trace of resorcinol) were present. If the eluates of a certain batch were shown to contain more than a trace of impurities, they were subjected to further preparative paper chromatographic purification in a partitioning solvent

(n.-B.A.W.). The final eluates were concentrated under partial vacuum in desiccators, over $CaCl_2$. Small crystals formed after 3 to 4 days, which were allowed to grow for four more days. The crystals were sucked off and washed with ice water. The solubility of the substance decreases in the sequence acetone, iso-propanol, ether, sec.-butanol, methanol, ethanol, ethanol/water, and water. At elevated temperatures (60 -70°C), methanol and ethanol are excellent solvents for the crystalline pigment.

A total of 680 mg of crystalline, orange pigment generated from 50 g ethyl acetate extract of fresh black wattle bark, was isolated (yield 1.4%). When compared with the crystalline orange pigment generated from pure constituent D, the substances were identical.

Attempted identification of the orange pigment.

The crystal structure of the brownish material was kindly analysed by Prof. E. Mountain of Rhodes University, The pigment crystallises in triclinic, (anorthic) platy prisms. The terminal faces are parallel, and not symmetrical. The colour is deep and pleochroic (different for different vibration directions), i.e. golden brown in the one direction and brown red in the other direction. The refraction indices are about 1.605. The crystalline material did not melt at 330°C. Microscopic examination

of the product, after the attempted m.p. determination, showed that the crystal shape was still intact.

The orange pigment was found to have acidic properties, when examined by the bicarbonate/ether method, the substance being quantitatively liberated from its sodium salt by acetic acid.

The colours produced by the orange pigment, on spraying paper chromatograms with various selective spray reagents, were not conclusive, due to the inherent orange colour of the substance. Spraying with silver nitrate reagent reflected the presence of strongly reducing ortho-dihydroxy- or orthotrihydroxy-groups. The benzidine reagent shifted the inherent orange colour towards claret-maroon, while the vanillin-toluene-p-sulphonic acid reagent gave no visible colour change. Spraying with ferric alum produced a mauve, which was similar to that formed on spraying β-resorcylic acid with the same reagent. The colour of the pigment under U.V. light was deep pink to red, which did not change on fuming with ammonia, while in visible light a colour shift from orange to claret-maroon occurs, indicating a highly mesomeric structure with ionisable hydroxyl groups.

Microfusion of the crystalline substance, gave re-

sorcinol and gallic acid as main, and β-resorcylic acid as minor degradation products.

The U.V.- and visible light absorption spectrum (1.21 mg/100 ml ethanol) over the range 250-575 mμ (Fig. 6), showed peaks at $\lambda_{max}$ 282 ($E^{1\%}_{1cm}$ 301), 342 ($E^{1\%}_{1cm}$ 463) and 468 mμ ($E^{1\%}_{1cm}$388). In ethanolic $AlCl_3$ solution, these maxima were shifted to $\lambda_{max}$ 284, 380 and 548 mμ. The absorption spectrum of the substance in 0.002 M sodium ethoxide had $\lambda_{max}$ 289, 391, and 546 mμ. The infra-red spectrum had strong bands at 3400 (-OH), 1680 (pyrone C=O or carboxylic C=O), 1600, 1500, 1420 (aromatic C=C), 1220 (aromatic C=O) and at 1120 $cm^{-1}$(aryl-alkyl-ether), (Fig. 12).

The orange pigment was dried for two hours at 110°C, under vacuum. No changes of the $\lambda_{max}$ and $E^{1\%}_{1cm}$ values occurred as compared with the behaviour of anthocyanidin chlorides, which, on drastic drying under vacuum, lose Cl and consequently alter their $\lambda_{max}$ and $E^{1\%}_{1cm}$ values. Found: C, 61.88 and 61.34; H, 4.34 and 5.0%. The values of a third sample had to be corrected for ash; found C, 59.88; H, 3.92; ash, 2.3%, corrected: C, 61.28, H, 4.01%. $C_{15}H_{12}O_6$ requires: C, 62.48; H, 4.20%. $C_{15}H_{14}O_6$ requires: C, 62.07; H, 4.86%.

Acetylation of orange pigment.

The substance (100 mg) was acetylated with acetic anhydride and sodium acetate giving 107 mg amorphous light

brown acetyl derivative after two precipitations. Found: C, 59.93; H, 4.80; $COCH_3$, 36.73%. $C_{23}H_{22}O_{10}$ requires C, 60.26; H, 4.80 and $COCH_3$, 37.55%. The molecular weight of the acetylated product was found to be 792. Recalculated for an acetyl content of 36.73%, the above estimated value reflects a M.W. at 506 for the parent substance.

Methylation of orange pigment.

The substance (140 mg) was methylated with excess diazomethane, giving 100 mg of the amorphous light yellow O-methyl-derivative. Found: C, 64.91; H, 6.86; $OCH_3$, 35.79%. $C_{19}H_{22}O_6$ requires: C, 65.89, H, 6.36, $OCH_3$, 35.83%. The methylated product is a neutral substance. The infra-red absorption spectrum of the methylated product shows distinctive differences when compared with that of the parent substance (orange pigment). The strong peak at 3400 $cm^{-1}$(OH) is virtually absent in the spectrum of the methylated pigment, thus indicating complete methylation of the substance. The methyl derivative shows a prominent peak at 2900 $cm^{-1}$ which indicates strong C-H stretching frequencies, due to CH, $CH_2$ or $CH_3$ groups. The shift of the peak at 1680 $cm^{-1}$(parent substance), indicating pyrone or carboxylic C=O, to 1750 $cm^{-1}$ cannot be explained. (Fig. 13).

The molecular weight of the methylated orange pigment (ebulliometric method in acetone) was found to be 493. Recalculated for a methyl content of 35.79%, the above estimated value reflects a M.W. of 413 for the parent substance.

