ANALYTICAL PROCEDURES FOR THE DETERMINATION OF WATTLE POLYPHENOLS IN WASTEWATERS

by

Antony John Hendry

A thesis submitted to Rhodes University
in partial fulfilment
of the requirements for the Degree of

Master of Science

Leather Industries Research Institute
Rhodes University
Grahamstown

June 1983

ACNKOWLEDGEMENTS

This work was carried out at the Leather Industries Research Institute, Grahamstown. I wish to thank the following people who made this project possible.

Dr N.P. Slabbert, for his continued interest, inspiration and advice.

Dr D.R. Cooper, Director, for his support and making the facilities at Leather Industries Research Institute available.

Dr C. Whiteley, for his assistance.

All the members of Leather Industries Research Institute staff, for their help and encouragement.

Professor D.G. Roux of the University of the Orange Free State, for his advice and samples of reference standards.

Bernadette, my wife, for the exacting task of typing this thesis.

This work was financed by the annual grants of the Wattle Industry Fund and the Council for Scientific and Industrial Research.

CONTENTS

LIST OF FIGURES

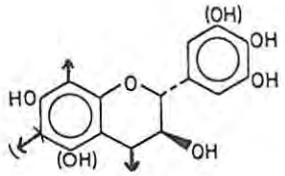
LIST OF TABLES

1 INTRODUCTION

1.1 General

Black wattle ('Mimosa') extract, a product from the bark of *Acacia mearnsii*, is used mainly as a tanning material. It is a complex mixture of condensed polyphenolic components (70%), nitrogenous compounds (3%) and carbohydrates (27%).[1] It owes its tanning action to hydrogen-bond formation between the hydroxyl groups of the condensed phenolic tannin oligomers and the polypeptide groups of hide protein, collagen.[2] Recently, however, its range of uses has been extended to the fields of ore flotation depressants, water treatment flocculants and adhesives.[3,4]

Vegetable tannins in general, are divided into two classes, *viz.* condensed and hydrolysable. Both classes consist of polymeric phenolic compounds. The condensed type is based on flavanoid units joined by



Repeating flavanoid unit

C-C linkages, for example, wattle and quebracho extracts. The hydrolysable tannins, for example, chestnut extract, are based on gallic acid units joined by ester linkages. Under acidic conditions, condensed tannins can polymerize to form compounds with higher molecular mass whereas hydrolysable tannins fragment as a result of acid hydrolysis.[5] The complex phenolic composition of vegetable tannins has posed major analytical problems.[5] Quantitation has classically been based on the combination of tannins with proteins, for example, collagen, gelatin, haemoglobin and casein.[6] The standard hide-powder method of tannin analysis,[7] is univerally used by vegetable tannin producers and tanners alike, despite the problems experienced in obtaining standardized hide-powder.[8] This simple bulk method, however, gives no information on the constitution of the wattle tannins (oligomeric flavanoids) and phenolic non-tannins (dimeric and monomeric flavanoids and associated flavonoids).

The phenolic non-tannins, not adsorbed by the hide during the tanning process, are a potential nuisance in discharged wastewaters. While most

of the tannin is removed from solution, some phenolic tannin material is inevitably discharged. These phenols are toxic to aquatic life due to their ability to bind to proteinaceous matter, notably the gills of fish and to aquatic microfauna.[9] In addition, the impact of these organic constituents on the aquatic environment, especially the avidity for oxygen consumption which can give rise to eutrophic conditions in receiving waters,[10] is of growing ecological concern. Additional problems may arise from disinfection of such waters. Chlorination leads to the formation of a variety of organochlorine compounds,[11] such as haloforms, chlorinated phenols and phenolic acids, chlorinated quinones and heterocyclic compounds,[12] which are toxic and persistent.

Wastewaters containing phenolic residues undergo oxidative browning via biotic and abiotic mechanisms,[13] and the complexity of wattle phenols in wastewater can be further compounded by the formation of oxidation-degradation products. The determination of such phenolic related substrates by standard effluent oxygen-based methods (*viz.* COD, PV and BOD determinations),[14] is non-specific and is open to misinterpretation.[15] Hence, the need for more specific analytical methods for phenols which is the subject of this thesis.

1.2 Chromatography and composition of wattle extract

Paper chromatography has been a useful qualitative analytical and separation technique; together with nuclear magnetic resonance (n.m.r.) and mass spectrometry, it has been largely responsibly for most of the knowledge pertaining to phenols in the plant kingdom,[5,16] including black wattle extract.[17] While the higher oligomers themselves give poorly defined chromatograms, the method is ideal for separating mono-, di- and trimeric flavanoids and co-occurring flavonoid components. The mechanism of migration on cellulose is dependant on the solubility/partitioning and adsorption characteristics of the solutes in the different mobile phases. These characteristics are in turn dependant on the number and pattern of hydroxyl groups on the relevant flavonoid skeleton, as well as its spatial configuration.[18]

A popular method for the analysis of wattle extract has been two-dimensional paper chromatography using water-saturated butan-2-ol as the mobile phase in the first direction,[18] where the partitioning mechanism

predominates. In the second direction with 2% (v/v) aqueous acetic acid, the adsorption mechanism is important where structural differences in flavonoid isomers, epimer or optical, results in resolution. This is often marginal[19] and where different solutes are present in the same starting spot, variable amounts of mutual interference can result in anomalous R_f values.[20]

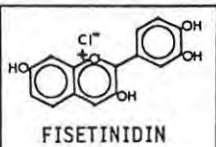
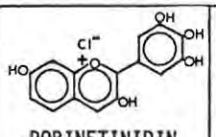
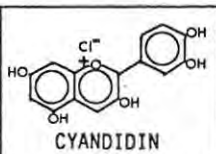
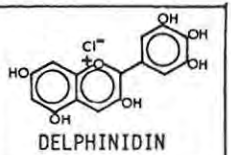
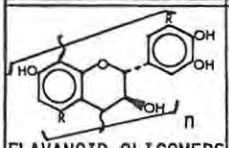
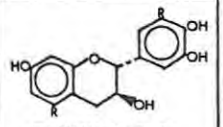
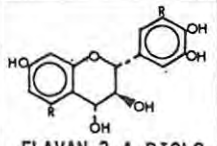
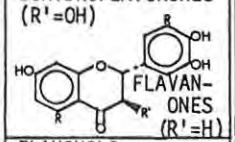
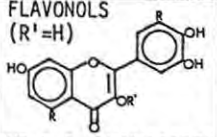
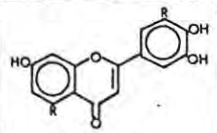
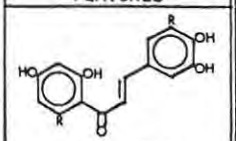
Extensive studies have been carried out on the composition of wattle tannin.[17] The major portion is comprised of an heterogeneous group of condensed flavanoid oligomers. These proanthocyanidins are accompanied by a minor fraction of monomeric flavonoid analogues and related phenolic precursors.[21,22] Generation of the anthocyanidins from the complex flavonoid mixture in wattle extract (see Fig. 1) produces four major components,[23] *viz.* robinetinidin (70%), fisetinidin (25%), delphinidin and cyanidin (5%). These results indicate that of the phenolic material, the prorobinetinidin oligomers and related flavonoids are the most abundant.

Robinetinidin and fisetinidin analogues occur mainly in the heartwood and stem bark while delphinidin and cyanidin analogues occur mainly in the chlorophyl rich portions of black wattle.[22] The last two analogues do not form a full series of flavonoids as do the first two analogues, but for the sake of completeness, Fig. 1 shows the full series of analogues with their relevant phenolic hydroxylation patterns.

Analytical techniques for direct quantitation of the above flavonoid monomers are a recent development. However, past results from qualitative and semi-quantitative procedures,[19] have indicated that the simple monomeric flavonoids collectively represent a small percentage of the phenolic material in wattle extract. Densitometry estimations from paper chromatograms showed that the three predominant flavan-3-ols, (+)-catechin (3), (+)-gallocatechin (4) and (–)-robinetinidol (2), occur in the 1% range.[24] The trace quantities of (+)-leucorobinetinidin (6) and (+)-leucofisetinidin (5) found in wattle extract provided the clue to the biogenesis of the oligomers.[25]

Due to the complexity of the extract composition, Roux and co-workers have recently concentrated on synthesis of oligomers.[26] Control over reaction conditions and substrates has shown that there is a selective condensation sequence resulting from steric constraints and hyperconjuga-

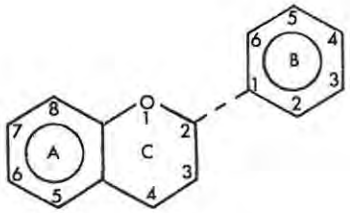
Figure 1. Anthocyanidin series of wattle-type flavonoid analogues.

ANTHOCYANIDIN SERIES AND FLAVONOID ANALOGUES ↓ →	 FISETINIDIN	 ROBINETINIDIN	 CYANDIDIN	 DELPHINIDIN
 FLAVANOID OLIGOMERS	CONDENSED PROANTHOCYANIDINS			
 FLAVAN-3-OLS	(1) (–)-Fisetinidol	(2) (–)-Robinetinidol	(3) (+)-Catechin	(4) (+)-Gallocatechin
 FLAVAN-3,4-DIOLS	(5) (+)-Leuco-fisetinidin	(6) (+)-Leuco-robinetinidin	((+)-Leuco-cyanidin)**	(+)-Leuco-delphinidin*
DIHYDROFLAVÓNONES (R'=OH)  FLAVAN-ONES (R'=H)	(7) (+)-Fustin	(8) (+)-Dihydro-robinetin	(9) Dihydroquercetin*	(10) Dihydromyricetin*
	(11) (+)-Butin	(12) (+)-Robtin	(13) Eriodictyol*	(5,7,3',4',5'-Pentahydroxy-flavanone)**
FLAVONOLS (R'=H)  AND 3-O-RHAMNOSIDES	(14) Fisetin	(15) Robinetin	(16) Quercetin*	(17) Myricetin*
	Fisitrin*	Robinitrin*	(18) Quercitrin	(19) Myricitrin
 FLAVONES	7,3',4'-Tri-hydroxyflavone*	(7,3',4',5'-Tetrahydroxy-flavone)**	(20) Luteolin*	(21) Tricetin*
 CHALCONES	(22) (–)-Butein	(23) (–)-Robtein	2',4',6',3,4-Pentahydroxy chalcone*	(2',4',6',3,4,5-Hexahydroxy-chalcone)**

* Not identified in wattle.

** Not yet adequately identified in nature.

tive effects at competing reactive centres.[27-34] These *in vitro* biomimetic-type condensation reactions have provided synthetic proof of the coupling sequence and absolute configuration of the reaction products. These condensed proanthyocyanidins consist of multiple flavan-3-ol units with 4,6- or 4,8-interflavan linkages. They originate from

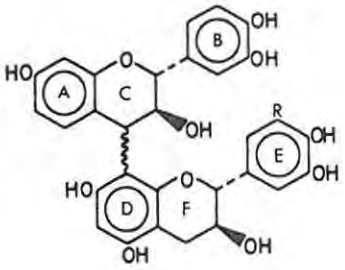


Flavanoid skeleton

the condensation of either (+)-leucorobinetinidin (6) or (+)-leucofisetinidin (5) with (+)-catechin (3) or (+)-gallocatechin (4). The flavan-3,4-diols (with nucleophilic 6- and 8-positions; electrophilic 4-position) are potential electrophiles and participate in electrophilic substitution reactions with the flavan-3-ols (with nucleophilic 4-, 6- and 8-positions). Substitution at C-8 precedes that at C-6.[31,33] The flavan-3-ols cannot initiate electrophilic addition reaction due to their inability to form 4-carbocations and, in this sense, act as 'terminators' to these oligomeric condensations.[35,36] The flavonoids with a 4-carbonyl functional group (see Fig. 1) are excluded from these condensations since the 4-carbonyl group prevents condensation at this point and deactivates the A-ring towards electrophilic attack.[33] The molecular gradation of oligomers ranges from 300 to 3 000 a.m.u. with a number-average mass of approximately 1 250 a.m.u.[37] This indicates an average oligomer consisting of approximately four flavan-3-ol units.

To date, some of the synthetic products enumerated below have been correlated with their natural counterparts in wattle extract by comparison of their high resolution n.m.r. spectra.[30,33] The comparisons have proved feasible only after tedious chromatographic separations of components. Methylation and acetylation of these components, to give the methyl ether diacetates, are used in these n.m.r. comparisons. No direct identifications have been made of the free phenolic oligoflavanoids because of their susceptibility to oxidative degradation during separation.

Paper chromatograms of wattle extract show a definitive pattern (see Fig. 3, p. 23). The spots locate discrete areas of homogeneous isomers. Investigations have revealed that areas F/A, B and D are biflavanoids,[30] while C and E are triflavanoids.[33] Area F/A contains two (—)-fisetinidol—(+)-catechin biflavanoids, (24) and (25).[30]

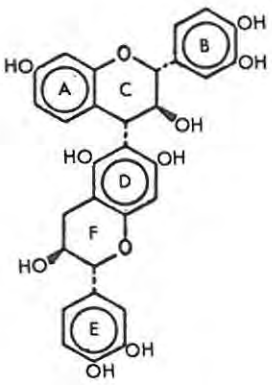


§ = ┆ or ▲

			Abbreviation
(24)	▲	,R = H	[4,8]-*F-cis-C*
(25)	┆	,R = H	[4,8]-*F-trans-C*
(26)	▲	,R = OH	[4,8]-*F-cis-G*
(27)	┆	,R = OH	[4,8]-*F-trans-G*

where :
F = (—)-Fisetinidol (1),
C = (+)-Catechin (3), and
G = (+)-Gallocatechin (4).

The [4,8]-2,3-*trans*-3,4-*cis*:2,3-*trans*-(—)-fisetinidol-(+)-catechin (24) biflavanoid is the major while the all-*trans*-isomer (25) is the minor component of A. The [4,6]-all-*trans* structural isomer (28), produced under synthetic conditions, has not been observed in area A.[30]

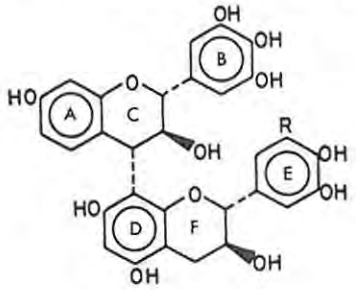


Abbreviation

(28) [4,6]-*F-trans-C*

No (—)-fisetinidol-(+)-gallocatechin biflavanoids, (26) and (27), have been synthesized or identified in the natural extract.

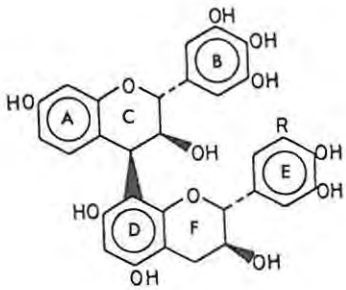
Areas B and D contain the [4,8]-all-*trans*-(—)-robinetinidol-(+)-catechin (29) and the [4,8]-all-*trans*-(—)-robinetinidol-(+)-gallocatechin (30) biflavanoids, respectively.[30]



		Abbreviation
(29)	R = H	[4,8]-*R-trans-C*
(30)	R = OH	[4,8]-*R-trans-G*

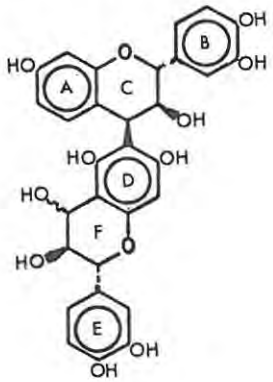
where :
R = (—)-Robinetinidol (2).

Neither the [4,8]-3,4-*cis*-diastereoisomer (31), nor the [4,6]-all-*trans*-diastereoisomer, analogous to [4,6]-*F-trans-C* (28) have been isolated from areas B and D although these are major products of the synthetic reaction of (+)-leucorobinetinidin (6) and (+)-catechin (3).[30] The related (+)-gallocatechin (4) reaction has not been reported.



		Abbreviation
(31)	R = H	[4,8]-*R-cis-C*
(32)	R = OH	[4,8]-*R-cis-G*

Four diastereoisomeric biflavanoid homologues, [4,6]-(—)-fisetinidol-(+)-leucofisetinidins, have been isolated from the (+)-leucofisetinidin rich heartwood of the black wattle tree.[32] These are unique in both their terminal 3,4-diol function and 4,6-linkage. Of these, the two major homologues have been shown to possess 2,3-*trans*-3,4-*trans*:2',3'-*trans*-3',4'-*trans* (33) and 2,3-*trans*-3,4-*cis*:2',3'-*trans*-3',4'-*cis* (34) configurations. These have not been previously detected in bark extract.



		Abbreviation
(33)	{ = ¦	[4,6]-*F-cis-LF*
(34)	{ = ▌	[4,6]-*F-cis-LF'*

where :
LF = (+)-Leucofisetinidin (5).