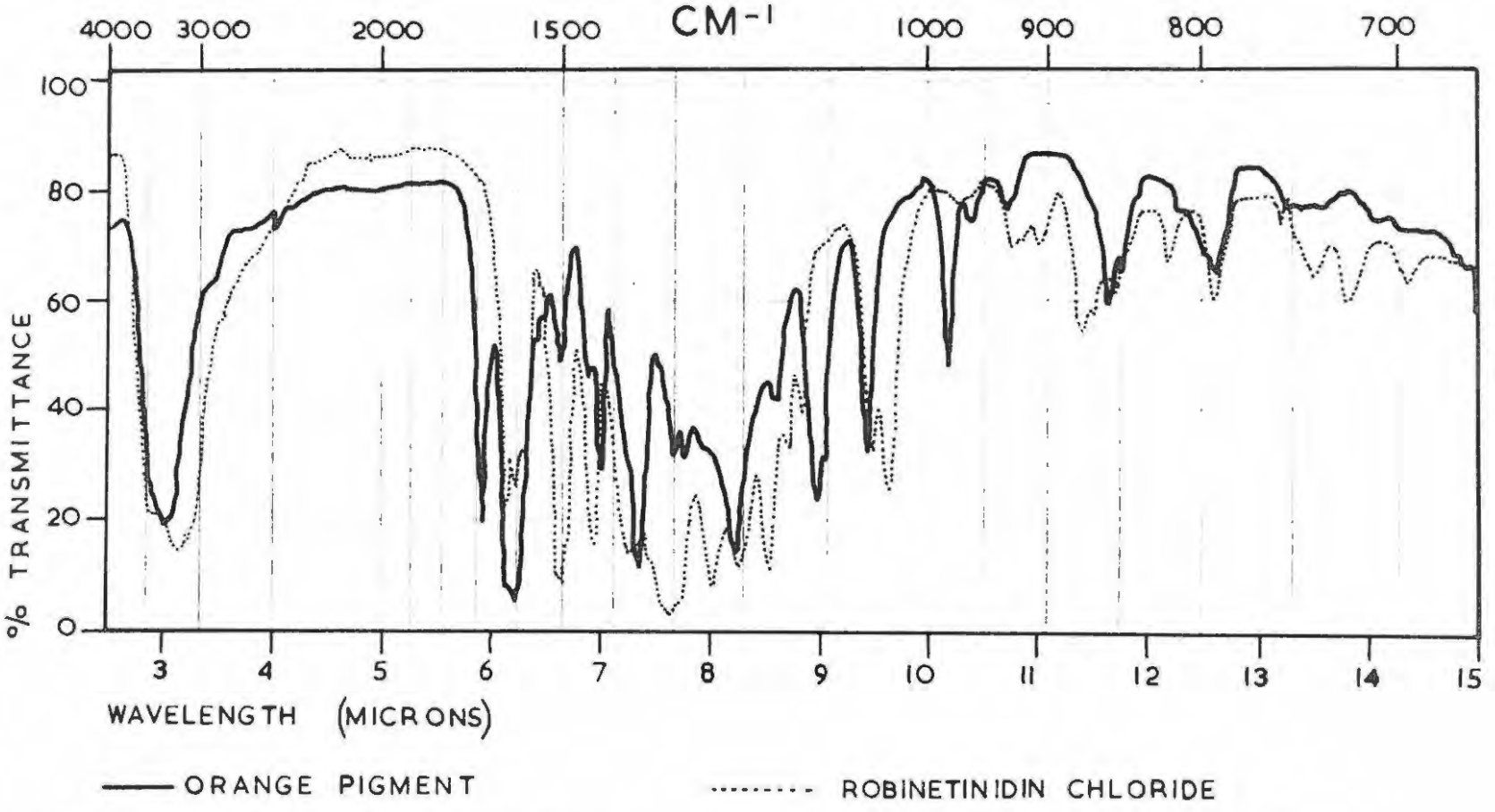



FIG. 12 INFRARED ABSORPTION CURVE OF ORANGE PIGMENT

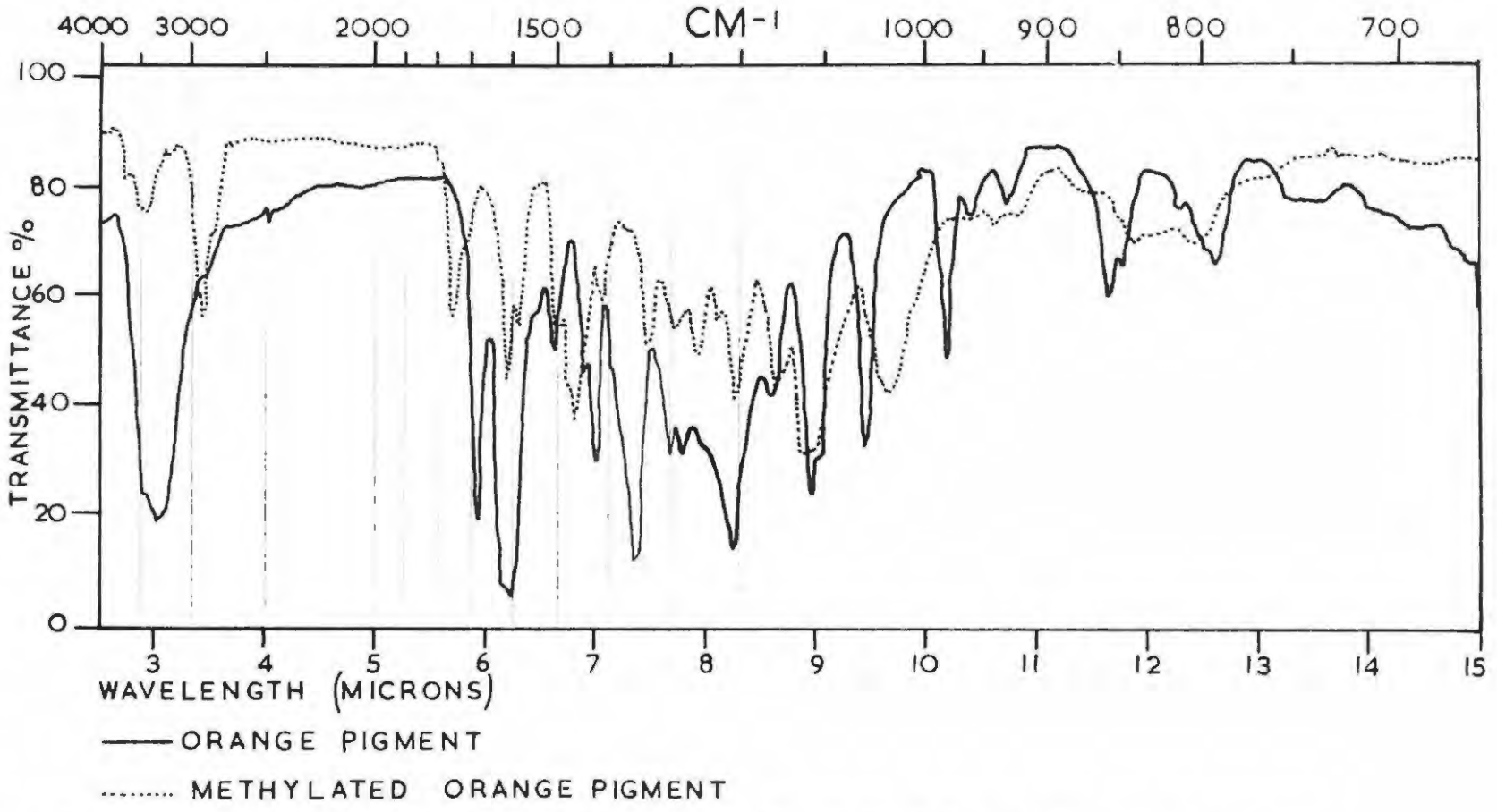



FIG. 13 INFRARED ABSORPTION CURVE OF ORANGE PIGMENT.