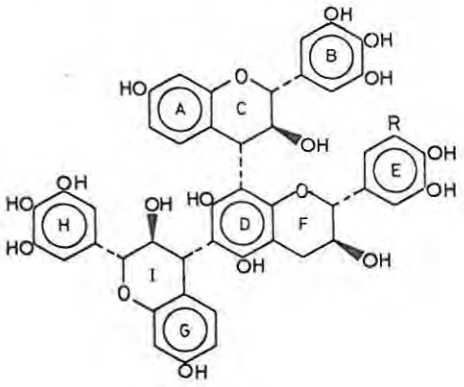
The triflavanoid area C consists of a mixture of three 'angular' triflavanoid diastereoisomeric bi-[(–)-robinetinidol]-(+)-catechins (35), (36) and (37), in the following proportions:[33]

	Relative Amounts
(35) [4,6:4,8]-all-*trans*-isomer	3,2
(36) [4,6:4,8]-2,3-*trans*-3,4-*cis*:2',3'-*trans*: 2",3"-*trans*-3",4"-*trans*-isomer	3,6
(37) [4,6:4,8]-2,3-*trans*-3,4-*cis*:2',3'-*trans*: 2",3"-*trans*-3",4"-*cis*-isomer	1,0

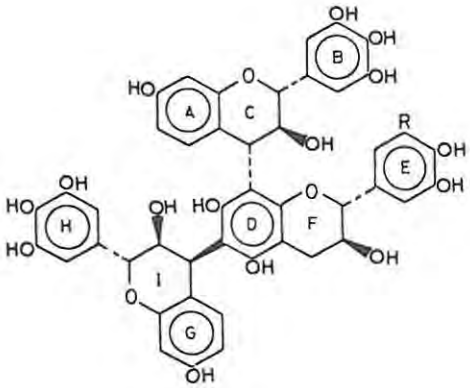
The homogeneous area E consists of the two 'angular' triflavanoid diastereoisomeric bi-[(–)-robinetinidol]-(+)-gallocatechins (38) and (39), in the following proportions:

	Relative Amounts
(38) [4,6:4,8]-all-*trans*-isomer	1,8
(39) [4,6:4,8]-2,3-*trans*-3,4-*cis*:2',3'-*trans*: 2",3"-*trans*-3",4"-*trans*-isomer	1,0

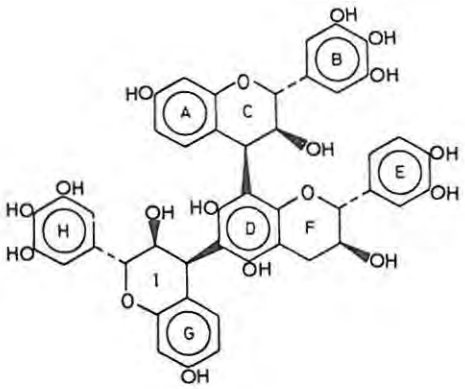
The all-*cis*-isomer does not occur, probably due to steric interactions of the B-moiety of (+)-gallocatechin.[33]



		Abbreviation
(35)	R = H	[*4,6:4,8*]-*R-trans-C-trans-R*
(38)	R = OH	[*4,6:4,8*]-*R-trans-G-trans-R*



		Abbreviation
(36)	R = H	[*4,6:4,8*]-*R-trans-C-cis-R*
(39)	R = OH	[*4,6:4,8*]-*R-trans-G-cis-R*



(37) Abbreviation [4,6;4,8]-*R-cis-C-cis-R*

The triflavanoids are hence made up exclusively of prorobinetinidins (35) - (39), while the biflavanoids include both profisetinidins (24) and (25) and predominant prorobinetinidins (29) and (30). A profisetinidin tetramer has recently been synthesized.[34] The analogous prorobinetinidin tetramer has not yet been synthesized or identified in the natural product.

Gel chromatography has found recent application in preparative separations of synthetic oligomers (D.G. Roux, personal communication). Sephadex polydextran gels generally evoke a molecular sieving mechanism and the solutes should elute in order of decreasing molecular size. However, aromatic compounds, and particularly phenols, experience gel-solute interactions and separations are achieved by an adsorption mechanism.[38] If Sephadex G-25 is used to separate flavonoids, a basic aqueous eluent is required to provide solute mobility.[39] Organic solvents can, however, be used with Sephadex LH-20, the hydroxypropylated form of G-25. The fractionation range is 200 to 3 000 although the exclusion limit can vary from 2 000 to 10 000, depending on the mobile phase composition.[40] With methanol, the degree of adsorption of flavonoid aglycones depends on the number of hydroxyl groups.[41] The later eluting solutes are, therefore, more highly hydroxylated. A similar correlation has been found on ethanolic elution of plant procyanidins.[42] The order of elution was found to be first flavan-3-ols, then procyanidin dimers and finally procyanidin oligomers.

In general, paper chromatography lacks the resolution, speed, and quantitative accuracy needed for the fast, reliable analysis of complex mixtures. While TLC offers greater resolution and speed it too lacks quantative features. Both gas and gas liquid chromatography are better techniques, provided an analyte is volatile enough. The plant phenols,

however, are relatively non-volatile and require derivitization. Monoflavonoid derivatives, notably the trimethylsilyls, are amenable to gas chromatography,[43-46] but oligomers have too high a molecular mass to be derivitized to suitable volatility. Thermal degradation and isomerization is also possible with such heat labile compounds.

The complimentary high-performance liquid chromatography (HPLC) provides a versatile means of rapid separation, identification and quantitation of non-volatile labile analytes.[47,48] Derivitization is obviated and numerous detector systems are available,[48,49] with UV being best suited for flavonoids.[50] Highly polar substrates are often irreversibly retained on normal-phase (silica) stationary media. Reversed-phase columns, particularly the popular octadecylsilyl silica, (*e.g.* μBondapak C_{18}) solves this problem as it allows for the alternate use of a broad spectrum of mobile phases which enable the column to be cleaned and re-used.[51]

Reversed-phase HPLC has resulted in the resolution and analysis of a wide range of flavonoids and related phenolic compounds,[52-55] notably in tea,[56] flavouring compounds,[57] ciders and wines,[58] apple juices,[59] propolis,[60] and plant ellagitannins.[61] While a wide data base has been accumulated for retention time and relative elution sequences of numerous flavonoid monomers,[62,63] only limited information is available on condensed flavanoid oligomers.[58,64]

Mobility has been interpreted as a function of flavonoid shape and resultant solvent interactions.[54] For example, planar molecules should prove more difficult to solvate, resulting in longer retention times. Studies with polymethoxylated flavones showed that adsorption, not size, is operative.[63,65] Elution sequence has been interpreted on the grounds that the compound is first adsorbed on the hydrophobic stationary phase by 'hydrophobic interaction' with subsequent elution being governed by the extent of hydrogen-bond formation with the mobile phase.[62] This theory is based on the important solvent studies pioneered by Rohrschneider[66] and Snyder,[67] who developed empirical quantitative relationships for the strength ('polarity') and selectivity of 84 solvents. Each solvent has the ability to interact with the solute as a proton-donor, proton-acceptor or dipole. These values, represented on the Snyder solvent selectivity triangle, are useful for selecting solvents and solvent mixtures that will exhibit different chemical interactions

with the solute. Techniques for the interactive mixture-design of multi-component mobile phases, have resulted in selectivity and solvent strength optimizations.[68]

The major drawback to acceptance of HPLC in official methods has been the wide variation in the properties of reversed phase media with the 'same' composition.[69] This variation is due to the different silylation techniques which are used to fix the bonded phase and to cap the residual silica hydroxy groups.[70] A retention index scale has been proposed to define the retention specifications of a column and to quantitatively differentiate between phases coated with different alkyl chains and phenyl groups.[71] The retention index values of homologous series of the alkylarylketones (PhCOR) on the column, can be compared with a theoretical hexane-water partition chromatograph to give a set of retention constants. Despite this variation in retention for different reversed-phase columns, reported elution sequences of flavonoids indicate that, under isocratic conditions, elution sequence remains constant.[62,63,65] Hence, if standards are available, the variation in retention times is not a serious problem. No HPLC study on wattle extract has as yet been published.

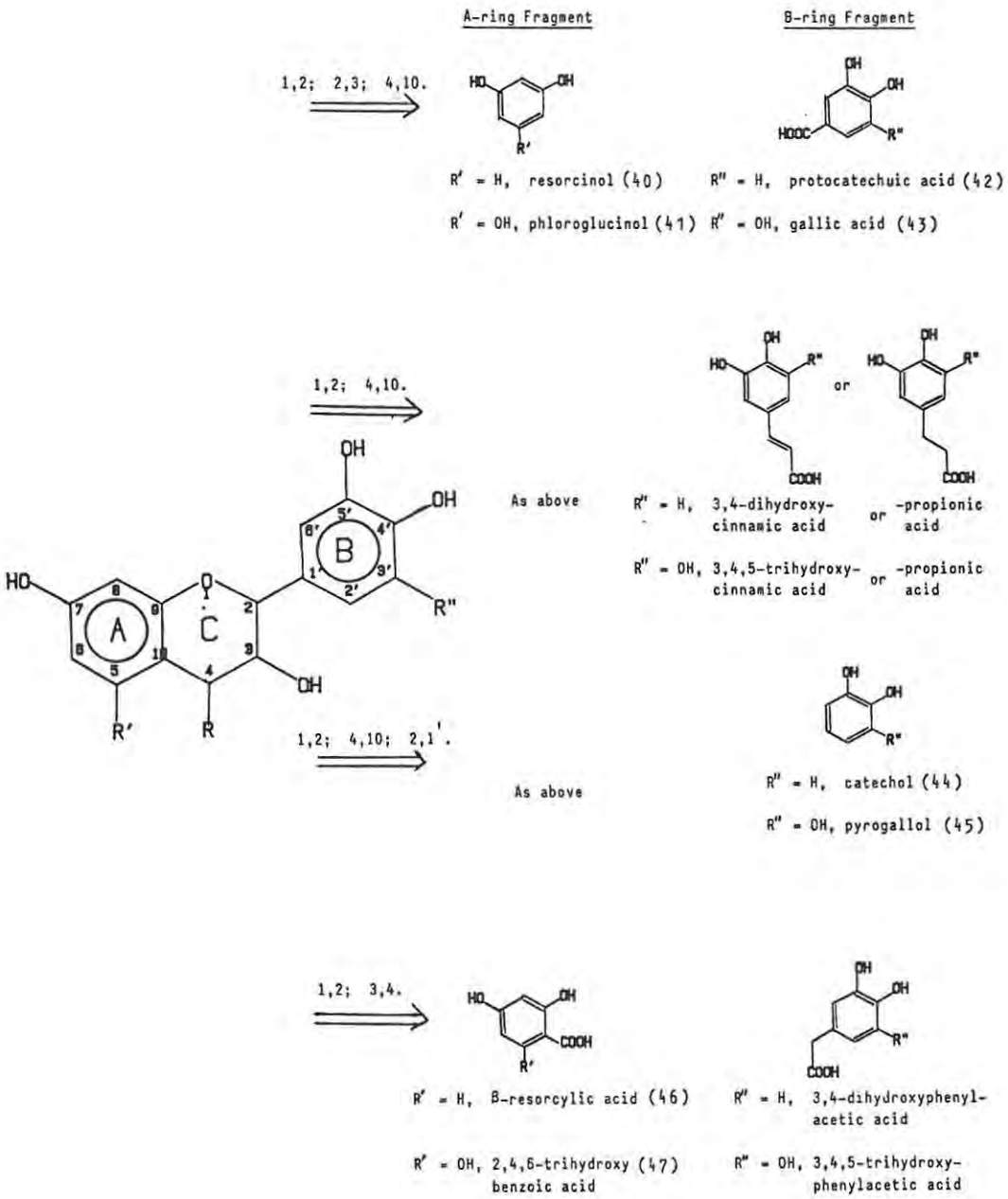
Hence, a preliminary HPLC investigation on wattle extract was initially carried out prior to assessing HPLC as a method for monitoring wattle-based wastewaters.

1.3 Polyphenolic degradations and classical wastewater analytical methods

Ultra-violet irradiation of flavonoids leads mainly to photochemical opening of the heterocyclic ring or to complete photofragmentation.[72] Chemical degradation, *e.g.* alkali fusion, produces simpler phenols and phenolic acids.[73]

Biological degradation of monomeric flavonoids is well documented with most metabolic studies having been performed with soil microbes and intestinal microflora.[74-77] Evidence shows that although only a few degradative mechanisms have evolved in micro-organisms, these are sufficient for the catabolism of a large variety of different phenolic structures.[78,79] This normally leads to simpler phenols, phenolic and dibasic acids, which are themselves eventually degraded to CO_2 (see Fig. 2). All flavonoids possessing free 5- and/or 7- hydroxyl groups in the A-ring and a free 4'-hydroxyl group in the B-ring, give ring fission products.[75]

Figure 2. Mononuclear phenolic cleavage products from feasible intra-flavan bond disconnections.



Absence of a hydroxyl group or methylation in these positions gave no degradation products in this intestinal microflora study.

Catabolic studies on condensed tannins and other polymers have largely been neglected although one study showed that a fungal type (*Penicillium adametzi* Zaleski strain) is capable of degrading low-molecular mass procyanidin oligomers.[9] A study on a biological treatment of a wastewater containing condensed tannins also indicated that degradation by fungi took place.[80]

An alternative 'degradation' pathway is abiotic oxidative polymerization resulting in larger or different polymers.[13] With dissolved catechol (44) and other catechol and pyrogallol (45) derivatives, a variety of transition metal oxides and cations, typically found in natural ecosystems, promote polymerization. These 'browning reactions' were found to be enhanced in natural stream waters, probably due to oxidative catalyzing effects of additional cations or oxides not identified in the above study. The mechanism appears to incorporate the radical $^{\bullet}OH$ from the activation of dissolved O_2. This in turn reacts with phenols to give various phenolic radicals which initiate polymerization. Sephadex G-25 gel chromatography with water as eluent estimated the 'red-brown' polymers from oxidative polymerization of phenols to have a molecular range from 500 to 1 500 a.m.u.[13] Tannin polymers, on the other hand, require a basic eluent to achieve mobility on the G-25 gel,[39] indicating that they are different from the 'red-brown' polymers.

Catechols polymerized by oxidation have been postulated as a prototype for humic material,[81] although they have an excess of aromatic carbon-hydrogen bonds relative to natural humic materials.[13] Photo-oxidation of phenol, catalysed by trivalent iron, also produces polymeric material.[82] Similar polymerization occurs on ageing red wines, with the red anthocyanin pigments, *ca.* 500 a.m.u., forming brick-red and tawny pigments, with average molecular masses as high as 50 000 a.m.u..[83]

It is possible that non-specific humic acid-type polymers could form on discharging spent phenolic wastes to natural waters. The non-specific analytical methods, chemical oxygen demand (COD), permanganate value (PV) and biological oxygen demand (BOD), of conventional water analysis would be suitable for monitoring such humic material. Whilst single parameter values, such as COD, PV or BOD, may lead to oversimplification or mis-

interpretation of the pollutant constitution, a combination of these analyses is able to typecaste the pollutant characteristics of a wastewater and give an indication of biodegradability.[14,84-87]

The chemical oxidation methods, *viz.* COD[88] and PV[89] measure the oxygen equivalent of the oxidizable constituents in a wastewater sample. The COD method (which utilizes acidic dichromate), is more vigorous than the PV method, (which utilises acidic potassium permanganate) and oxidizes phenols to CO_2 with ease. Many organic compounds are not oxidized by permanganate but phenols are susceptible, although the reaction does not go to completion.[90] These tests, however, do not validate the biodegradability of phenols.

The biological oxygen demand (BOD) method,[88] originally developed as an indicator of the effect of treated sewage upon the dissolved oxygen in receiving waters,[91] is also important as a measure of the biodegradable substrate concentration. It has been argued that the BOD value is as meaningful and useful as a complicated direct determination of the various components of a mixed substrate.[87] However, the BOD method has been found to underestimate substrate concentrations.[14,87] This method is dependent on the substrate being oxidized, the inoculated microbes involved and the initial dilution of the sample. The major criticism is that the method relies on a static-degradation oxidation process.[92] In reality, the reaeration of a stream or biological reactor is a dynamic oxidation process. Misinterpretation or abuse of the test occurs when a low BOD alone classifies an effluent as good.[15] The substrate may be non-biodegradable, which would reflect in the BOD:COD ratio. In addition, acclimatized water used for dilution in the BOD method could result in a higher value due to the resilience of the inoculated bacteria.[15] The method is also potentially open to interference from auto-oxidation of phenols, thereby abiotically removing oxygen from the system.[93]

Corning[95] has adapted Roux's spectrophotometric ultra-violet (UV) method[94] for vegetable phenolic determinations in tannery wastewaters. Roux established a direct relationship between the wattle flavanoid Band II chromophore at 280 nm[96] and the tannin content as determined by the standard hide-powder method from numerous UV/tannin correlations.[97,98] This method is, however, open to interference from the presence of other wastewater organic compounds which absorb at 280 nm. Corning's variation is based

on this 280 nm measurement before and after the filtered samples have been treated with lead acetate to precipitate out the vegetable phenols. This difference is expressed in terms (mg/ℓ) of a standard vegetable tannin. The major drawback inherent in this method is that the average extinction coefficient of the phenolic substrate rises with oxidation,[17] giving inflated results.

A method that is sensitive to phenols has been adopted by the American Public Health Association for tannins and lignins.[88] Not to be confused with Slabbert's colorimetric molybdate method for tannins,[99] this method involves the reduction of Folin-Ciocalteu (F-C) reagent,[100] containing the 2:18 molybdotungstophosphoric heteropoly anionic species,[101,102] by phenolic material to form the mixed valence heteropoly blue species.[103,104] Utilizing a wattle calibration curve, Corning found that the F-C method on vegetable tannery wastewater samples, gave results which were less than half the values determined by the UV method.[95] Together with possible UV interferences, this lower result can be ascribed to heteropoly anions not being reduced by oxidized phenols. The 2:18 heteropoly anions only oxidize phenols to their quinoidal stage.[105] Hence the UV and F-C methods are not ideal monitoring methods for phenolic wastewaters.

Determination of volatile phenols (steam distillable) in wastewaters containing various vegetable tannins have shown negligible contents (less than 0,1 mg/ℓ).[106] The standard hide-powder method[7] and the newly proposed alternative cellulose method[8] for tannin analysis were developed specifically to analyse the tannin content of solutions containing approximately 0,45% (m/v) of phenolic tannins, whereas integrated tannery wastewaters typically contain less than 1 000 mg/ℓ (*i.e.* 0,01%) of phenolic constituents, mainly non-tannin. Hence these methods would also not be suitable for monitoring wastewaters containing wattle extract.

The present investigation is concerned with the development of specific techniques for the analysis of wattle polyphenols in solutions and the application of classical analytical methods to wattle-based wastewaters in order to establish a meaningful basis for interpretation.

2 EXPERIMENTAL

2.1 Materials

Distilled, de-ionized water and analytical grade chemicals and solvents were used throughout this investigation.

The following simple mononuclear phenols were purchased from commercial sources: resorcinol (Merck, extra pure), phloroglucinol (Merck, extra pure), catechol (Merck, analytical reagent), pyrogallol (Merck, analytical reagent), gallic acid (May and Baker, analytical reagent), protocatechuic acid (Kock-Light, pure), 2,6-dihydroxybenzoic acid (Kock-Light, pure), 3,5-dihydroxybenzoic acid (Koch-Light, pure), quinhydrone (BDH, analar), hydroquinone (May and Baker, pure) and *p*-benzoquinone (BDH, analar).