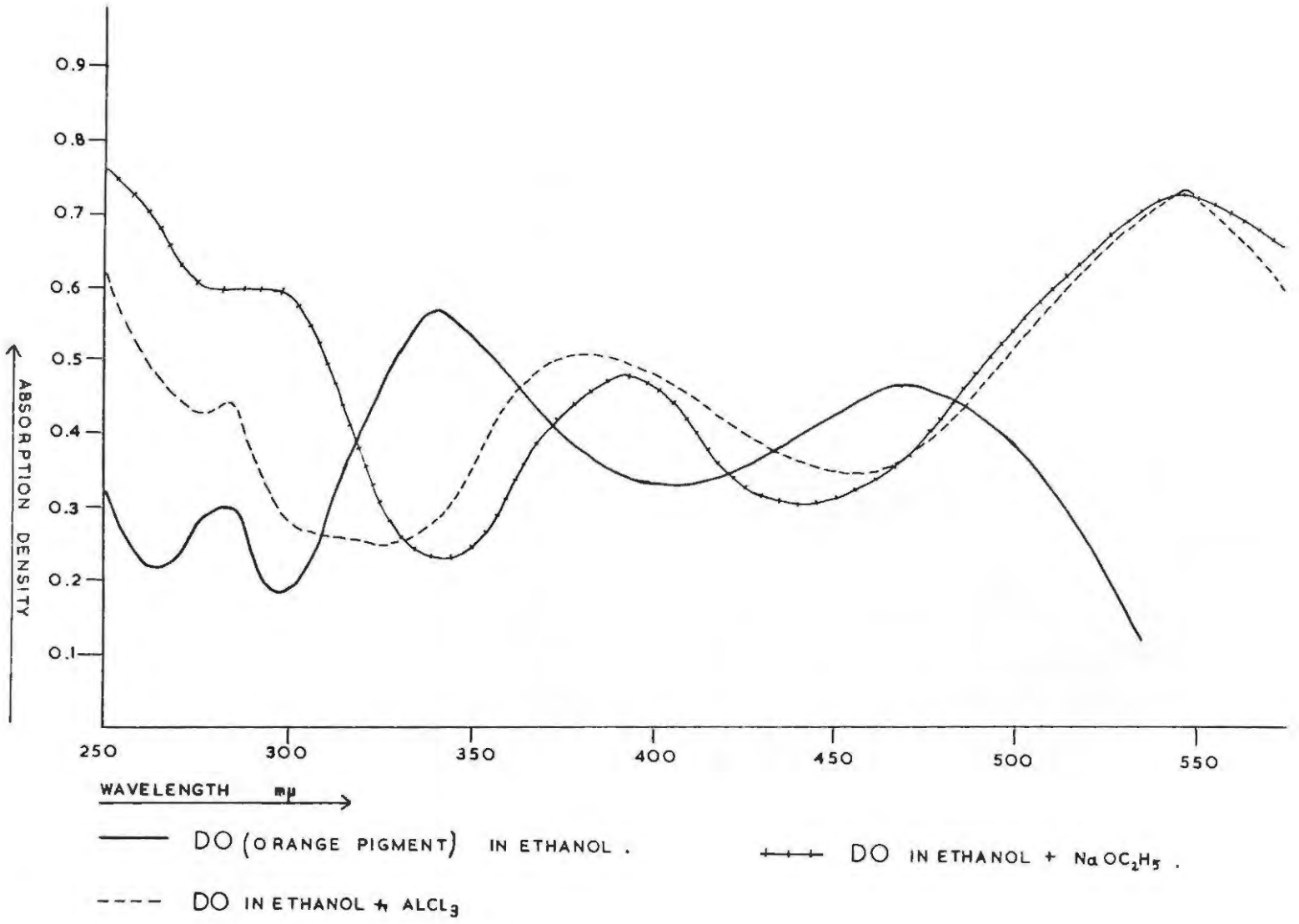
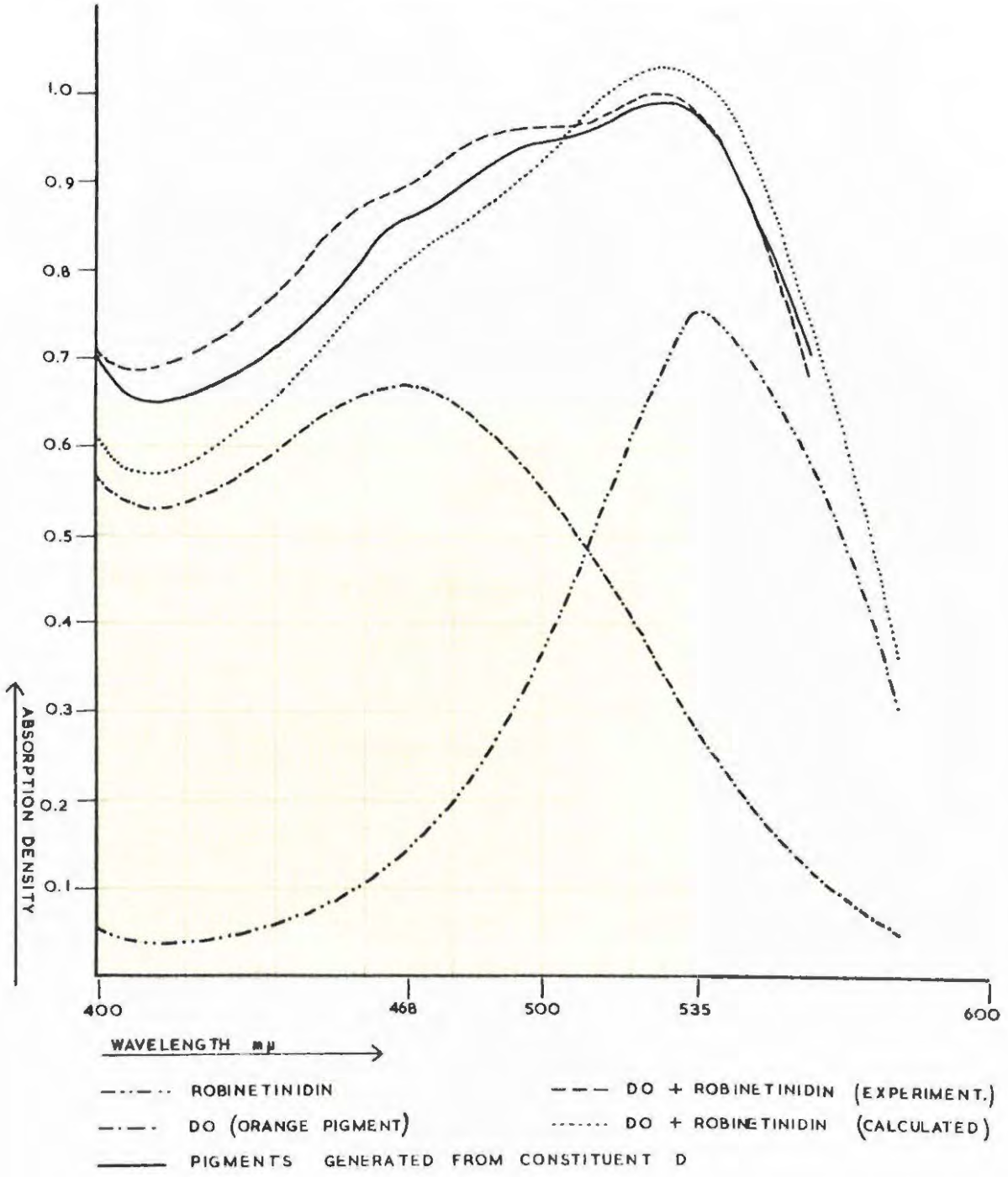



FIG. 14 ABSORPTION CURVES OF AN ORANGE PIGMENT GENERATED FROM BLACK WATTLE TANNIN.

FIG. 15 ABSORPTION CURVES OF PIGMENTS GENERATED FROM BLACK WATTLE CONSTITUENTS.



Quantitative estimation of robinetinidin and of the orange pigment in the reaction products of constituent D.

1.73 mg orange pigment was dissolved in 50 ml of the mixture iso-propyl alcohol : 3 N HCl (4:1). 10 ml of the solution was diluted 1:1 with the same solvent and the light absorption measured over the range $\lambda$ 400 to 580 mμ. Robinetinidin chloride (3.235 mg) was dissolved in 250 ml of the above solvent mixture. 10 ml of this solution was diluted 1:1 with the same solvent, and the light absorption measured over the above range. (Fig. 15).

The absorption curves of the two pigments overlap over the whole range measured ($\lambda$ 400 to 580 mμ). Robinetinidin has an absorption density of 0.750 at its maximum ($\lambda$ 535 mμ) and an absorption density of 0.140 at the maximum of the orange pigment ( $\lambda$ 468 mμ), while the orange pigment has absorption densities of 0.670 and 0.280 at is maximum ($\lambda$ 468 mμ) and at the maximum of robinetinidin ($\lambda$ 535 mμ) respectively.

It has been found that the absorption densities of each pigment, measured at the maximum of the pigment and at the maximum of the second pigment, bear a constant ratio to each other, irrespective of concentration. Thus the absorp-density of robinetinidin at $\lambda$ 468 mμ (max. orange pigment)

is $\frac{0.140}{0.750} = 0.187$ of the absorption density measured at $\lambda$ 535 mμ. Similarly the absorption density of the orange pigment at $\lambda$ 535 mμ (max. robinetinidin) is $\frac{0.280}{0.670} = 0.417$ of the absorption density measured at $\lambda$ 468 mμ. Where (X) and (Y) are the absorption densities of robinetinidin and of the orange pigment at their individual maxima, the absorption density of robinetinidin at $\lambda$ 468 mμ is 0.187 (X), while the absorption density of the orange pigment at $\lambda$ 535 mμ is 0.417 (Y).