The following wattle-type flavonoids were available, (+)-catechin (Koch-Light), (±)-dihydroquercetin (Fluka AG) and quercetin (L. Light). Samples of the reference compounds (+)-dihydrorobinetin, (+)-leucorobinetinidin, (+)-fustin and fisetin were kindly supplied by Prof. D.G.Roux. Impure samples of fisetin, (±)-fustin, robinetin and (+)-leucofisetinidin were available at Leather Industries Research Institute. These samples were purified by dissolving in ethanol and chromatogramming on Sephadex LH-20 with ethanol eluent. Concentrations were calculated from published molar extinction coefficients.[107] All compounds (pure by HPLC) were used without further purification.

Wattle extract, prepared by methanolic extraction of fresh mature black wattle bark, followed by concentrating to dryness by rotary evaporation,[107] was found to contain 74% of wattle tannin both by the hide-powder,[7] and UV photometric methods.[94] Mimosa M E (*ex.* NTE), the commercial spray-dried aqueous extract of wattle bark contained 66% of wattle tannin. This commercial extract, aqueously leached at high temperatures (90°C) is more likely to contain some decomposition products than the methanolic extract (64°C), which also contains less gums. Hence, the methanolic extract was used in the detailed chromatographic study whereas Mimosa M E and spent wattle tanning liquors from a pilot plant process at Leather Industries Research Institute were used in the wastewater study.

2.2 Chromatographic Separations

2.2.1 Two-dimensional paper chromatography

Whatman No. 3 paper (44 cm square) was used for the two-dimensional chromatography of the wattle extract and its separated flavonoids (see Sections 2.2.2 and 2.2.3). The two dimensions were achieved by first a downward migration using water-saturated butan-2-ol (20 h) followed by an upward migration with 2% (v/v) aqueous acetic acid (4 h). After each development, the chromatograms were dried.

The following selective spray reagents were used:

(i) Ammoniacal silver nitrate. Silver nitrate (14 g) was dissolved in water (100 mℓ) and ammonium hydroxide (6*M*) added until the precipitated silver oxide produced, had dissolved.

(ii) Bis-diazotized benzidine. This reagent consisted of two solutions: (a) Benzidine hydrochloride (6 g) was stirred with conc. HCl (14 mℓ) and the suspension made up to 1 ℓ with water. (b) Sodium nitrite (10 g) was dissolved in water and made up to 100 mℓ. Two parts (b) were added to three parts (a) and the reagent used immediately.

Sample spots of the separated wattle components were extracted for HPLC identification as follows: a matching chromatogram, sprayed to identify the pattern, was used to locate the required areas on an unsprayed chromatogram. The spot was cut out and macerated in ethanol-water (2 mℓ of 1:1). After immersion in an ultra-sound bath and subsequent syringe filtration (0,45 μm teflon filter), clean samples were obtained.

2.2.2 Gel chromatography

A glass column (1,6 x 90 cm) was packed with Sephadex LH-20 (40 g), previously swollen with ethanol (3 h). Concentrated wattle extracts (0,5 g/10 mℓ ethanol) were placed on the column and the whole eluted with ethanol (0,3 mℓ/min). Fractions (3 mℓ) were collected *via* a Beckman Model 132 refrigerated (0°C) fraction collector and their absorbance monitored (60 μl mircocuvette flow-cell housed in a Beckman ACTA CII spectrophotometer interfaced with a W & W 1 100 flatbed recorder) at 282 nm. When absorbance exceeded 1,5,the relevant fractions were diluted with ethanol and remeasured. After elution, the Sephadex LH-20 gel was washed with acetone-water (1:1), dried and re-used. The void volume, V_o, was deter-

mined with polydextran blue, which is sparingly soluble in ethanol.

2.2.3 Liquid-liquid solvent extraction

Gel chromatography was performed on the total wattle extract and on three separated wattle extracts, prepared by liquid-liquid solvent extraction. Dry methanolic wattle extract (5 g) was dissolved in water (200 mℓ) and consecutively extracted with ether (4 x 100 mℓ). The aqueous phase was then consecutively extracted with ethyl acetate (4 x 100 mℓ). The three respective phases were vacuum concentrated to dryness in a Büchner rotary evaporator. The excess moisture was azeotropically removed by redissolving these extracts in ethanol (50 mℓ) and further vacuum concentrating with the water-bath temperature maintained below 40°C to minimize oxidation.

2.2.4 High-performance liquid chromatography

The HPLC apparatus comprised of Waters Associates modules, *viz.* two solvent delivery systems, Models 6 000A and M45, a Model 660 solvent programmer, a Model 440 dual absorbance detector which monitored the eluate at 280 and 254 nm and a μBondapak C_{18} steel column (30 cm x 4 mm i.d.). Retention times and chromatograms at both wavelengths were recorded with the Waters Data Module which had the facility of integrating the absorption counts on either one of the detection channels.

All solvents and samples were filtered through a Millipore teflon filter (0,45 um). The filter was initially wet with methanol to facilitate aqueous filtrations. Samples (10 - 25 μℓ) were injected using a Hamilton syringe. Peak spreading was minimized by 'sandwiching' the sample between two 25 μℓ water injections. Concentrations of sample constituents were determined by integration using catechin as a standard.

On each day, prior to any sample injecting, the column was washed with 50 mℓ of water and then equilibrated with the mobile phase. Depending on the composition of the mobile phase, the operating pressure range was 2 000 to 2 700 p.s.i. at a flow rate of 2 mℓ/min. All mobile phases contained 1% (v/v) acetic acid. At the end of each period of operation, the column was washed exhaustively with, and then stored in methanol-water (80:20). For chromatography terms and symbols, see Appendix 1.

2.3 Spectrophotometric Methods

A Beckman Model 35 Spectrophotometer and a matched pair of 1,000 cm quartz cuvettes were used for all UV and visible spectrophotometric measurements.

2.3.1 The ultra-violet (UV) method

Absorbance values were determined at 20°C against a water blank. The relevant extinction coefficients were utilized on both a molar and mg/ℓ basis with

$$A = \varepsilon cd \qquad \text{or} \qquad A = \alpha cd$$

where :

A = absorbance,
ε = molar extinction coefficient, $\ell.mol^{-1}.cm^{-1}$,
α = specific extinction coefficient, $\ell.mg^{-1}.cm^{-1}$, and
d = path length, cm.

α represents the absorbance of a 1 mg/ℓ solution of analyte. For aqueous wattle solutions,[99] α = 0,01283.

2.3.2 The Folin-Ciocalteu (F-C) method[105]

Folin-Ciocalteu reagent was prepared by dissolving sodium tungstate (100 g $Na_2WO_4.2H_2O$) plus sodium molybdate (25 g $Na_2MoO_4.2H_2O$) in distilled water (700 mℓ) in a 2 ℓ round-bottomed pyrex flask, which contained a few glass beads. 85% Phosphoric acid (50 mℓ) and conc. hydrochloric acid (100 mℓ) were added and a reflux condenser attached *via* an ungreased ground-glass joint. The solution was refluxed (10 h) and then cooled, after which distilled water (50 mℓ) was used to rinse down the inside of the condenser. With condenser removed, lithium sulphate (150 g $Li_2SO_4.H_2O$) was added. To oxidize any traces of molybdo-tungstophosphoric heteropoly blue, bromine (0,25 mℓ) was added and the solution boiled (15 min) without condenser to remove the excess bromine. The solution was cooled, diluted to 1 ℓ, filtered through sintered glass and stored in a glass stoppered bottle.

For the F-C method a measured volume of sample was introduced to a 100 mℓ volumetric flask and water was added to give a total of 75 mℓ. F-C reagent

(5,00 mℓ) was added and after 30 sec but before 8 min, sodium bicarbonate (15 mℓ of a 15% (m/v) solution) was added and the contents of the flask made to volume. The absorbance was measured at 765 nm after 2 h at 25°C. The total polyphenol content was calculated in catechin equivalents from a standard analytical calibration curve prepared with 0-300 μg catechin.

2.4 Carbonaceous Oxygen Demand Methods

2.4.1 Chemical oxygen demand (COD)[88]

A sample (20,0 mℓ) was pipetted into a 250 mℓ refluxing flask containing several glass beads. The following reagents were added in this order; mercuric sulphate (0,20 g), standard potassium dichromate (0,250*N*, 10,0 mℓ) and sulphuric acid reagent (30 mℓ of sulphuric acid that contained dissolved silver sulphate (0,30 g)).

After refluxing (2 h), the mixture was allowed to cool, then distilled water (100 mℓ) was added through the condenser. Cooled to room temperature, the excess of potassium dichromate was titrated with standard ferrous ammonium sulphate (0,250*N*) using ferroin indicator (3 drops), with the endpoint at the colour change from blue-green to reddish brown. A blank determination was also made, using distilled water (20,0 mℓ).

Calculation:

$$\text{mg/ℓ COD} = \frac{(A - B) \times N \times 8\,000}{\text{mℓ sample}}$$

where : A = volume of ferrous ammonium sulphate used for blank, mℓ,
B = volume of ferrous ammonium sulphate used for sample, mℓ, and
N = normality of ferrous ammonium sulphate.

2.4.2 Permanganate value (PV)[89]

A sample (100 mℓ) was pipetted into a 250 mℓ glass-stoppered bottle to which 25% (v/v) sulphuric acid (10 mℓ) and standard potassium permanganate (0,0125*N*, 20,0 mℓ) had been added. The bottle was maintained at 27°C in a constant-temperature water bath (4 h). Thereafter, potassium iodide (0,5 g) was added and the sample immediately titrated with standard sodium thiosulphate (0,0125*N*) to a pale straw colour. Starch solution (1 mℓ) was then added and the titration continued to the first disappearance of the blue colour. A blank determination was also made using distilled water (100 mℓ).

Calculation :

$$mg/\ell\ PV = (A - B)$$

where : A = volume of thiosulphate used for blank, mℓ, and
B = volume of thiosulphate used for sample, mℓ.

2.4.3 Biological oxygen demand (BOD) [88]

Dilution water was prepared by aeration of distilled water (20 ℓ) in a 25 ℓ plastic drum to which the following solutions (20 mℓ of each) had been added:

(a) Phosphate buffer solution (pH of 7,2) consisting of KH_2PO_4 (8,5 g), K_2HPO_4 (21,8 g), $Na_2HPO_4.7H_2O$ (33,4 g) and NH_4Cl (1,7 g) diluted to 1 ℓ.

(b) Magnesium sulphate solution consisting of $MgSO_4.\ 7H_2O$ (22,5 g) diluted to 1 ℓ.

(c) Calcium chloride solution consisting of $CaCl_2$ (22,5g) diluted to 1 ℓ.

(d) Ferric chloride solution consisting of $FeCl_3.6H_2O$ (0,25 g) diluted to 1 ℓ.

Prior to aeration, this dilution water was seeded with effluent (0,5 mℓ) from an activated sludge system, used to treat solutions of wattle extract.

Before performing the BOD method, the COD of the sample was first determined. The sample was diluted to give a COD of 5 - 10 mg/ℓ and then transferred to the 250 mℓ glass-stoppered BOD bottles. Enough sample was diluted to afford duplicate dissolved oxygen (DO) determinations.

The azide modification of the titrimetric iodometric method was used to determine DO. The following reagent solutions were used:

(a) Manganous sulphate solution consisting of $MnSO_4.4H_2O$ (480 g) diluted to 1 ℓ.

(b) Alkali-iodide-azide reagent consisting of sodium hydroxide (480 g) and sodium iodide (750 g) which were dissolved in a solution (500 mℓ) which contained sodium azide (10 g).

Manganous sulphate solution (1 mℓ), followed by alkali-iodide-azide reagent (1 mℓ), were added to the 250 mℓ BOD bottle. After carefully stoppering to exclude air, the sample was mixed by inverting several times. When the precipitate had settled, conc. sulphuric acid (1 mℓ) was added, the bottle restoppered and mixed by inverting several times until the precipitate had dissolved. The sample was titrated with standard sodium thiosulphate (0,0125*N*) to a pale straw colour. Starch solution (1 mℓ) was then added and the titration continued to the first disappearance of the blue colour.

Calculation :

For a titration of 200 mℓ sample,
1,00 mℓ 0,0125*N* sodium thiosulphate = 0,50 mg/ℓ DO.

For the BOD method, an initial DO was determined immediately after preparation on both the samples and the seeded dilution water controls. The remaining BOD bottles containing the desired dilutions and seeded dilution water controls were incubated at 20 ± 1°C; at the end of the required period (t) their DO's were determined.

Calculation :

$$\text{mg/ℓ } BOD_t = \frac{(D_1 - D_2) - (B_1 - B_2)f}{P}$$

where : D_1 = DO of diluted sample immediately after preparation (t = 0), mg/ℓ,
D_2 = DO of diluted sample after period (t = no. of days) of incubation at 20°C, mg/ℓ,
B_1 = DO of seeded dilution water immediately after preparation (t = 0), mg/ℓ,
B_2 = DO of seeded dilution water after incubation (t = no. of days), mg/ℓ,
f = ratio of seeded dilution water in sample : seeded dilution water in the control, and
P = decimal volumetric fraction of sample used.

3 RESULTS and DISCUSSION

3.1 Chromatographic Separations

3.1.1 Paper chromatography

Two-dimensional paper chromatography has traditionally been the main separation technique for wattle tannins. This technique was also used to study the constituents of process liquors during and after vegetable tanning with wattle extract.[17] A typical two-dimensional paper chromatogram of wattle extract showing the reported spots and labelling system used by previous workers is shown (Fig. 3) while the relevant R_f values of the wattle constituents that have been identified are listed (Table 1). The constituents with greater mobility are mainly the flavonoid monomers and biflavanoids,collectively known as the phenolic non-tannins.[17] The oligomeric tannins that have a higher affinity for cellulose (and collagen) are less mobile.

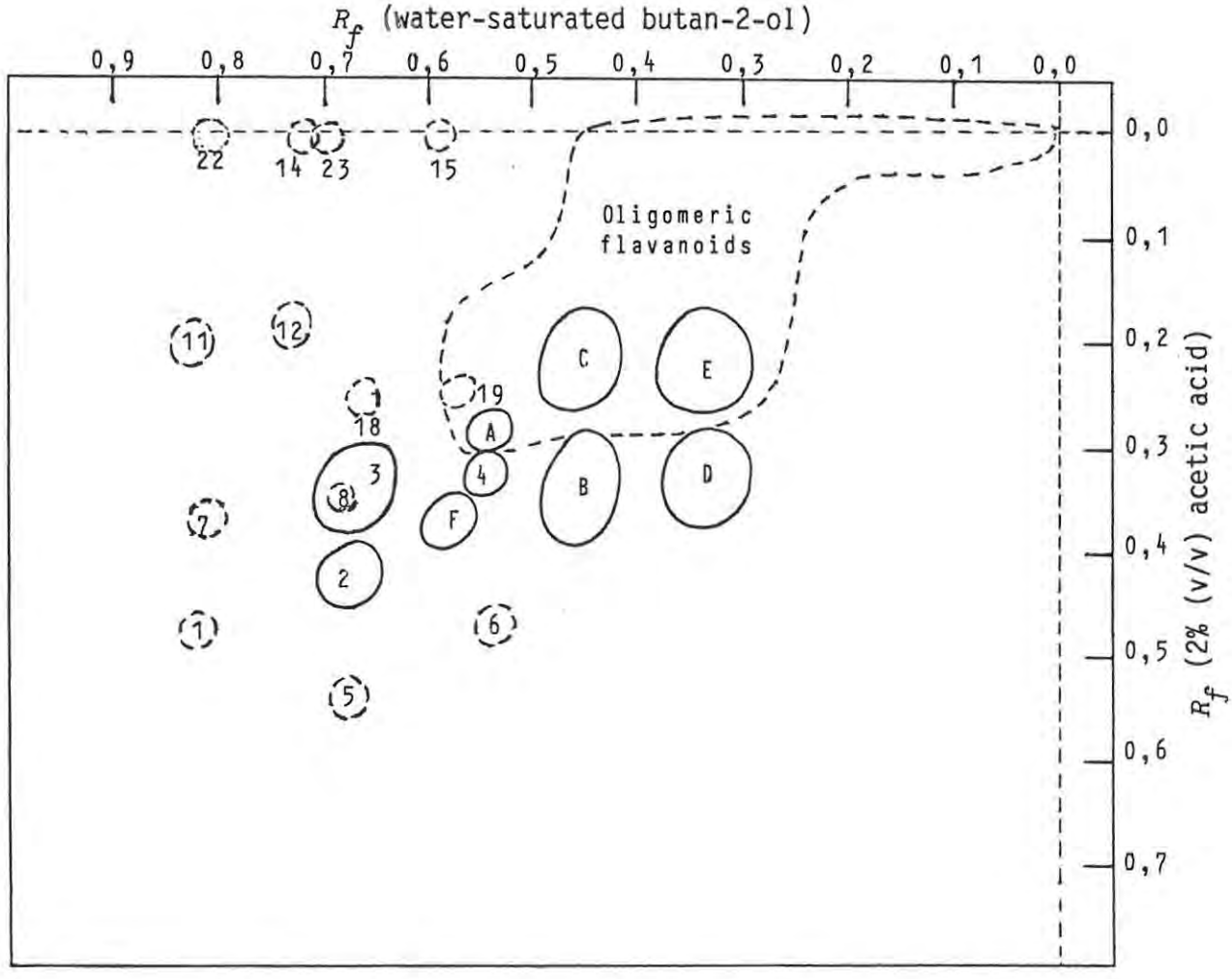


Figure 3. Relative positions of the wattle bark flavonoid components on a two-dimensional paper chromatogram.

Table 1. Paper R_f co-ordinates of wattle flavonoid constituents.

Compound	R_f Co-ordinates[a]	Reference
1. (—)-Fisetinidol	(0,82 ; 0,48)	109, 20
2. (—)-Robinetinidol	(0,67 ; 0,42)	19
3. (+)-Catechin	(0,67 ; 0,35)	18, 111
4. (+)-Gallocatechin	(0,54 : 0,32)	18
5. (+)-Leucofisetinidin	(0,68 ; 0,54)	19
6. (+)-Lecuorobinetinidin	(0,54 ; 0,47)	18, 110
7. (+)-Fustin	(0,81 ; 0,37)	18, 19
8. (+)-Dihydrorobinetin	(0,68 ; 0,35)	18, 110
11. (+)-Butin	(0,83 ; 0,20)	110
12. (+)-Robtin	(0,73 ; 0,18)	Estimated
14. Fisetin	(0,71 ; 0,00)	18
15. Robinetin	(0,59 ; 0,00)	18
18. Quercitrin	(0,66 ; 0,25)	110
19. Myricitrin	(0,57 ; 0,25)	110
22. (—)-Butein	(0,80 ; 0,00)	110
23. (—)-Robtein	(0,70 ; 0,00)	110
A. Biflavanoid	(0,54 ; 0,30)	17
B. Biflavanoid	(0,46 ; 0,34)	111
C. Triflavanoid	(0,46 ; 0,22)	17
D. Biflavanoid	(0,34 ; 0,33)	111
E. Triflavanoid	(0,34 ; 0,22)	17
F. Biflavanoid	(0,58 ; 0,37)	110

a (R_f with water-saturated butan-2-ol; R_f with 2% acetic acid).

The use of this method to monitor treated or untreated wastewaters from vegetable tanneries proved impractical as it failed to meet the requirements of rapid quantitative detection and identification of wattle constituents, especially in dilute solutions. Paper chromatography and other variants of chromatography reported below, were used to isolate and identify wattle constituents not readily available as standards.

3.1.2 HPLC of wattle extract

Although HPLC is a rapid separation technique, ideally suited for moni-

toring complex biological samples, like wattle extract, it has not yet been used to examine polyphenols from wattle extract. Before this technique could be applied to wattle phenols in wastewaters, therefore, a study on HPLC separations of wattle extract was carried out. Before any wattle polyphenols were subjected to HPLC, the μBondapak C_{18} column was typecaste with the alkylarylketone (PhCOR) series, acetophenone (R = Me), propiophenone (R = Et) and butyrophenone (R = Pr). With a mobile phase containing 50% methanol, the slope of this retention index scale was 0,00315, close to the 0,00319 reported[71] for an ODS-Hypersil column.

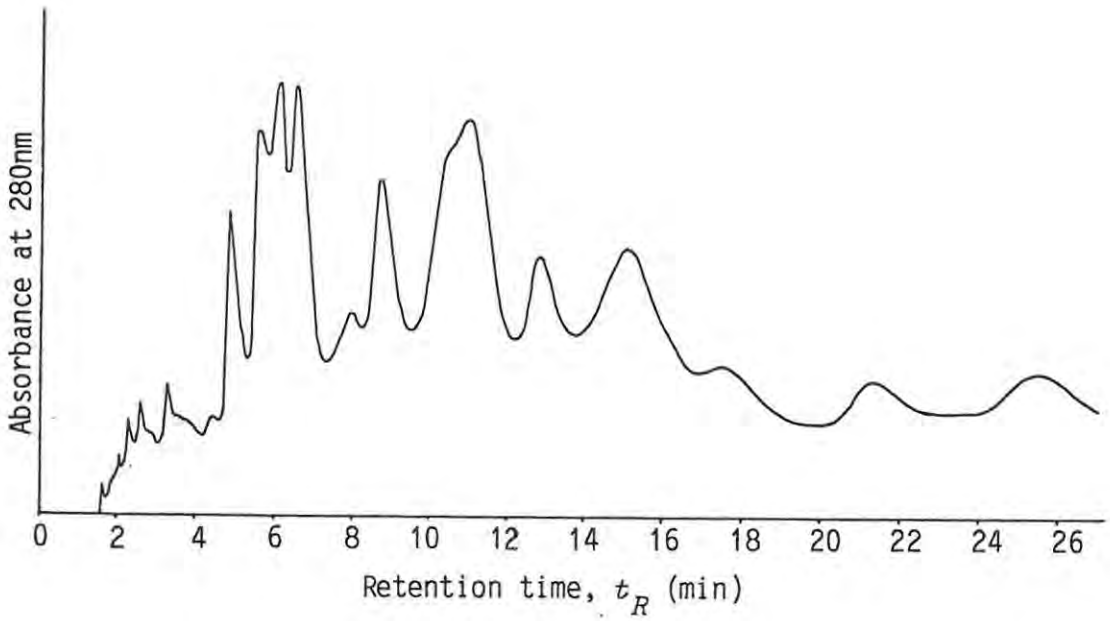


Figure 4. High-performance chromatogram of methanolic wattle extract. Column: μBondapak C_{18} steel column (30 cm x 4 mm i.d.). Sample: 15 ℓ of 6 010 mg/ℓ aqueous wattle extract. Mobile phase: methanol-water-acetic acid (15:84:1). Flow rate: 2 mℓ/min. UV detection channel: 280 nm. Sensitivity: 0,02 AUFS.

The chromatogram of wattle extract (Fig. 4) shows a separation pattern that is repeatable, with numerous semi-resolved peaks overlapping a wide, unresolved zone. Using the Snyder solvent selectivity triangle,[68] two mobile phases of similar strength but different selectivity were used. Both dioxane-water-acetic acid (10:89:1) and DMF-water-acetonitrile-acetic acid (15:83:1:1), however, did not simplify the separations.

3.1.3 Liquid-liquid solvent extraction

Solvent extraction with ether is known to concentrate the mobile, so-called

phenolic non-tannin, constituents of wattle extract,[112] with the oligomeric tannins remaining in the aqueous phase. To simplify the assignments of HPLC peaks to specific components, sequential extraction of an aqueous solution of wattle extract with ether and then ethyl acetate gave 4,2 and 70,2% dry matter, respectively. This was followed by gel chromatography of these extracts.

3.1.4 Gel chromatography

Although Sephadex LH-20 is an exclusion gel, adsorption is an important property on elution of phenolic components,[38] and flavanoids.[41]

Table 2. Elution characteristics of wattle-type flavonoids on Sephadex LH-20.

Compound	Substituents	V_R/V_o [a]	Reference
(+)-Catechin(3)	3,5,7,3',4'-OH	5,25	This work[b]
		5,2	41[c]
(+)-Leucofisetinidin(5)	3,4,7,3',4'-OH	5,27	This work
(+)-Leucorobinetinidin(6)	3,4,7,3',4',5'-OH	6,72	This work
(+)-Fustin(7)	3,7,3',4'-OH	4,88	This work
(±)-Dihydroquercetin(9)	3,5,7,3',4'-OH	5,57	This work
		5,5	41
Fisetin(14)	3,7,3',4'-OH	6,71	This work
		6,6	41
Robinetin(15)	3,7,3',4',5'-OH	7,45	This work
		7,4	41
Quercetin(16)	3,5,7,3',4'-OH	8,43	This work
		8,3	41
Myricetin(17)	3,5,7,3',4',5'-OH	9,2	41

a V_R/V_o = ratio of retention volume to void volume.

b Ethanol eluent.

c Methanol eluent.

The number of hydroxyl substituents influences the extent of adsorption and hence retention time and elution order.[40] Results for wattle-type flavonoids (Table 2) support this theory provided similar classes of flavonoids are compared; the latter component of the following related flavonoid pairs elute at a slower rate: (+)-leucofisetinidin/(+)-leucorobinetinidin, (+)-fustin/(±)-dihyroquercetin, fisetin/robinetin and quercetin/myricetin. Both methanol and ethanol gave similar results.

Intramolecular hydrogen-bonding also influences rate of elution.[41] The planar structure of the flavonols promotes intramolecular hydrogen-bonding between the 5-hydroxyl and 4-carbonyl groups, which decreases solubility in the polar alcoholic media. Therefore, quercetin and myricetin elute later than fisetin and robinetin, although robinetin and quercetin have the same number of hydroxyl substituents, indicating solubility is also an important elution factor. Lack of conjugation in the C-ring, *e.g.* for the flavanone (±)-dihydroquercetin, reduces this intramolecular hydrogen-bonding and hence it precedes the less soluble quercetin. (+)-Catechin, which has no 4-carbonyl functionality and therefore greater solubility, elutes just prior to (±)-dihydroquercetin. Interestingly, (+)-leucofisetinidin, with 2 alcoholic and 3 phenolic groups, and (+)-catechin, with 1 alcoholic and 4 phenolic groups, elute simultaneously indicating it is the total number and not the type of hydroxyl groups that is important.

The cyanidin and robinetinidin flavanoid analgoues should elute at about the same time, as both have 5 hydroxyl groups. The fisetinidin analogues, with 4 hydroxyls should elute first while the delphinidin analogues, with 6 hydroxyls, last. Overlap between the different flavonoid classes of these analogues occurs due to the different factors which influence rate of elution. For wattle, further complications can be expected with the oligomeric material. Ethanolic elution of plant procyanidins showed the order of elution to be first flavan-3-ol, then procyanidin dimers and finally the larger oligomers.[42] This also substantiates the importance of the adsorption property on gel chromatography of flavonoids.

When wattle extract was separated through a Sephadex LH-20 column with ethanol, a chromatogram containing numerous semi-resolved and unresolved bands was obtained (Fig. 5). Peak *15* marks the start of the poorly

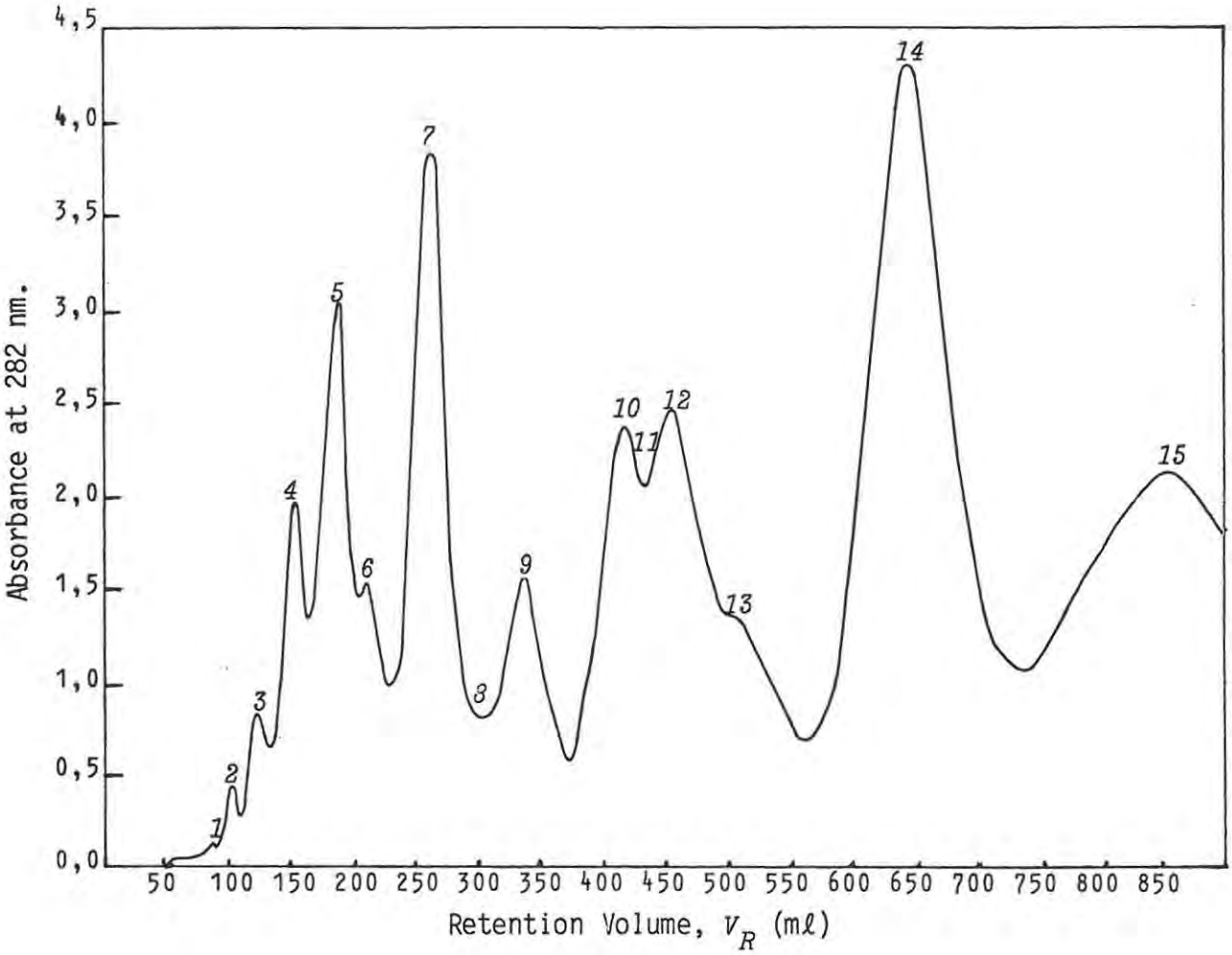


Figure 5. Gel chromatogram of methanolic wattle extract. Column: 1,6 x 90 cm contained 40 g Sephadex LH-20. Sample: 459,0 mg wattle extract dissolved in 5 mℓ ethanol. Eluent: ethanol. Flow rate: 0,3 mℓ/min. Fraction time: 10 min. V_o = 50 mℓ.

resolved region. During the first 50 h (V_R = 900 mℓ) and the next 150 h, 27,1 and 35,2%, respectively, of the phenolic material was eluted. The residue, stripped with acetone-water (1:1), amounted to 35,1% which gives a net loss of 2,6%. The regenerated Sephadex LH-20 was off-white in colour, accounting for some of the loss.

Relative quantities of the ether, ethyl acetate and aqueous extracts (*cf.* Section 3.1.3) of the total wattle extract were also separated by gel chromatography (Fig. 6). Three additional peaks, not obvious in the chromatogram of the total extract (Fig. 4) were resolved, *viz.* peak *11* appeared from the aqueous phase while the ether phase produced peak *8* and accentuated peak *13*.

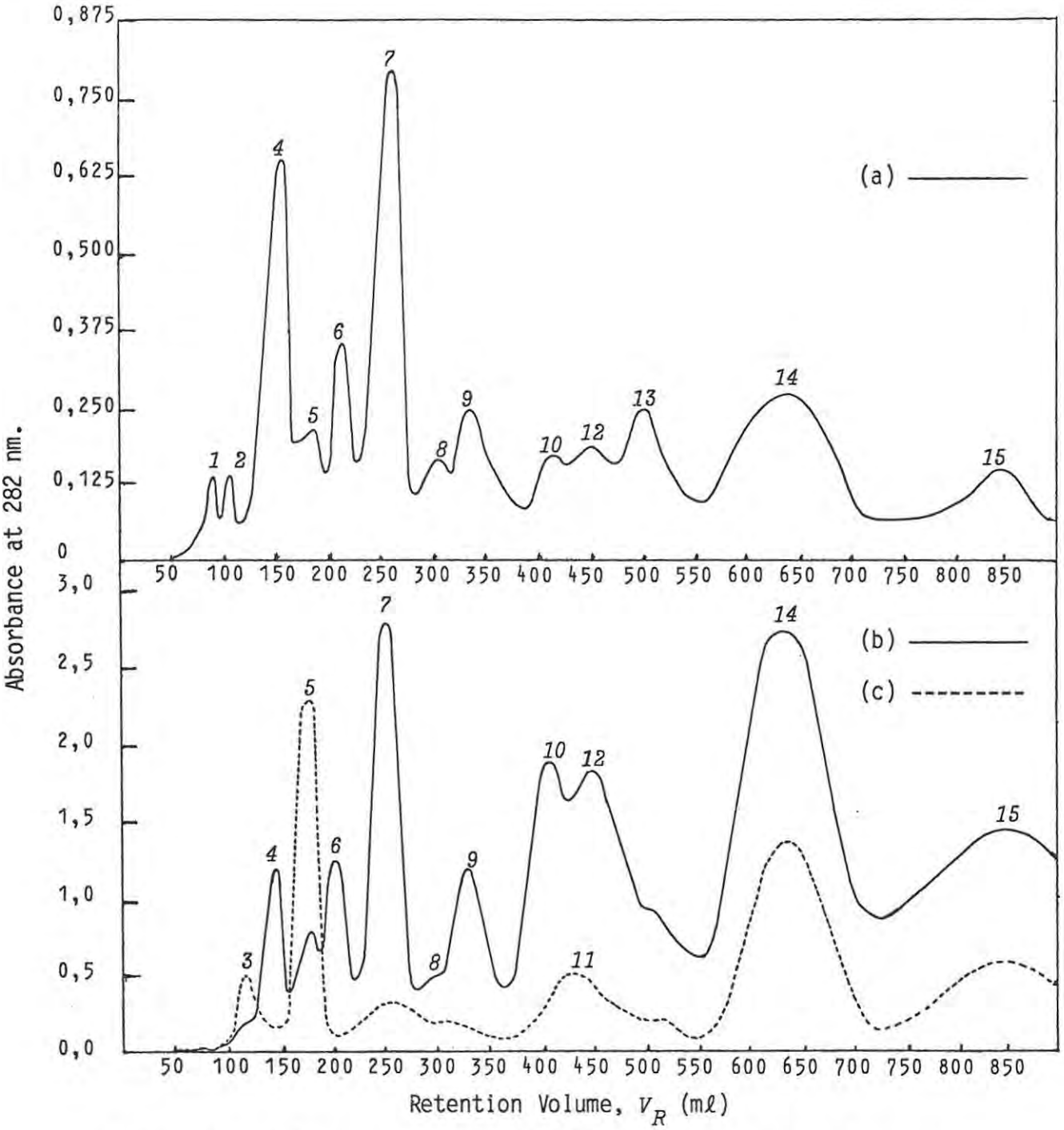


Figure 6. Gel chromatograms of solvent partitioned wattle extract. Separations of:

(a) ether soluble extract. Sample: 19,3 mg dissolved in 2 mℓ ethanol.
(b) ethyl acetate soluble extract. Sample: 321,9 mg dissolved in 5 mℓ ethanol.
(c) remaining aqueous extract. Sample: 117,4 mg dissolved in 5 mℓ ethanol.