The original solutions of robinetinidin and of the orange pigment (20 ml of each) were mixed and the light absorption measured over the range $\lambda$ 400 to 580 mμ (Fig. 14). The absorption curve had a shoulder at $\lambda$ 468 mμ ($\delta$, 0.860), a peak at $\lambda$ 530 mμ ($\delta$, 0.990) and was found to be the sum of the individual absorption curves of the two pigments.

In a mixture the absorption, when measured at the maximum of one of the two components, is therefore the sum of the absorption of the specific compound and the absorption which the second pigment exhibits at that wavelength.

The absorption density of robinetinidin and of the orange pigment in a mixture of the two may therefore be calculated from the following equations:

Instrument reading at $\lambda$ 468 mμ = (Y) + 0.187 (X)....(1)

Instrument reading at $\lambda$ 535 mμ = (X) + 0.417 (Y)....(2)

Where:

Instrument reading = absorption density of the mixture,

(X)=absorption density of robinetinidin at $\lambda_{max.}$ 535 mμ and

(Y)=absorption density of the orange pigment at $\lambda_{max.}$ 468 mμ.

From the above formula the concentration of robinetinidin and of the orange pigment in reaction mixtures of constituent D (leuco-robinetinidin tannin) may be calculated, provided no other substances absorbing at $\lambda$ 468 and 535 mμ are present.

Chromatographically pure constitutent D (1.88 mg) was dissolved in 10 ml of the mixture iso-propyl alcohol : 3N HCl (4:1) and heated on a boiling water bath for one hour, under pressure. The light absorption curve of the generation products was measured over the range $\lambda$ 400 to 580 mμ and was found to be nearly identical to the absorption curve of the artificial mixture of robinetinidin and of the orange pigment (Fig. 15). The disagreement between these two light absorption curves, found experimentally, and a calculated curve ( $\delta$ , robinetinidin + $\delta$ , orange pigment) can,however, not be explained.

TABLE VII.

Percentage of pigments formed on heating leuco-robinetinidins with 5 ml iso-propanol:3N HCl (4:1) mixture

| Sample: | % orange pigment. | % robinetinidin chloride. |
|---|---|---|
| 1.5 mg monomolecular leuco-robinetinidin: | nil | 29.3 |
| 2.35 mg constituent D: | 10.3 | 3.2 |
| 6.00 mg constituent D: | 10.8 | 3.7 |
| 2.43 mg constituent D: | 11.2 | 3.6 |

The values are expressed as percentage of chromatographically pure constituent D (dried for 2 hours at 110°C under vacuum), and are calculated for absorption densities of 0.387 (at 468 mμ) for 1.0 mg orange pigment and 0.750 (at 535 mμ) for 0.647 mg robinetinidin chloride, both in 100 ml of the mixture iso-propylalcohol:3N HCl = 4:1.

Attempted identification of "constituent B".

The amorphous light brown substance (1.6g) from the C-5 fraction which appeared to be chromatographically pure has $R_F$ values of 0.46 in sec.-B.A.W.; 0.42 in n.-B.A.W. and 0.33 in 2% aqueous acetic acid (Whatman no. 1).
On spraying with different spray reagents constituent B developed the following colours: silver nitrate reagent - black brown; benzidine reagent - deep yellow; vanillin-toluene-p-sulphonic acid reagent - deep yellow; toluene-p-sulphonic acid reagent - orange; and ferric alum reagent,

blue with green edge. The substance does not fluoresce under U.V. light. A two-dimensional paper chromatogram of B, run in the water direction for a prolonged period (10 instead of 5 hours), showed the presence of two overlapping bodies on spraying the chromatogram with the ferric alum reagent. The main spot gave a blue colouration overlapping a green crescent in the higher $R_F$ area of the spot. After 30 seconds the whole spot turned green. The other spray reagents did not differentiate between these two overlapping spots. In spite of the fact that B obviously consists of two bodies which did not separate sufficiently to allow their isolation, an attempt was made to identify B as far as possible.

Alkali microfusion of "constituent B" gave resorcinol, $\beta$-resorcylic acid, protocatechuic acid and gallic acid as the main degradation products. These findings agree with the colour reactions of "constitutent B", which indicated resorcinol (A), and catechol and pyrogallol (B) nuclei. The positive leuco-anthocyanidin reaction of B with toluene-p-sulphonic acid was confirmed by the formation of three pigments when the substance was heated with dilute mineral acid in alcoholic solution under pressure. The three pigments were separated on one dimensional paper chromatograms (3N HCl:90% formic acid = 1:1, Whatman no. 1)

having $R_F$ values of 0.26 (purple red pigment), 0.43 (pink pigment) and 0.56 (orange pigment). The purple red pigment of $R_F$ 0.26 (robinetinidin chloride) and the orange pigment of $R_F$ 0.56 were shown to be identical with the two pigments generated from constituent D by paper chromatographic comparison. The third pigment of pink colour (bright orange under U.V. light) was identified as fisetinidin chloride by its $R_F$ value, absorption spectra and the shift of its $\lambda_{max.}$ 525 mμ to 545 mμ on formation of the aluminium complex (for reference, Table I). To gain further proof, the two anthocyanidin chlorides were run simultaneously with synthetic robinetinidin and fisetinidin (synthesized and kindly supplied by Dr. Roux) on one dimensional paper chromatograms. The $R_F$ values and colour reactions of the two anthocyanidin chlorides generated from constituent B and those of the synthetic substances were identical.