Column: 1,6 x 90 cm contained 40 g Sephadex LH-20. Eluent: ethanol. Flow rate: 0,3 mℓ/min. Fraction time: 10 min. V_o = 50 mℓ.

3.1.5 HPLC of fractions from gel chromatography

In a preliminary study of the fractions, a strong mobile phase (containing 50% methanol) was used to detect components of high retention. A weaker mobile phase (*i.e.* of lower methanol concentration, typically 15%) effected separation of components with low retention.

HPLC of fractions obtained by gel chromatography showed that while most samples included a number of components, the resultant chromatograms were simple in comparison to that of total wattle extract (*cf.* Fig. 4). As consecutive fractions were analysed by HPLC, the various components indicated a gradient in concentration from fraction to fraction, thereby positioning the HPLC resolved component in the gel chromatogram peak. These separations were best enumerated by a system analogous to a two-dimensional paper chromatogram. The HPLC dimension was expressed as the catechin relative retention, r_{Cat}, where (+)-catechin had a k value of 3,0 with the mobile phase methanol-water-acetic acid (15:84:1). The gel chromatographic dimension was expressed as the ratio of retention volume to void volume, V_R/V_o. The components were labelled by a system which utilized the number of the gel chromatographic peak and the HPLC relative retention order of the specific component, *e.g.*, component *5c* is from gel peak *5* and elutes third (*c*) on HPLC.

The resultant two-dimensional chromatogram (gel-HPLC) of wattle extract shows that a total of 77 components were separated (Fig. 7). Most of these components, however, were minor, the majority being detected in the initial gel peaks of the ether extract, which itself amounted to only 4% of the total extract. The identification of these minor components were considered beyond the scope of this study. The present work concentrated on using HPLC to identify and quantitate the major flavanoid components present in wattle extract.

To establish these major components of the total wattle extract (Fig. 5), the fractions for each peak were combined and the components resolved by HPLC. The relative concentrations of the major components in the consecutive gel chromatographic peaks evidenced band spreading (Fig. 8). The sensitivity, determined by 25 $\mu\ell$ injections with absorbance set at 0,005 AUFS, was sufficient to detect 40 components (Table 3). Of these, 29 individually exceeded 0,1% and 12 exceeded 0,5% of the total wattle extract.

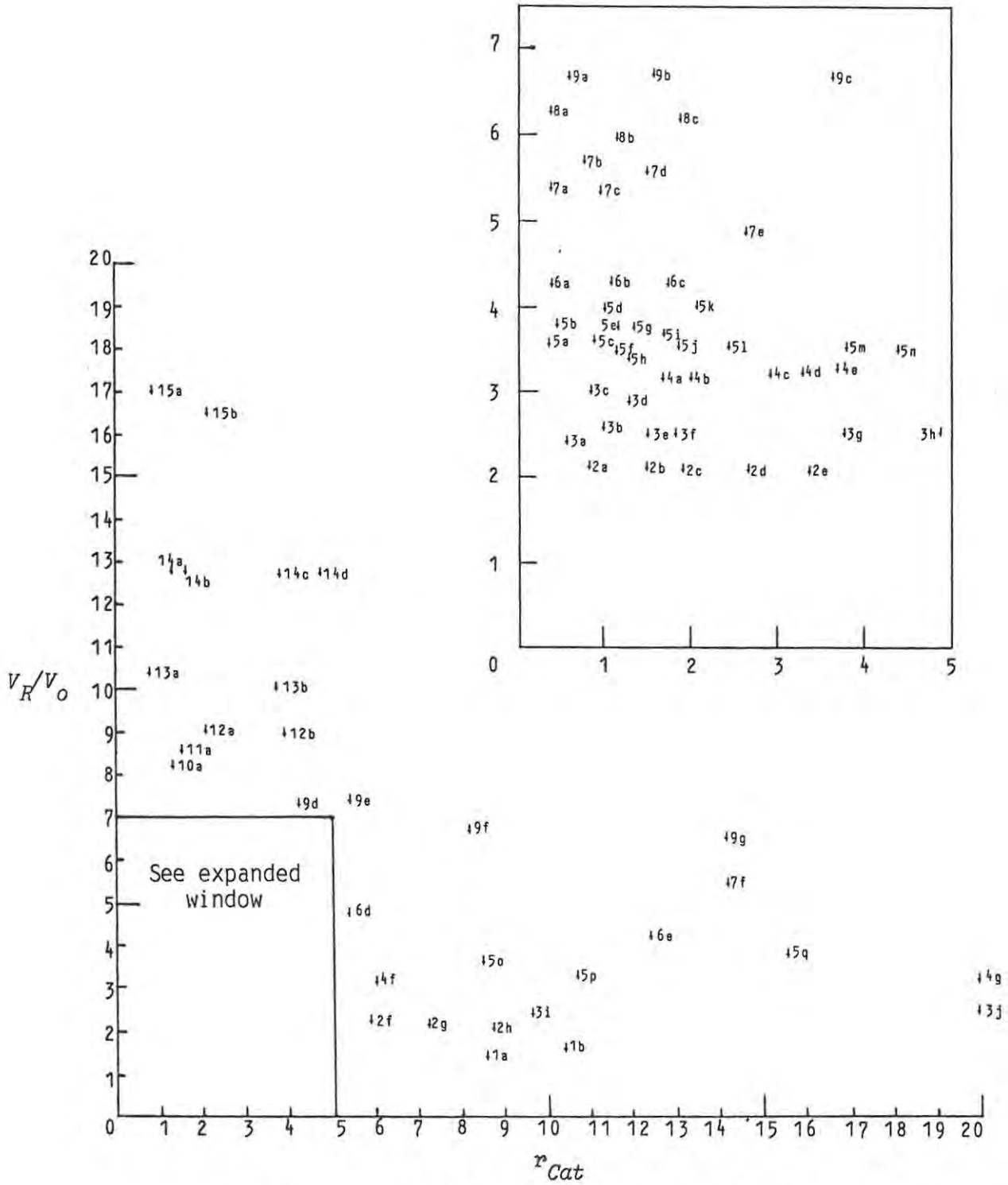


Figure 7. Two dimensional gel-HPLC chromatogram of wattle components. Y-dimension: Gel V_R/V_o value. X-dimension: HPLC r_{Cat} value.

Table 3. Comparison of gel peak integrations with the HPLC resolved component integrations for the total wattle extract.[a]

Gel Peak No.	Gel Peak Integration[b]	HPLC Component Integration[c]
1	0,04%	Not detected
2	0,11%	Not detected
3	0,31%	*3a* (0,025%); *3b* (0,153%); *3d* (0,030%); *3f* (0,025%); Loss (0,077%)[d]
4	0,84%	*4a* (0,097%); *4b* (0,305%); *4f*, *4g* (trace amounts) Loss (0,437%)
5	1,63%	*5a* (0,144%); *5b* (0,047%); *5c* (0,047%); *5d*, *5e*, *5f* unresolved 0,616%); *5g* (0,070%); *5h* (0,073%); *5i* (0,227%); *5j* (0,196%); *5k* (0,135%) Loss (0,077%)
6	0,67%	*6a* (0,168%); *6b* (0,230%); *7b* (0,051%); *7c* (0,051%); *6c* (0,065%) Loss (0,106%)
7	2,96%	*7a* (0,297%); *7b* (0,699%); *7c* (1,153%) *7d* (0,362%); *8b* (0,272%) Loss (0,177%)
9	1,44%	*8b* (0,128%); *9a* (0,266%); *9c* (0,802%); *10a* (0,126%); *11a* (0,033%) Loss (0,165%)
10	2,05%	*10a* (1,150%); *11a* (0,151%); *12a* (0,157%) Loss (0,232%)
12	2,42%	*10a* (0,272%); *11a* (0,286%); *12a* (1,408%); *13a* (0,266%); Loss (0,189%)
13	1,41%	*12a* (0,208%); *13a* (0,756%); *13b* (0,219%); *14c* (0,134%); Loss (0,093%)
14	7,79%	*14a* (0,763%); *14b* (5,264%); *14c* (0,612%); *14d* (0,394%) Loss (0,757%)
15	5,44%	*15a* (1,645%); *15b* (3,341%)[e] Loss (0,454%)

Footnotes P.T.O.

(Table 3 footnotes)

a The gel peaks of Fig. 5.

b Using the catechin equivalent.

c These components were detected using a maximum of 25 µl samples at 0,005 AUFS, the most sensitive detector setting. Comparison with a (+)-catechin standard enabled integration.

d Loss expressed as the difference between gel peak integration and sum of the HPLC component integrations.

e Peak *15* encroached beyond 900 ml so that these percentage values are underestimated, especially for *15a*.

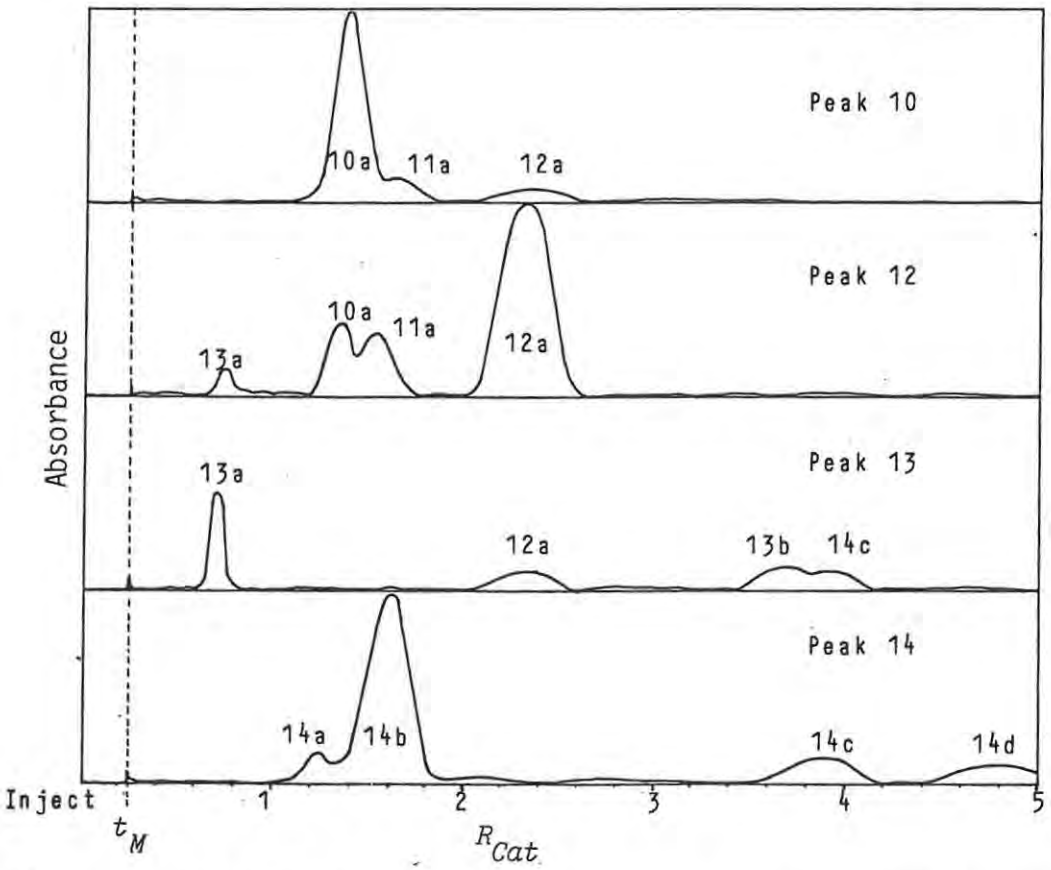
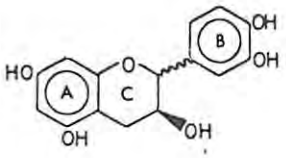


Figure 8. High-performance chromatograms of consecutive gel peaks. Mobile phase: methanol-water-acetic acid (15:84:1). Flow rate: 2 ml/min. Detector Channel: 280 nm. Sensitivity: 0,01 AUFS. Injection volume: 25 µl.

3.1.6 Identification of the monoflavonoids

In reversed-phase HPLC of flavonoids, polarity and concomitant solubility in the aqueous mobile phase are the predominant factors responsible for the mobility of a substrate.[62,63,65] These factors are in turn determined by the flavonoid shape, hydroxylation number and substitution pat-

tern. The following flavanoids, (–)-fisetinidol (1), (+)-leucofisetinidin (5), (+)-fustin (7), (–)-robinetinidol (2), (+)-leucorobinetinidin (6), (+)-dihydrorobinetin (8) and (+)-gallocatechin (4) (Fig. 1, p. 4), previously isolated and identified in wattle extract, all have the same absolute configuration at the C-2 and C-3 atoms as (+)-catechin (3),[19] *i.e.* 2R:3S with resultant 2,3-*trans* configuration.[113]



(3) ,(+)-Catechin

(48) ,(–)-Epicatechin

The aryl B-group is likely to have an axial position,[114] presenting a non-planar structure. In contrast, (–)-epicatechin (48) with 2,3-*cis* configuration, has an equatorial 2-aryl substituent, which results in a near planar structure. (–)-Epicatechin (48) elutes after (+)-catechin (3) on C_{18} columns,[56] despite their hydroxylation number and substitution pattern being equivalent. The easier solvation of the non-planar (+)-catechin (3) explains this. The near planar (–)-epicatechin would require more highly ordered solvation to effect the same solubility.

Since the wattle-type flavanoids have equivalent configuration, only hydroxylation number and substitution pattern should affect retention. This indicates that flavanoids with higher hydroxyl substitution should elute early on HPLC as they are more polar. The same flavanoids would elute later on gel chromatography, due to their adsorption characteristics. Table 4 summarises the identification of the wattle monoflavonoids which are discussed below.

6b : (–)-Fisetinidol (1) Despite a reference compound not being available, since it possesses one less hydroxyl group than (+)-catechin, (–)-fisetinidol would predictably elute before (+)-catechin in gel chromatography and after in HPLC. Component *6b* complied. When this component, enriched in the ether extract was subjected to paper chromatography with (+)-catechin as reference, the spot had R_f co-ordinates of (0,80; 0,49) close the those reported (0,82; 0,48).[20] The bis-diazotized benzidine reagent afforded a pale yellow spot, typical of (–)-fisetinidol.[19]

Table 4. Flavonoid monomers identified by HPLC.

Component	Identification	r_{Cat} [a]	a_r [b]	Quantitation (%)[c]
9a	(+)-Gallocatechin (4)	0,54 ± 2,8%	0,76 ± 4,3%	0,26
	(+)-Leucorobinetinidin (6)	0,54 ± 2,3%	2,46 ± 4,6%	trace[d]
7d	(–)-Robinetinidol (2)	0,81 ± 1,9%	3,41 ± 4,2%	0,74
7c	(+)-Catechin (3)	1,00 ± 1,8%	4,62 ± 4,1%	1,20
	(+)-Leucofisetinidin	1,00 ± 1,7%	6,64 ± 3,5%	trace
6b	(–)-Fisetinidol (1)	1,15 ± 2,6%	4,39 ± 3,7%	0,23
8b	(+)-Dihydrorobinetin (8)	1,50 ± 1,6%	2,65 ± 3,7%	0,40
7e	(+)-Fustin (7)	2,65 ± 1,7%	3,91 ± 3,4%	trace
9d	Robinetin (15)	4,21 ± 2,8%	0,36 ± 4,8%	trace
9f	Fisetin (14)	8,31 ± 2,7%	0,33 ± 4,5%	trace

a The average relative standard deviation of the relative retentions was 2,2% over a 3 month period (n = 10 for each component).

b The average relative standard deviation of the peak absorbance ratios was 4,1% over a 3 month period (n = 10 for each component).

c From Table 3.

d Identified from ether extract.

7b : (–)-Robinetinidol (2) No reference compound was available but *7b* was easily identified by the position in two-dimensional paper chromatography of this fraction relative to (+)-catechin. An extracted sample, from the same spot of a total wattle paper chromatogram gave an identical HPLC chromatogram, with the 280/250 nm peak area ratios agreeing with *7b*. With bis-diazotized benzidine, the spot of the fraction was canary-yellow, with R_f co-ordinate (0,65; 0,40), typical of (–)-robinetinidol.[19]

7c : (+)-Catechin (3) The V_R/V_o value of 5,25 for the (+)-catechin standard (*cf.* Table 2) corresponds to peak *7*. The *7c* component had identical HPLC and two-dimensional chromatographic parameters. The claret-maroon colour of the spot of this fraction with bis-diazotized benzidine had R_f co-ordinates (0,69; 0,35), typical of (+)-catechin.[18,19] An interesting comparison between (–)-robinetinidol and (+)-catechin is that while both substrates have four aromatic and one aliphatic hydroxyl groups, (–)-robinetinidol has a more asymmetric substitution pattern (with a trihydroxy B-ring), and therefore is more polar than (+)-catechin (with a

dihydroxy B-ring). (–)-Robinetinidol, therefore, elutes before (+)-catechin in HPLC and in keeping with the reversed logic (p. 34), elutes after (+)-catechin in gel chromatography.

(+)-Leucofisetinidin (5) This is structurally isomeric with both (–)-robinetinidol and (+)-catechin. When a sample of the reference compound was subjected to gel chromatography and HPLC, in both systems it behaved like (+)-catechin with no difference in the retentions. When the central *7c* fraction of the ether extract was subjected to paper chromatography, besides the (+)-catechin and (–)-robinetinidol spots, a very weak spot, pale yellow with bis-diazotized benzidine, with R_f co-ordinates (0,70; 0,55) was obtained. The spot compared favourably with published values for (+)-leucofisetinidin.[19] The fact that the a_r value of (+)-leucofisetinidin is 6,7 accounted for the component *7c* value of 4,8 compared to the usual 4,6 for (+)-catechin.

For a sample containing reference compounds of (+)-leucofisetinidin and (+)-catechin in the same concentration range, the alternative mobile phases, dioxane-water-acetic acid (10:89:1) and DMF-water-acetonitrile-acetic acid (15:83:1:1), both effected partial resolution with a baseline resolution (R_s) of 1,12 (for baseline resolution, $R_s \geq 1{,}5$). When, however, either of the reference compounds was more than 5 times in excess, the resolution was lost. In addition, both mobile phases gave no resolution between (–)-robinetinidol and (+)-catechin, so that HPLC of component *7c* was optimum with the methanolic mobile phase. The methanolic mobile phaseswere, therefore, used for the remaining HPLC separations.

7e : (+)-Fustin (7) This component of the ether extract was identified by HPLC with a reference standard. On gel chromatography, *7e* was the first component to emerge in peak *7*. Since (+)-fustin has a total of four hydroxyl groups, adsorption factors indicate that it should elute before (+)-catechin. In addition, (+)-fustin was found to elute after (–)-fisetinidol which agrees with the analogous (+)-catechin/(±)-dihydroquercetin elution order (*cf.* Table 2). The two-dimensional paper chromatogram of *7e* located it ahead of (+)-catechin at R_f co-ordinates (0,80; 0,38) with a pale yellow colour with bis-diazotized benzidine.[19]

8b : (+)-Dihydrorobinetin (8), eluted from gel just after (–)-robinetinidol, in the same order as the (–)-fisetinidol/(+)-fustin and (+)-catechin/(±)-dihydroquercetin pairs. Component *8b* was enriched in the ether extract. It was identified by comparison with a reference sample, and the spot on two-dimensional paper chromatogram overlapped (+)-catechin. Alone, the spot produced a golden-yellow colour with bis-diazotized benzidine.[19]

9a : (+)-Gallocatechin (4) This compound has a molar extinction coefficient of about 1 700 at 272 nm ,[111] while (+)-catechin has an ε of 3 800 at 280 nm in aqueous solutions.[107] As gel chromatography detection was made at 282 nm and HPLC detections at 280 and 254 nm , the (+)-gallocatechin absorbance maximum lay between these wavelengths, which decreased the detection sensitivity. Therefore, (+)-gallocatechin proved difficult to locate as no reference standard was available. The similar eluting characteristics, however, of the (+)-catechin/(+)-leucofisetinidin pair, discussed above, indicated that (+)-gallocatechin and (+)-leucorobinetinidin, both possessing 6 hydroxyl groups, should elute together in both HPLC and gel chromatography. When (+)-leucorobinetinidin was subjected to gel chromatography, it had a V_R/V_o value of 6,72 (*cf.* Table 2), identical with component *9a*. With HPLC, an r_{Cat} value of 0,55 was the same for (+)-leucorobinetinidol and *9a*. The a_r values, however, differed substantially; *9a* with a value of 0,76 and (+)-leucorobinetinidol a value of 2,5. A sample of component *9a* from the ethyl acetate enriched extract , separated on a two-dimensional paper-chromatogram, gave a deep claret-maroon colour with bis-diazotized benzidine at R_f co-ordinates (0,55; 0,33), typical of (+)-gallocatechin.[18,19] A very weak canary-yellow colour bis-diazotized benzidine spot at R_f co-ordinates (0,55; 0,48) ran ahead of the (+)-gallocatechin, typical of (+)-leucorobinetinidol (6).

9d : Robinetin (15) and *9f* : Fisetin (14) Reference standards of both of these flavonols enabled the identification of *9d* and *9f* in the ether extract. Their respective gel chromatography and HPLC characteristics (Tables 2 and 4) were identical and two-dimensional chromatography of the fractions for these components showed 254 nm flourescent spots at R_f co-ordinates (0,60; 0,00) and (0,70; 0,00), indicative of robinetin and fisetin, respectively.

Results in Table 4 and the above discussion have shown that while HPLC is capable of resolving even pairs of structural isomers, multicomponent samples are usually difficult to resolve. The relative retentions and the absorbance ratio values are a precise paired technique for flavonoid identification. The relative retentions showed a long term relative standard deviation of 2,2%. Short term deviation was 1,3%, not as good as a typical GLC value of 0,6%.[115] While a long term variation of 21% was found for UV peak height ratios (h_r) in a study which utilized dual detection,[50] during this work the average long term relative standard deviation for a_r was 4,1%. This improved precision is due to the area of the peaks being used in the calculation and not peak heights.

3.1.7 Identification of biflavanoids

Eight components of wattle extract from gel chromatography and HPLC were found to occur in the F/A, B and D areas on a two-dimensional chromatogram (Fig. 9). These are the general areas where 4,8 linked biflavanoids

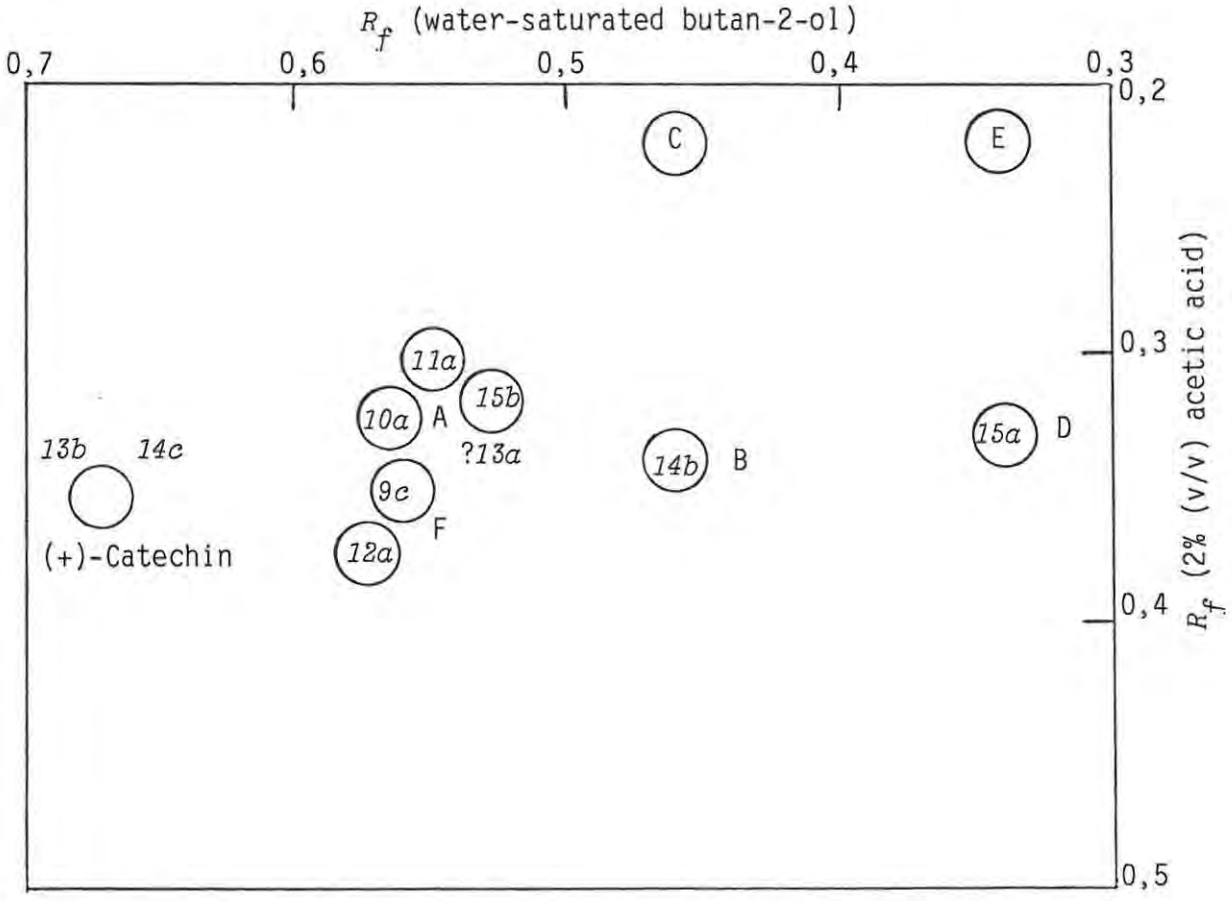


Figure 9. Expanded window of two-dimensional paper chromatogram showing the relative positions of the biflavanoids.

of wattle extract are known to occur.[30] Proposed identifications of some of these components are given (Table 5) and discussed below.

Table 5. Biflavanoids identified by HPLC.

Component	r_{Cat}	a_r	Identification	No. of OH groups	Calculated a_r [a]	Quantitation (%)[b]
13a	0,73	1,81	[4,8]-*R-cis-G* (32)[d]	11	1,61	1,02
15a	0,83	1,72	[4,8]-*R-trans-G* (30)			1,65[c]
14b	1,60	3,11	[4,8]-*R-trans-C* (29)			5,26
10a	1,36	3,12				1,91
11a	1,49	2,68	[4,8]-*R-cis-C* (31)[e]	10	3,54	0,47
15b	2,15	3,13				3,34
12a	2,15	5,73	[4,8]-*F-cis-C* (24)[d]	9	5,63	1,77
9c	3,12	5,32	[4,8]-*F-trans-C* (25)[d]			0,80
13b	3,67	6,43	[4,6]-*F-?-LF*[d]	10	6,64	0,22
14c	3,83	6,49	[4,6]-*F-?-LF*[d]			0,75

a Using experimental monomer a_r values from Table 4; for calculations see text.

b From Table 3.

c Underestimated due to component eluting beyond 900 mℓ.

d Tentative identifications.

e Any one of these components is a possibility.

15a : [4,8]-all-*trans*-(–)-robinetinidol-(+)-gallocatechin (30) The HPLC retention time of the compound extracted from spot D of a two-dimensional chromatogram and that of component *15a* were identical. In addition, component *15a* gave a single spot that coincided with D on two-dimensional chromatography, proving that *15a* is the biflavanoid [4,8]-*R-trans-G (30)*, which has been previously isolated and identified.[30]

14b : [4,8]-all-*trans*-(–)-robinetinidol-(+)-catechin (29) Component *14b* and the compound extracted from spot B of a two-dimensional paper chromatogram had identical HPLC and two-dimensional paper chromatographic parameters. This compound has previously been isolated and identified as [4,8]-*R-trans-C*.[30]

9c and *12a* : [4,8]-(–)-fisetinidol-(+)-catechin biflavanoids Although these two biflavanoids are known to occur in the F/A area of the two-dimensional paper chromatogram, definite identifications with two specific components were not possible as a mixture of six were found in this general area (see Fig. 9). The a_r values of these components, however, proved useful in making tentative identifications.

The sum of the 280 nm molar extinction coefficients (ε) of the A- and B-moieties of mono- and biflavanoids approximate the ε value of the relevant flavanoid itself.[107] This indicates that the UV spectrums of the two monoflavanoids, which comprise the biflavanoid, are additive. The sum of the individual a_r values, therefore, should also be additive. This hypothesis was tested on the known biflavanoids, [*4,8*]-*R-trans-C* (29) and [*4,8*]-*R-trans-G* (30). Better agreement was obtained using flavan-3,4-diol (*i.e.* (+)-leucorobinetinidin) instead of the flavan-3-ol (*i.e.* (–)-robinetinidol) a_r values for the 'upper' half. This is understandable since in the biflavanoid, the 'upper' flavanoid has a substituent at C-4. Using relevant monoflavanoid a_r values (*cf.* Table 4, p. 35), expressed in fraction form, and summing the individual numerators and denominators, gives:

$$\text{Calculated } a_r \text{ (29)} = \frac{246 + 462}{100 + 100} = 3{,}54\longrightarrow$$

$$\text{Calculated } a_r \text{ (30)} = \frac{246 + 76}{100 + 100} = 1{,}61\longrightarrow$$

These values compare favourably with experimental values of 3,1 and 1,7, respectively, which indicates that tentative identifications on this basis are possible. Only components *9c* and *12a* have a_r values close to that calculated for the (–)-fisetinidol-(+)-catechin biflavanoids (*cf.* Table 5).

The major component *12a* is tentatively identified as [*4,8*]-*F-cis-C* (24) since it has previously been identified as the more predominant isomer.[30] In addition, the relative positions on the two-dimensional paper chromatogram of the *cis-* and *trans-* isomers,[33] agree with those for components *12a* and *9c*, respectively. Component *9c* is, therefore, tentatively assigned as [*4,8*]-*F-trans-C* (25).

13a : [4,8]-3,4-*cis*-(−)-robinetinidol-(+)-gallocatechin (32) Unfortunately no pure fraction of *13a* was obtained from gel chromatography. A mixed fraction of components *12a* and *13a* gave one spot in the F/A area on a two-dimensional paper chromatogram. Only component *13a* has an a_r value (Table 5) similar to that of [4,8]-*R-trans-G* (30) *i.e.* component *15a*. Component *13a* is therefore tentatively identified as the related *cis*- isomer, [*4,8*]-*R-cis-G* (32).

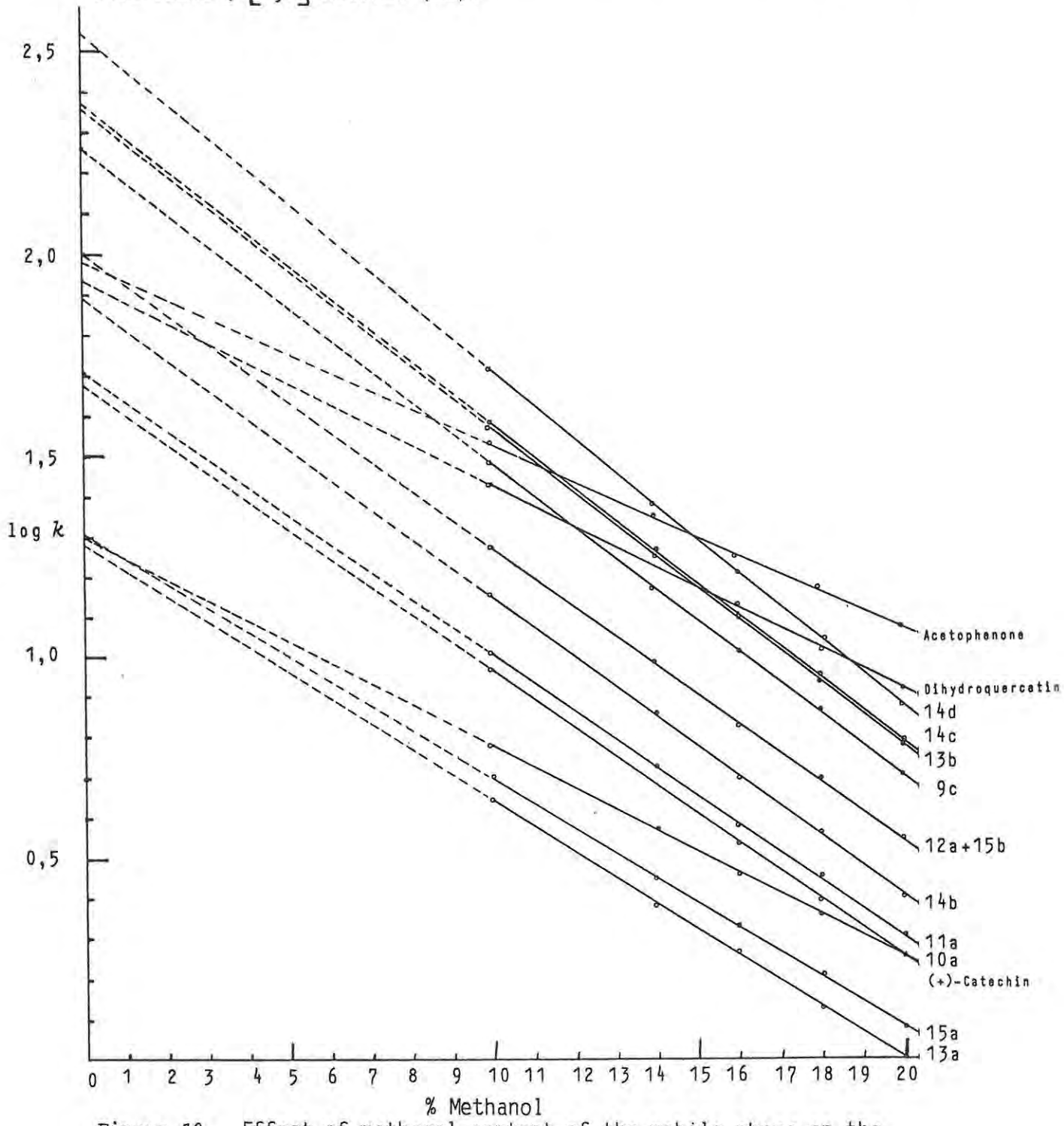


Figure 10. Effect of methanol content of the mobile phase on the capacity factor, k, of the biflavanoids.

Table 6. HPLC linear regression parameters of the biflavanoids and reference standards in the mobile phase concentration range of 10 - 20% methanol.[a]

Compound	Slope	Intercept	Correlation Coefficient (r)
13a	-0,0638	1,28	0,9997
15a	-0,0611	1,30	0,9999
(+)-Catechin (3)	-0,0523	1,30	0,9998
10a	-0,0714	1,68	0,9998
11a	-0,0700	1,71	0,9992
14b	-0,0744	1,89	0,9999
15b	-0,0729	2,01	0,9999
12a	-0,0722	2,00	0,9999
9c	-0,0776	2,26	0,9998
13b	-0,0797	2,36	0,9996
14c	-0,0793	2,36	0,9998
(±)-Dihydroquercetin (9)	-0,0505	1,93	0,9998
Acetophenone	-0,0458	1,98	0,9997

a For the equation log k = a (conc.) + b where conc. = % methanol.

A second determinant that proved useful for indirect and, therefore, tentative identification of component *13a* was HPLC retention under different isocratic conditions. Linear plots of log k (derived from results in Table 18, Appendix II, p. 79) against the methanol concentration in the mobile phase (Fig. 10) should be similar for compounds of similar structure.[54] The plots show that only component *13a*, already tentatively identified as [*4,8*]-*R-cis-G* (32), has a slope value and intercept close to that of the related *trans*-isomer (39), *i.e.* component *15a*. This assignment is despite the fact that no [*4,8*]-*R-cis-G* (32) has been previously isolated. Steric reasons have been cited for its non-formation.[30]

Where flavanoids have different slope values which sometimes result in the intersection of the linear plots (Fig. 10), the point of intersection determines the methanol content at which retention will be indentical for the two compounds. By changing the methanol concentration in the mobile

phase from one side of the intersection to the other, the elution order of the compounds will reverse. This type of behavious would explain the discrepancy that, while quercetin (16) has been reported[60] to precede fisetin (15) in HPLC, the present work and another study[62] found the reverse order.