"Constituent B"(250 mg) was acetylated with acetic anhydride in pyridine to give 350 mg of a light brown amorphous acetyl derivative (acetyl value 41.39%). Molecular weight estimations of acetylated B by the improved ebulliometric method of Evelyn (164, 165, 166) reflected a M.W. of 1,135. Calculated for an acetyl content of 41.39% this value reflects a M.W. of 676 for"constituent B." "Constituent B"precipitates gelatine salt reagent and is therefore a tannin.

Discussion of the orange pigment.

The substance is generated from polymeric but not from monomeric leuco-robinetinidins under the conditions of the leuco-anthocyanidin reaction (hot dilute mineral acid in alcoholic solution under pressure) or on enzymic degradation with tannase. The generation of the pigment by enzymic degradation, its migration in water on paper chromatograms ($R_F$ 0.26) and the absence of chlorine when analysed, indicate that the substance is not an anthocyanidin chloride. As in the case of other conjugated flavonoid compounds, the pigment has a high melting point (above 330°C).

The colours produced on spraying the substance with various selective spray reagents were not conclusive, due to the inherent orange of the pigment. Spraying with the silver nitrate reagent showed the presence of strongly reducing orthodihydroxy- or orthotrihydroxy-groups. Alkali microfusion gave resorcinol and gallic acid as main degradation products, thus indicating the presence of a resorcinol (A) and a pyrogallol (B) nucleus. The U.V.- and visible light absorption spectra showed absorption at $\lambda$ 282 and 342 mμ, indicating the presence of phenolic OH- and conjugated carbonyl-groups respectively (Fig. 14). On fuming with ammonia

or on the addition of sodium ethoxide the pigment showed a big colour shift towards longer wavelength (468 mμ to 546 mμ) (Fig. 14). These colour shifts indicate ionisable hydroxyl groups in conjugated systems with carbonyl groups as electron acceptors (Geissmann, 205; Klages, 213). A similar colour shift on addition of ethanolic aluminium chloride showed the presence of vicinal orthodihydroxy- or orthotrihydroxy groups, or a carbonyl group so placed with respect to a phenolic hydroxy group as to allow the formation of metal complexes (Fig. 14). The infrared absorption spectrum of the pigment (Fig. 12) indicated the presence of OH groups, strong benzoid C=C conjugation (C=C stretching frequenices), possible absorption due to aryl-alkyl ether groups and aryl ketone or quinonoid C=O groups. (Fig. 12).

The peaks at 342 mμ (U.V.-spectrum) and at 1685cm$^{-1}$ (infrared spectrum) indicating strong absorption due to conjugated carbonyl groups are of special interest, because they are absent in the parent substance (constituent D). The presence of carbonyl groups in the orange pigment therefore indicates that it is a transformation product, and not a simple hydrolytic fraction of D. There is also little similarity between the infrared-absorption

spectrum of the orange pigment and of robinetinidin chloride, with the exception of those peaks due to OH groups, and stretching frequencies of the benzenoid rings due to C=C (Fig. 12).

The orange pigment was found to have acidic properties. The acidic nature of the substance must not necessarily indicate the presence of a carboxylic group, but may be due to conjugated tertiary OH- and carbonyl groups as in the case of ascorbic acid (acidity due to O=C-C=C-OH; Klages 213), or may be due to the presence of two oxygen atoms on one C atom (-O-C-OH) as in the case of the recently isolated alphitonin (Birch, Ritchie and Speake 230).

On the basis of a $C_{15}$ structure ($C_{15}H_{14}O_6$) acetylation of the orange pigment affords an acetyl derivative containing the exact equivalent of four acetyl groups. Diazomethane reacts to the equivalent of four methoxyl groups, corresponding to the methylation of four acidic, possibly phenolic hydroxyl groups. The infrared absorption spectrum of the methylated pigment (Fig. 13) shows that all free hydroxyl groups have been methylated. Of the remaining two oxygen atoms per $C_{15}H_{14}O_6$, one probably belongs to a carbonyl group, while the other is presumably involved in an ether link. This leaves two alternative

structures for the orange pigment. The first would correspond to a molecule with a total of four phenolic OH groups in the A and B nuclei, and a carbonyl group in the heterocyclic ring. The second alternative corresponds to a structure with a quinonoid carbonyl group in one of the two phenolic rings, and an acidic hydroxyl group in the heterocyclic ring.

Molecular weight estimations of the acetylated and of the methylated orange pigment reflect values, which are too high to fit a monomolecular $C_{15}$ structure (M.W.290), and too low to explain a dimolecular structure. Freudenberg *et al.* (37), on acetylating anthocyanidins, found these highly resonating phenolic compounds to give both monomeric and polymeric acetyl derivatives, due to acid-induced condensation, under the conditions of the acetylation. Similar condensation on acetylation may explain the high molecular weight of the acetylated product.

Summing up these findings it may be assumed that the orange pigment probably represents a conjugated flavonoid system with resorcinol (A) and pyrogallol (B) nuclei. The (B) nucleus may have a paraquinonoid structure. The two nuclei are likely to be conjugated by an at least partially unsaturated heterocyclic ring. Continued work is needed to elucidate the structure of this pigment.

Discussion of black wattle bark constituent D.

While the three polyphenolic constituents (+)-catechin, (+)-gallocatechin and (-)-robinetinidol, isolated from black wattle bark extract so far, were phenolic non-tannins, constituent D represents the first flavonoid tannin isolated in chromatographically pure form from a commercial vegetable tannin source.

From analytical figures D is surmised to be built of two $C_{15}$ units (each $C_{15}H_{14}O_7$), at least one of which has a flavan-3:4-diol structure capable of conversion into robinetinidin. The second pigment or orange colour has not yet been fully identified, but is surmised to have a highly resonating flavonoid structure with the same phenolic elements as robinetinidin chloride.

Quantitative estimations showed that 3.6% robinetinidin chloride and 10.8% orange pigment were generated from D under the conditions of the anthocyanidin reaction. Monomeric leuco-robinetinidin on the other hand yields up to 29% robinetinidin chloride, but no orange pigment under the same conditions. The finding that monomeric leuco-anthocyanidins on treatment with hot dilute mineral acid genrate a higher proportion of anthocyanidin chlorides than corresponding complex leuco-anthocyanidins has also been observed by Roux (238). This may be due to an easier conversion of complex polyphenols into insoluble phlobaphenes.

Enzymic degradation of constituent D with tan-

nase furnishes the orange pigment as the only reaction product, while monomeric leuco-robinetinidin generates traces of several minor polyphenolic substances, but no orange pigment under identical conditions.

Alkali microfusion of D gave resorcinol, β-resorcylic acid and gallic acid as main, and phloroglucinol as minor degradation products. Selective spray reagents did not reflect the presence of phloroglucinol, only of resorcinol and pyrogallol nuclei in the parent substance. Acidic breakdown under the conditions of the leuco-anthocyanidin reaction furnished β-resorcylic acid, resorcinol, gallic acid and the two pigments (robinetinidin and the orange pigment) as the main reaction products. Some samples of D also furnished traces of phloroglucinol and delphinidin under the above conditions. Alkali microfusion of the orange pigment and of robinetinidin, on the other hand, gave only resorcinol, β-resorcylic acid and gallic acid as degradation products. The presence of phloroglucinol among the products of alkaline and acidic degradation of the tannin (constituent D), must therefore originate from a trace of an associated leuco-delphinidin.

Calculated to a $C_{15}$ basis, constituent D reacted with diazomethane to the equivalent of four methoxyl groups, corresponding to the methylation of all phenolic hydroxyls in the suggested 7:3':4':5'-tetrahydroxyflavan-3:4-diol. Direct acetylation afforded an acetyl deriva-

tive containing the equivalent of five acetyls per $C_{15}$ unit, and presumably represents the substitution of the equivalent of four phenolic and one aliphatic hydroxyl group. On the assumption that D is a dimer of 7:3':4':5'-tetrahydroxyflavan-3:4-diol ($C_{15}H_{14}O_7$), it is apparent that one of the two remaining oxygen atoms per $C_{15}$ unit which neither methylates nor acetylates possibly belongs to the aryl-alkyl-ether of the pyrone ring. The remaining equivalent of one aliphatic hydroxyl group per $C_{15}$ unit is presumably involved in direct condensation, or has undergone conversion into tertiary alcohol as a result of condensation, or alternatively has become sterically hindered or hydrogen bonded following condensation. An exact parallel was found by the isolation of a trimeric leuco-fisetinidin tannin from the heartwood of A. *mearnsii* by Roux (238).

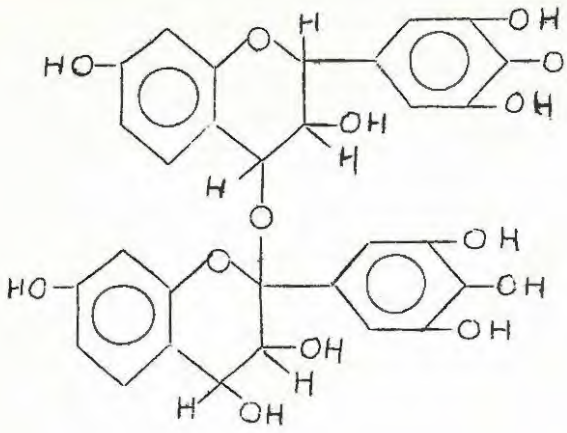
The ultraviolet absorption spectrum of D shows absorption of $\lambda$ 280 mu, indicating the presence of phenolic OH groups. The infrared absorption spectrum of D shows similarity with that of monomolecular (+)-leuco-robinetinidin (Fig. 10) over a wide range, having strong bands at 3450 ( -OH), 1610, 1530, 1500 and 1450 (aromatic -C=C- ), 1105 and a shoulder at 1120 (aryl-alkyl-ether) and at 1020 $cm^{-1}$ (*sec*.-aliphatic OH).

The infrared absorption curves of the methylated

tannin (D) (Fig. 10) and of methylated (+)-leuco-robinetinidin show similarity over a wide range. The additional peak at 2800 $cm^{-1}$ is due to stretching frequencies of the $CH_3$ groups.

Constituent D is laevorotary $[\alpha]_D$-269°, and so are the derivatives; acetylated D, $[\alpha]_D$-85.6°; methylated D, $[\alpha]_D$-103°.

Several factors must be taken into account when discussing a possible structure for the dimolecular tannin (D). The relative instability of the link between the two molecules makes a C-C type of bond unlikely. The fact that the parent compound and the two flavonoid degradation products show the same number of phenolic hydroxyl groups per $C_{15}$ unit, eliminates the possibility of an aryl-O-aryl or aryl-O-alkyl link between the two $C_{15}$ units, because fission of an aryl-ether link would result in a higher number of phenolic OH groups (per $C_{15}$) in the fission products, when compared with the parent compound. The linkage must therefore be entirely concerned with the alcoholic hydroxyl groups of the heterocyclic rings. The fact that tannase, an enzyme containing esterases and glycosidases (Maihs 240), is capable of splitting the tannin, makes a semiacetal link likely. The following structure is therefore proposed for constituent D:



Constituent D

A similar structure had been discussed by Forsyth and Roberts (136) for their leuco-cyanidin 1, but was ruled out by the difficulty of acetylation of the remaining alcoholic hydroxyl groups. In case of constituent D, however, two of these secondary alcoholic hydroxyl groups were easily acetylated, while the third was presumably sterically hindered.

The assumption that D is a complex of 7:3':4':5'-tetrahydroxyflavan-3:4-diol finds support by the results of the latest investigations on polymeric leuco-robinetinidins from the heartwood of Robina pseudacacia by Roux & Paulus (207), who found robinetinidin and an identical orange pigment as the only two pigments generated under the conditions used in this work.

Discussion of "constituent B".