10a, 11a or *15b* : [4,8]-3,4-*cis*-(−)-robinetinidol-(+)-catechin (31) The a_r values (Table 5) and the log k slope values (Table 6) for components *10a, 11a* and *15b* are all similar to that of [*4,8*]-*R-trans-C* (29), *i.e.* component *14b*, discussed above. Any of these components could be identified with the related *cis*-isomer which has been synthesized from (+)-leucorobinetinidin and (+)-catechin.[33] These components also occur in the F/A area on the two-dimensional paper chromatogram (see Fig. 9), in the same general position found for synthetic [*4,8*]-*R-cis-C* (31).[33]

13b and *14c* : [4,6]-(−)-fisetinidol-(+)-leucofisetinidin biflavanoids

Both components *13b* and *14c* were more concentrated in the ether extract. The only biflavanoids which would account for their high a_r values are the (−)-fisetinidol-(+)-leucofisetinidin biflavanoids, four of which have been isolated from wattle heartwood.[32] These form a unique class with 4,6-linkage and a 3,4-diol function in the 'lower' flavanoid unit. In two-dimensional paper chromatography, [*4,6*]-*F-LF* biflavanoids have similar R_f values to that of (+)-catechin.[116] Components *13b* and *14c* migrated with (+)-catechin (see Fig. 9) which suggests that these two components can be tentatively identified with the [*4,6*]-*F-LF*, (33) and (34), class of biflavanoids.

The fact that the above biflavanoids (with at least 9 hydroxyl groups) eluted well after the monoflavanoids (with at most 6 hydroxyl groups) in gel chromatography, conforms to the adsorption principle, and confirms that the exclusion principle is not important in gel chromatography of flavanoids. In addition, because these biflavanoids had similar HPLC retention to those of the monoflavanoids, polarity and solubility, and not molecular size or adsorption,[63,65] are the determining factors affecting elution properties of flavanoids on reversed-phase C_{18} columns.

3.1.8 Identification of triflavanoid and higher oligomers

Area C on the two-dimensional paper chromatogram is known to consist of a homogeneous mixture of three diastereoisomeric bi-[(−)-robinetinidol]-

(+)-catechin triflavanoids, (35), (36) and (37) and area E to consist of two bi-[(—)-robinetinidol]-(+)-gallocatechin triflavanoids, (38) and (39).[33] Such isomers possess several rotameric forms,[117] due to the restricted rotation about the inter-flavan $C(sp^2)$-$C(sp^3)$ hybridized bonds. This phenomenon results in the rate of interconversion between various conformers being too slow with respect to the rate of equilibration between the stationary and mobile phases,[47] resulting in a lack of resolution. This explains the broad unresolved peaks that were obtained in gel chromatography especially beyond peak *15*.

Samples of these peaks and of the triflavanoids extracted from spots C and E, of a two-dimensional paper chromatogram, also gave broad unresolved peaks in HPLC. The r_{Cat} values lay in the range 1 to 2, similar to the mono- and biflavanoids. The major difference between the mono-, bi- and triflavanoids is the degree of band-spreading in HPLC. The theoretical plate number, n, was in the range 500 - 1 000 for the monoflavanoids, and decreased to 150 - 500 for the biflavanoids which is well below the recommended value of 3 000. This efficiency was worse for the unresolved triflavanoids and higher oligomers, which eluted after peak *15* in gel chromatography and amounted to more than 70% of the total phenols (see Section 3.1.4).

3.1.9 HPLC of mononuclear phenols

Studies of chemical and biological degradation of flavonoids have indicated the formation of simpler mononuclear phenols (see Fig. 2, p. 12).[73-79] The separation of some of these wattle-related mononuclear phenols are shown (Fig. 11).

The sensitivity of HPLC at 0,005 AUFS, especially for the hydroxybenzoic acids, indicated that trace quantities of phenols were quantifiable (Table 7). Phloroglucinol (41) and pyrogallol (45) were least sensitive while compounds like resorcinol (40), catechol (44) and (+)-catechin (3) could be detected in solutions at 1 mg/ℓ (*i.e.* 0,1 µg/100 µℓ).

All of these phenolic standards had retentions less than (+)-catechin in HPLC. None of them could unfortunately be identified with the wattle components of the gel chromatographic fractions by only their retention and a_r or h_r values.

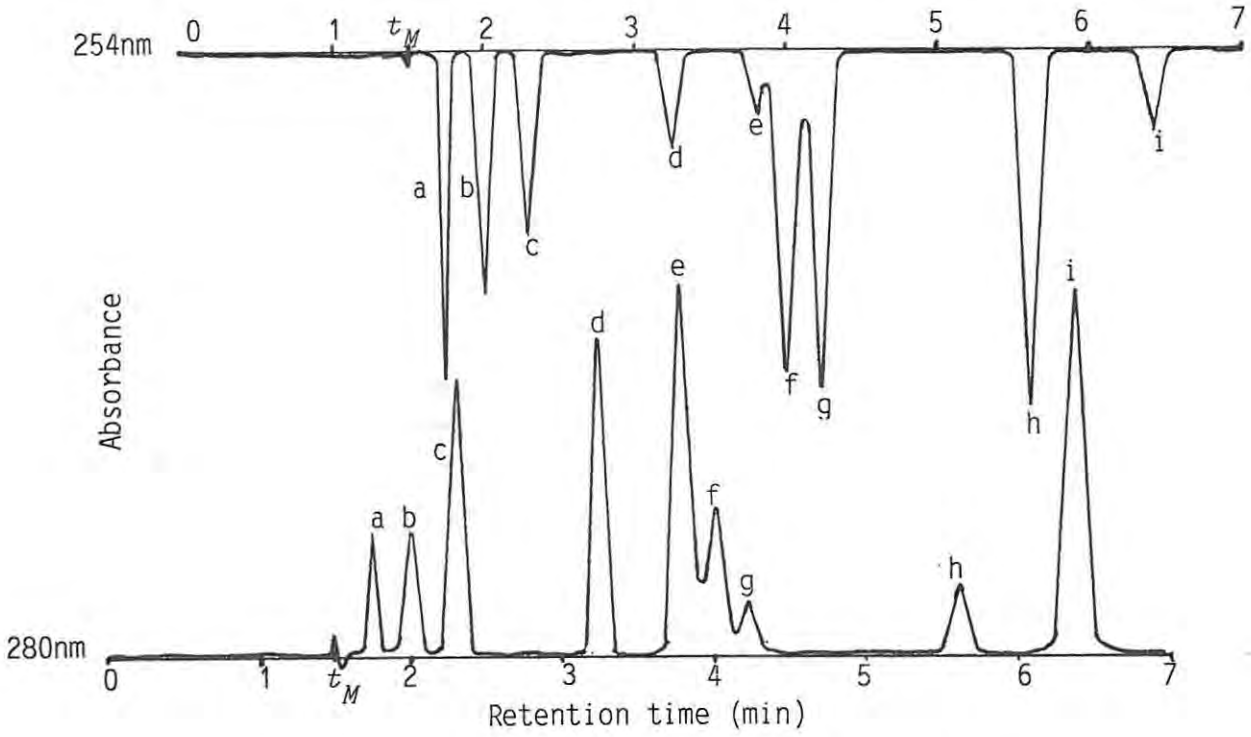


Figure 11. Paired chromatogram of the separation of mononuclear phenols. Mobile phase: methanol-water-acetic acid (15:1:84). Flow-rate: 2 mℓ/min. Sensitivity: 0,005 AUFS. 10 μℓ injections of: (a) 28.0 mg/ℓ phloroglucinol; (b) 23,4 mg/ℓ pyrogallol; (c) 1,22 mg/ℓ gallic acid; (d) 6,53 mg/ℓ resorcinol; (e) 6,21 mg/ℓ catechol; (f) 1,52 mg/ℓ protocatechuic acid; (g) 8,36 mg/ℓ 3,5-dihydroxybenzoic acid; (h) 3,20 mg/ℓ 2,6-dihydroxybenzoic acid; (i) 7,74 mg/ℓ (+)-catechin.

Table 7. HPLC parameters for the mononuclear phenolic standards.

HPLC Peak	Compound	r_{Cat}	h_r [a]	Response x 100[b]
a	Phloroglucinol (41)	0,27	0,37	1,54 (254nm)
b	Pyrogallol (45)	0,31	0,50	1,40 (254nm)
c	Gallic acid (43)	0,36	1,50	29,8 (280nm)
d	Resorcinol (40)	0,51	3,31	6,38 (280nm)
e	Catechol (44)	0,59	6,72	7,91 (280nm)
f	Protocatechuic acid (42)	0,63	0,46	27,8 (254nm)
g	3,5-Dihydroxybenzoic acid	0,66	0,16	5,29 (254nm)
h	2,6-Dihydroxybenzoic acid	0,88	0,20	15,0 (254nm)
i	(+)-Catechin (3)	1,00	4,62	6,23 (280nm)

a h_r approximates a_r for the sharp peaks of the mononuclear phenols.

b Response = height (cm) of peak per ng of substrate at 0,005 AUFS on the more sensitive channel.

3.1.10 Application of HPLC to wattle-based wastewaters

The HPLC chromatogram of total wattle extract shown in Fig. 12 (an expanded version of Fig. 4) shows the relative amounts of the semi-resolved components, some of which have now been identified. However, the similar retentions of the mono-, bi-, tri-and some of the higher oligoflavanoids indicates that HPLC is useful only as a comparative fingerprint and not for detailed analysis. Nevertheless, it was found that up to 27 min in the HPLC separation (Fig. 12), only 53,3% (relative standard deviation = 0,051, n = 3) of the wattle components were eluted. Thereafter, elution of the higher oligomers decreased monotonically.

The simplest wastewater that could be expected from a vegetable tannery would require tanning processes as efficient as that in the hide-powder method.[7] In the hide-powder method, there are none of the complications of tannin penetration experienced in full hide tannages. The comparative chromatograms of wattle components before and after treatment with hide-powder (Fig. 12) indicate that, in addition to the expected removal by adsorption of the higher oligomers, much of the mono- and biflavanoids (the so-called phenolic non-tannins) were also removed from solution. Up to 27 min in the HPLC separation of the hide-powder treated solution, 94,1% (relative standard deviation = 0,063, n = 3) of the phenolic non-tannins were found to be eluted. The bulk of the components were, therefore, part of the initial 53% of the wattle components that had been eluted in the first 27 min. These results show that the non-tannins amounted to 6,2% of the total wattle polyphenols.

Two-dimensional paper chromatography of such phenolic non-tannins, concentrated by ethyl acetate extraction, previously indicated that these phenolic non-tannins were mainly mono- and biflavanoids;[17] it was inferred that, as they were detected in the filtrate after hide-powder treatment, these low molecular mass components were not adsorbed by hide-powder. The present study, however, showed that while some of these constituents remained in solution, about 80% of these mono- and biflavanoids were in fact adsorbed by hide-powder.

A spent vegetable tanning wastewater from a pilot-plant LIRITAN process, showed similar chromatographic features to that of pure extract (Fig. 13).

This indicates less efficient tanning relative to that in the hide-powder method. There was, however, a notable accumulation of components with low retentions, similar to those of mononuclear phenols. Unfortunately their resolution was insufficient to enable identification with the standard phenols (Section 3.1.9). However, the decrease in the relative proportions of the mono- and biflavanoid peaks indicated their adsorption by the hides, as found with hide-powder.

HPLC was also used to monitor the treatment of such spent wastewaters by precipitation of the polyphenols with aluminium sulphate. Flavonoids have been extensively used as fluorimetric reagents for aliminium and the ability of this cation to chelate to the *ortho*-hydroxyl groups on the wattle flavanoid B-ring or alternatively, to the 3- or 5-hydroxyl and 4-carbonyl of the flavonoids,[118] assists in their insolubilization. Aluminium sulphate is a common coagulant for water treatment[119] and has been applied to vegetable tannery wastewaters.[120]

Use of various quantities of aluminium sulphate and wattle extract and monitoring the filtrate for tannins, indicated that 1 mole of Al(III) will precipitate about 2 moles of average wattle monomer ($C_{15}H_{12}O_{16}$), *i.e.* 1 mg of wattle polyphenol requires 0,533 mg of $Al_2(SO_4)_3.18H_2O$. Optimum precipitation was found to occur at a pH value of 7,5, which is economically achieved with lime addition.

For LIRITAN wastewaters, which (in addition to spent polyphenols and accumulated gums) contain high concentrations of polyphosphate, an excess of aluminium sulphate is required to precipitate the polyphenols since the aluminium phosphate which is formed is also insoluble. The acidic LIRITAN colour vat wastewater, which contained 2,5% (m/v) tannins, was treated with 2% (m/v) aluminium sulphate and 1,3% (m/v) lime to give a pH of 7,5. The HPLC comparison of the untreated and treated wastewaters (Fig. 13) is similar to that of the hide-powder comparison (Fig. 12). This treatment, however, visibly decreased the mono- and biflavanoids, and notably components *7b*,(–)-robinetinidol (1) and *7c*, (+)-catechin (3). The unprecipitated phenolic non-tannins, which had all eluted within 27 min, were found to amount to 6,1% of the total polyphenols present in the spent LIRITAN wastewater. Similar to the total wattle extract, about 55% of the components present in the untreated wastewater had been eluted within 27 min.

The chromatogram of this treated LIRITAN wastewater was compared with 1% (m/v) solutions of wattle extract and commercial Mimosa ME extract that had also been subjected to aluminium sulphate treatment (Fig. 14). The higher sensitivity enable comparisons to be made which indicate that the same components were present in all three samples. Relative sizes of peaks within the individual chromatograms show that the commercial extract contained higher concentrations of the components with low retentions (*i.e.* with $r_{Cat} < 1$) probably mononuclear phenols, than the methanolic extract. Together with the unknown component *5e*, these early peaks accumulated in the LIRITAN wastewater and are not wholly removed by aluminium precipitation.

A final study was performed on wastewaters from laboratory scale activated sludge units,[121] that had been acclimatized to Mimosa ME extract over a period of months, operating at a one day retention time. The influent consisted of domestic sewage which contained 1 000 mg/ℓ of Mimosa ME (*i.e.* 650 mg/ℓ tannin). Despite a distinct brown colour in the effluent after aerobic biological treatment (due to the presence of oxidized polyphenols) and spectrophotometric results (see Section 3.2.3, Table 13, p.60) which indicated that approximately 30% of the polyphenols had not been removed from the water, the HPLC chromatogram of the effluent surprisingly showed no component peaks at all. The oxidized polyphenols remained in the aqueous phase during extraction with ethyl acetate. After the ethyl acetate extractables were re-dissolved in methanol, affecting a 50-fold concentration, still no component peaks were observed with both weak and strong mobile phases.

Together with the adsorption of tannins on the proteinaceous sludge, both biotic degradation[75-79] and abiotic oxidative polymerizations[13] probably account for the removal of wattle polyphenols in an activated sludge reactor. The latter reaction has been observed for catechol,[81] phenol[82] and for polyphenols in wines,[83] to give polymers ranging from 500 to 50 000 a.m.u., with humic-type characteristics.[81] Similar products were probably formed in the above activated sludge treatment of wattle polyphenols which would account for the brown colour and the HPLC results of the treated effluent.

Since HPLC using a reversed-phase μBondapak C_{18} column was unfortunately not suitable for monitoring these oxidized wattle polyphenols, spectrophotometric and conventional analytical wastewater methods were investigated.

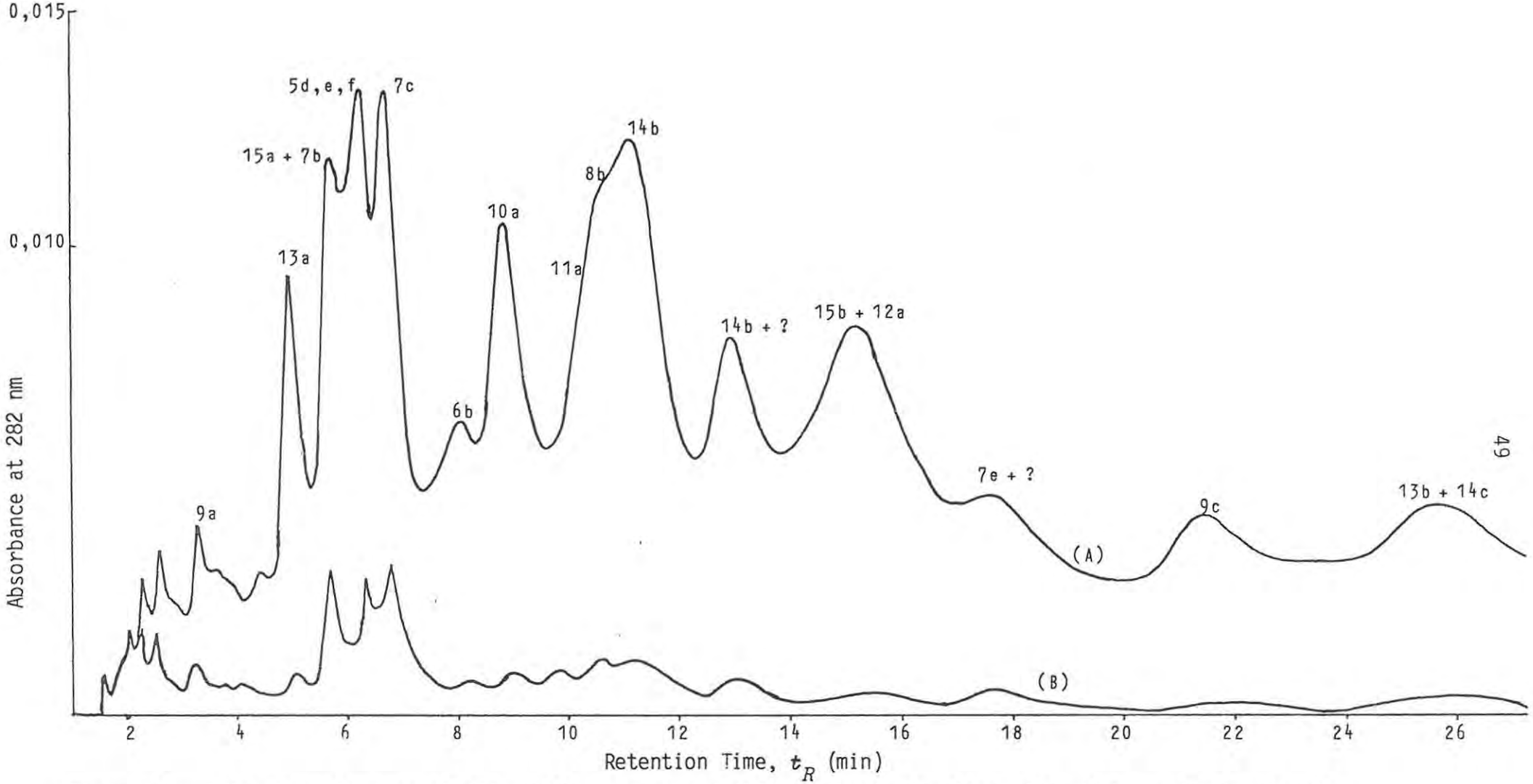


Figure 12. HPLC chromatograms of (A) wattle extract and (B) non-tannin fraction after hide-powder treatment. Wattle extract sample: 15 $\mu\ell$ of 6 010 mg/ℓ aqueous wattle extract. Non-tannin sample: 15 $\mu\ell$. Mobile phase: methanol-water-acetic acid (15:84:1). Flow rate: 2mℓ/min. UV detection channel: 280 nm. Sensitivity: 0,02 AUFS.