"Constituent B" is not a single substance but appears to consist of two substances, the separation of which has not been achieved. "Constituent B"is a tannin with an average molecular weight of 676 which indicates that both substances may be dimolecular flavonoids. The presence of resorcinol (A), and catechol and pyrogallol (B) nuclei, was established from colour reactions with selective spray reagents and by alkali microdegradation. On heating B with dilute mineral acid in alcoholic solution under pressure (leuco-anthocyanidin reaction) three pigments were formed, two of which were identified as fisetinidin chloride and robinetinidin chloride. The third pigment of orange colour has not yet been fully identified, but it has previously been shown that this pigment consists of a resorcinol (A) and a pyrogallol (B) nuclei, and is generated from polymeric leuco-robinetinidins under the conditions of the leuco-anthocyanidin reaction (with hot mineral acid) and on enzymatic degradation with tannase. It may therefore be assumed that"constituent B" consists of a mixture of complex leuco-robinetinidins and leuco-fisetinidins.

APPENDIX..

The isolation of a tannin from the bark of the golden wattle.

A leuco-robinetinidin tannin (80 mg) was isolated from the C band fraction C-7 of golden wattle (page 130). The constituent had identical $R_F$ values in different solvent systems and gave identical colour reactions and breakdown products as black wattle constituent D. Infrared absorption curves of the substances from black and from golden wattle were superimposable over the range 2.5 - 15 μ.

## CHAPTER 7

## COMPARATIVE STUDIES OF COMPLEX LEUCO-ANTHOCYANIDINS IN THE BARK EXTRACTIVES OF THE BLACK, GREEN, SILVER AND GOLDEN WATTLE TREE.

While Roux and Evelyn (55) made a comparative study of the complex leuco-anthocyanidins in quebracho heartwood- and black wattle bark extracts, a similar study of complex leuco-anthocyanidins in those wattle species which are closely related to black wattle is still outstanding.

Methanol extractives of authentic wattle samples from comparative studies of the catechin constituents of black, green, silver and golden wattle barks were used.

The methanol extractives (2mg per sample) were each dissolved in 5 ml of the mixture 3N HCl : iso.-propyl alcohol (1:4), and heated for one hour in 8 ml (total volume) glass pressure tubes on a boiling water bath. The black wattle bark constituent D, which is surmised to be built of two 7:3':4':5'-tetrahydroxyflavan-3:4-diol units, and a trimeric leuco-fisetinidin from black wattle heartwood (isolated, and kindly supplied by Dr. Roux) were treated under identical concentrations and conditions for reference. After cooling the reaction mixtures were streaked onto Whatman no. 1 paper and developed by the descending method for 4 hours in 3N HCl : 90% formic acid

(1:1). Synthetic robinetinidin and fisetinidin chloride (kindly supplied by Dr. Roux) was run simultaneously for reference. The pigments were well separated on the paper chromatograms and were identified by their $R_F$ values, their colours in ordinary and under U.V. light and by their $\lambda_{max}$. To estimate their absorption maxima the spots were cut out and their absorption curve measured against a piece of blank paper of the same paper chromatogram. The colour of the four anthocyanidins present, their $R_F$ values and their absorption maxima were identical to values reported by Roux (51) (Fig. 1).

The relative concentration of pigments on the paper chromatograms was estimated by visual comparison.

Discussion of Table XXVII.

Of the anthocyanidins generated, robinetinidin chloride was formed in high concentration from the extractives of black wattle species, and in lower concentration from golden, green, and silver wattles.

Fisetinidin chloride was generated from the extractives of all four wattle species, but the yield was much lower than robinetinidin chloride. The concentration of this pigment in the reaction products of black wattle bark extracts was considerably higher than in green, silver and golden wattle bark extracts.

Delphinidin chloride was present among the reaction products of all four wattle species, the highest concentration of this pigment being found in those wattle species (silver and green wattle) which also generated cyanidin chloride.

Roux (150) previously found the proportion of phloroglucinol, isolated from alkali degradation products of green wattle, significantly higher when compared with the yield from black wattle. This can now be explained by the higher concentration of leuco-delphinidin and the additional presence of leuco-cyanidin in green wattle bark.

An orange pigment, the structure of which has not yet been fully established, was generated in varying concentration from the extracts of all wattle species. There appears to be a parallel between the generation of robinetinidin and this pigment. This was confirmed by the isolation of a complex leuco-robinetinidin tannin from the wood of Robina pseudacacia by Roux & Paulus (207), and the isolation of a dimeric leuco-robinetinidin tannin from the bark of A. mearnsii. Under the conditions of the leuco-anthocyanidin reaction, these tannins from different sources all generate robinetinidin and an identical orange pigment.

TABLE XXVII.

Comparison of the relative concentration of pigments generated from the bark extractives of wattle species closely related to black wattle.

Abbreviations: Rob., robinetinidin chloride; Fis., fisetinidin chloride; Cyan., cyanidin chloride; Delph., delphinidin chloride; DO, orange pigment generated from complex tannins, which simultaneously generate robinetinidin chloride.

| Sample: | Rob.: | Fis.: | Cyan.: | Delph.: | DO: |
|---|---|---|---|---|---|
| Black wattle I. | ++++ | ++ | - | ++ | ++++ |
| II. | +++ | ++ | - | ++ | +++ |
| III. | +++ | + | - | + | +++ |
| Golden wattle | +++ | + | - | ++ | ++ |
| Green wattle | +++ | + | +++ | +++ | + |
| Silver wattle | + | + | +++ | ++++ | + |
| Constituent D | ++++ | - | - | - | ++++ |
| Trimeric leucofisetinidin | - | ++++ | - | - | - |

Note: Black wattle I, trees grown in Grahamstown (sandstone)
Black wattle II, " " " Natal
Black wattle III, " " " Natal ( on shale )

++++, +++, ++, + = decreasing concentration

- = absent

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Note: Journal of the Society of Leather Trade Chemists will be abbreviated J.S.L.T.C.


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