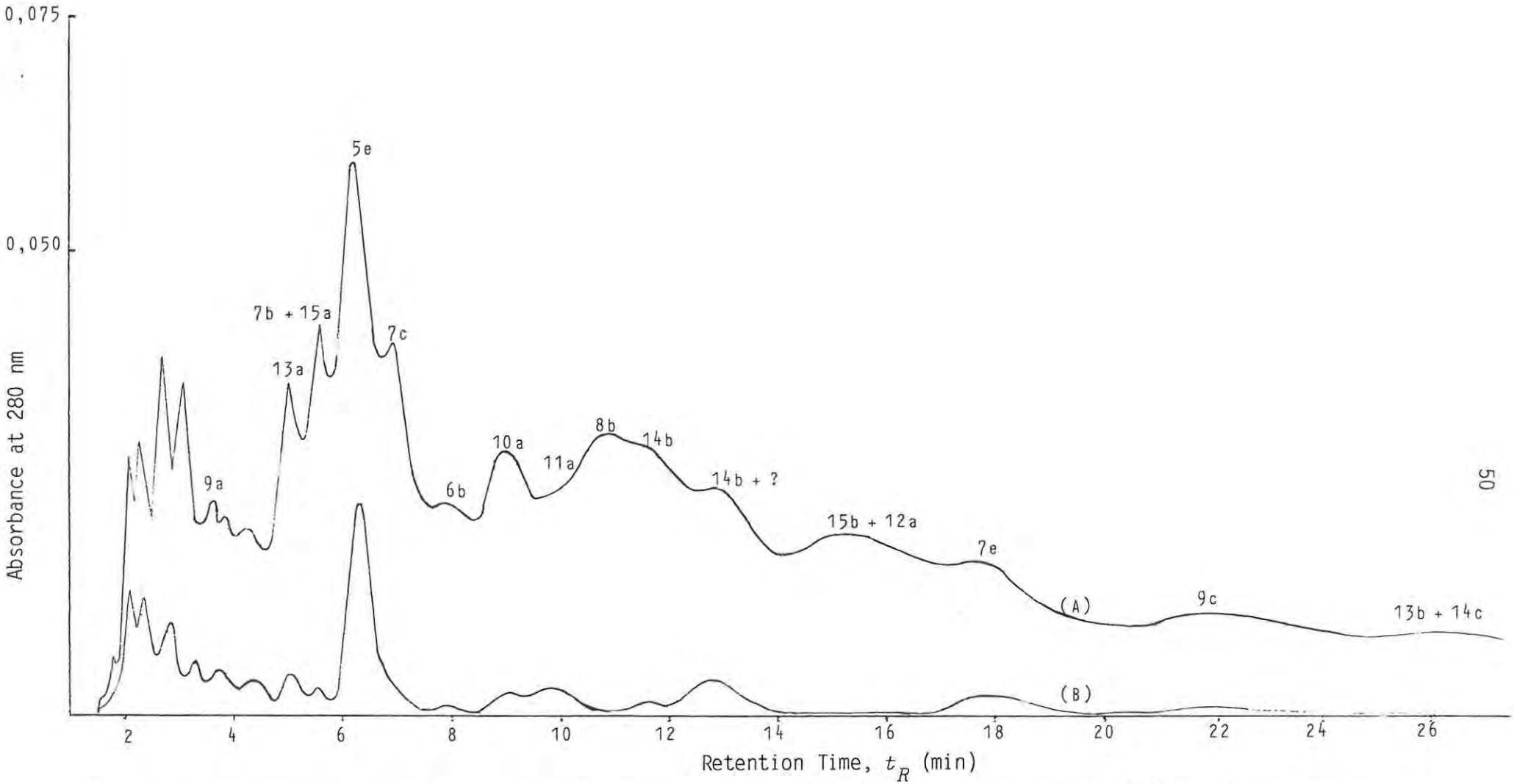


Figure 13. HPLC chromatograms of (A) LIRITAN colour vat wastewater (2,5% tannins) before and (B) after aluminium sulphate treatment. LIRITAN sample: 10 $\mu\ell$. Treated sample: 10 $\mu\ell$. Mobile phase: methanol-water-acetic acid (15:84:1). Flow rate: 2mℓ/min. UV detection channel: 280 nm. Sensitivity: 0,10 AUFS.

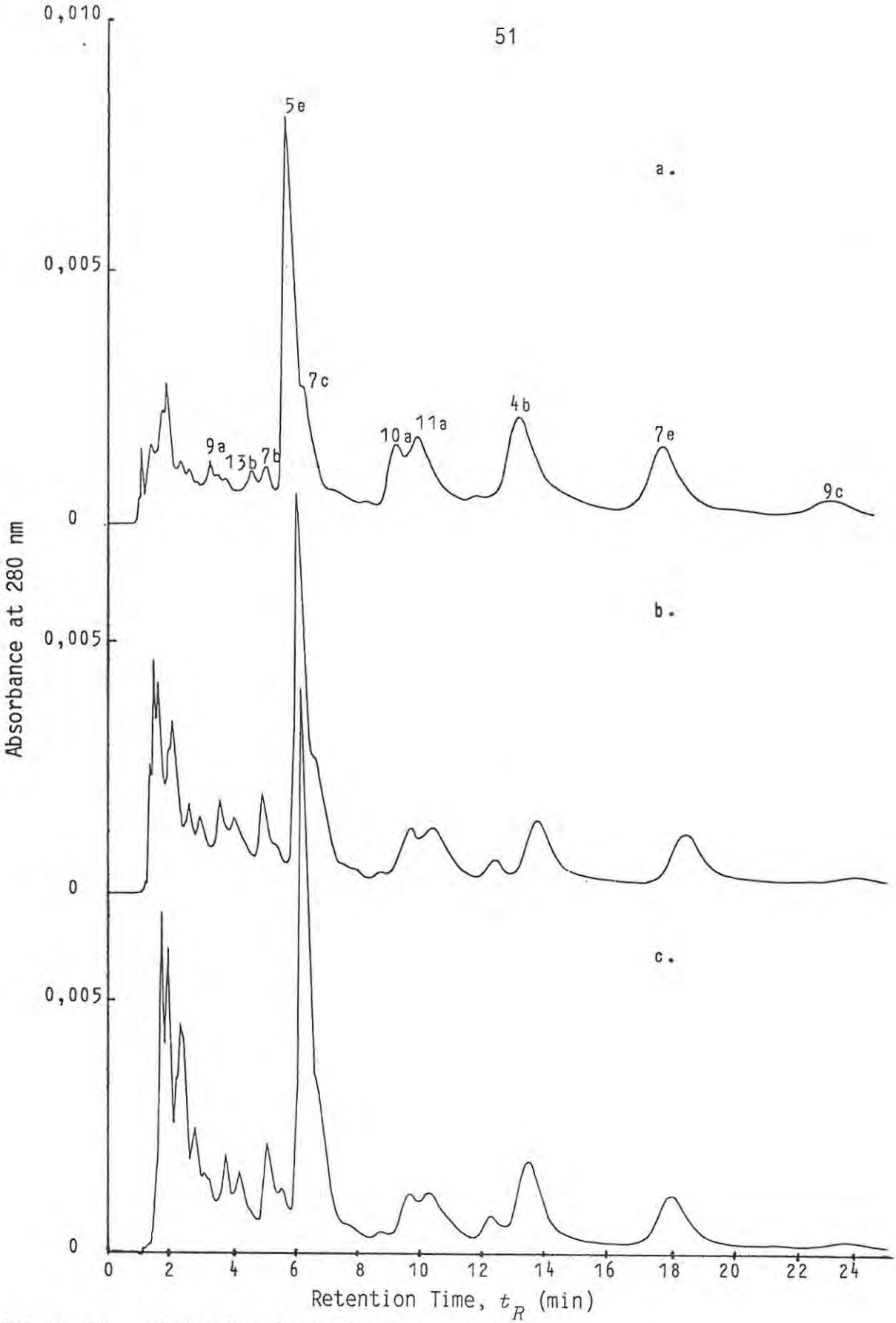


Figure 14. HPLC comparison of treated solutions.

a. 1% (m/v) wattle extract solution treated with aluminium sulphate. Sample: 25 µℓ.
b. 1% (m/v) Mimosa ME extract solution treated with aluminium sulphate. Sample: 25 µℓ.
c. LIRITAN colour vat wastewater (2,5% tannin) treated with aluminium sulphate. Sample: 5µℓ.

Mobile phase: methanol-water-acetic acid (15:84:1). Flow rate: 2 mℓ/min. UV detection channel: 280 nm. Sensitivity 0,01 AUFS.

3.2 Spectrophotometric Methods

3.2.1 The ultra-violet (UV) method

The 280 nm chromophore of wattle polyphenols forms the basis of the UV method for tannin analysis. This direct and easy method for quantitation of wattle tannins was developed by correlation between numerous hide-powder determinations and the 280 nm chromophore of wattle flavonoids in aqueous solutions.[94,97] The hide-powder method does not measure the so-called phenolic non-tannins, as discussed (Section 3.1.10), while the UV method measures all phenolic material. Commercial wattle extracts, however, normally contain 5 - 12% gums, of which at least 30% are adsorbed by hide-powder.[122] Fortuitously, on average, the non-tannin fraction equals the 'gum-tannin' fraction, thereby giving good correlation between the UV and hide-powder methods.[98]

UV absorbance of the phenolic chromophores was used in this study for the monitoring and quantitation in HPLC and gel chromatography. Wattle extract is, however, too complex a phenolic mixture to use as a reliable standard compound. The more specific catechin equivalent was, therefore, used in this work; this is analogous to the gallic acid equivalent, used in enological studies.[105]

Table 8. UV data for flavanoids in water and ethanol.

Description	λ_{max} (nm)	ε	a	References
Ethanolic wattle	282	NA	0,01366	This work
Aqueous wattle	280	NA	0,01283	94
Ethanolic (+)-catechin	281,5	4 020	0,01385	This work
	281	3 830	0,01319	111
Aqueous (+)-catechin	279	3 800	0,01309	107, 123
	280	3 810	0,01313	This work
Ethanolic (+)-leucofisetinidin	283	6 230	0,02148	This work
Aqueous (+)-leucofisetinidin	279	5 600	0,01930	107
Ethanolic (+)-leucorobinetinidin	281	3 860	0,01261	124
Aqueous (+)-leucorobinetinidin	279	3 720	0,01214	This work
Ethanolic [4,8]-*R-trans-G*	282	5 510	0,00927	This work
Aqueous [4,8]-*R-trans-G*	280	5 400	0,00909	111

Since ethanol was used in gel chromatography (Section 3.1.4), the change in the aqueous 280 nm chromophore characteristics was investigated (Table 8). By dissolving wattle and related flavanoids in ethanol instead of water, a slight bathochromic shift in wavelength of maximum absorption (λ_{max}) of about 2 nm occurs and extinction coefficients can increase up to about 6%, as in the case of wattle.

This ethanolic wattle α value was used to quantify the gel chromatography peaks. The α values of (+)-catechin and wattle extract in ethanol are almost identical and differ only slightly in aqueous solutions; therefore, quantitative comparisons of constituent components based on the catechin equivalent will approximate those in wattle extract.

3.2.2 The Folin-Ciocalteu (F-C) method

Phenols must be present as phenolates to reduce the yellow 2:18 molybdotungstophosphoric heteropoly anion of the F-C reagent to the blue form.[105] This requires basic conditions which unfortunately result in the rapid destructive hydrolysis of the 2:18 anion. Once the blue heteropoly anions form, however, they are stable even at pH of 9 - 10 ,[104] where most phenols are about 50% ionized. An excess of F-C reagent is thus required as the formation of the blue complex involves the competing reactions of destruction of the acidic yellow reagent by alkali, ionization of the phenols to phenolates and reduction of the reagent to produce the blue complex.

The F-C method, applied to mononuclear phenols (Table 9), indicates that phenolates undergo a 2-electron transfer per active hydroxyl group; for example, phenol gives an F-C molar extinction coefficient (ε) value of 12 700. This is close to the ε value of about 6 000 for each electron transferred, found for both the molybdenum and tungsten isostructural complexes.[105,125] These heteropoly anions of molybdenum and tungsten are able to undergo partial reduction with,

$$M(VI) + \bar{e} \longrightarrow M(V) \qquad (1)$$

where :

M = W or Mo.

The 2:18 heteropoly molybdophosphates are much more reducible than the corresponding tungstates but the latter exhibit 1-electron transfer steps at high pH whereas the molybdophosphates do not.[126] Otherwise the

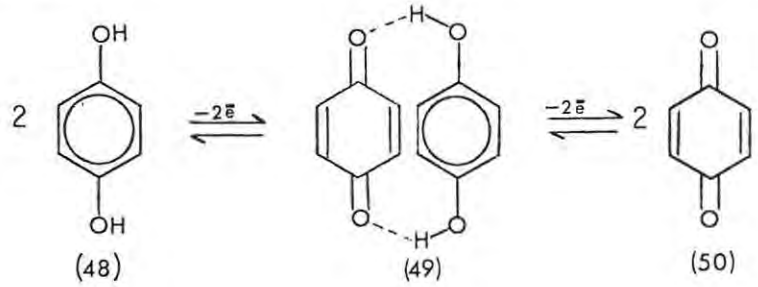
Table 9. Molar extinction coefficients of mononuclear phenols with F-C reagent.

Compound	ε	No. of phenolic OH groups	No. of active OH groups	Reference
Phenol	12 700	1	1	105
Hydroquinone(48)	12 600 12 800	2	1	This work 105
Quinhydrone (49)	14 700	2	1	105
Phloroglucinol (41)	13 400 13 300	3	1	This work 105
2,4,6-Trihydroxy benzoic acid (47)	13 600	3	1	105
Resorcinol (40)	19 500 19 800	2	2	This work 105
β-resorcylic acid (46)	14 600	2	1	105
Catechol (44)	22 400 22 500	2	2	This work 105
Protocatechuic acid (42)	17 600 17 300	2	1-2	This work 105
4-Methylcatechol	21 600	2	2	105
Pyrogallol (45)	24 600 24 800	3	2	This work 105
Gallic acid (43)	25 100 25 000	3	2	This work 105

electronic structure and behaviour of the two metals are similar.[127] The incorporation of both molybdenum and tungsten into the F-C reagent, therefore, gives an anion that has the molybdenum feature of being easily reduced and can simultaneously undergo the tungsten 1-electron transfers at high pH. The intervalence change-transfer transitions[103] that result from the mixed valence constitution of these heteropoly anions gives rise to the characteristic blue colour.

These results (Table 9) show that hydroquinone (48) and phloroglucinol (41) and its derivatives behave like phenol, with a 2-electron transfer from their one active hydroxyl group. Catechol (44) and pyrogallol (45) have ε values about twice that of phenol, indicating a 4-electron transfer. Resorcinol (40) and its derivatives, as well as the catechol derivatives, behave intermediate to the mono- and diphenols. Quinhydrone (49) gives

an ε value similar to that of hydroquinone which indicates that some phenols are not oxidized beyond their quinoidal stage,[105] as shown in reaction (2).



(48) (49) (50) (2)

No explanation has been proposed for the 'activity' of hydroxyl groups. However, since a phenolate is initially required, the dissociation constant (Ka) of the phenols is initially important. For example, catechol[128] (44) and hydroquinone[129] (48) have pKa_1 values of 9,37 and 9,85, and pKa_2 values of 13,7 and 11,4, respectively. Only the catechol (44), however, undergoes a 4-electron transfer indicating that the oxidation proceeds beyond the quinoidal stage. This is despite hydroquinone being a marginally stronger reductant than catechol,[105] and the pKa_2 value of hydroquinone being substantially lower than catechol. The fact that phenols

Table 10. Molar extinction coefficients (ε) of flavonoids with F-C reagent.

Compound	ε	Calculated ε values[a]	No.of phenolic OH groups	Reference
Myricetin (17)	48 500	38 100	5	105
Quercetin (16)	48 400	35 800	4	This work
	48 300			105
Robinetin (15)	47 300	35 000	4	This work
Fisetin (14)	42 800	32 700	3	This work
	42 700			105
(±)-Dihyroquercetin (9)	48 900	35 800	4	This work
	49 100			105
(±)-Dihyrorobinetin (8)	48 100	35 000	4	This work
(+)-Fustin (7)	43 800	32 700	3	This work
(+)-Catechin (3)	38 700	35 800	4	This work 105
(+)-Leucorobinetinidin (6)	35 400	35 000	4	This work
(+)-Leucofisetinidin (5)	33 200	32 700	3	This work
3-Hydroxyflavone	3 500	0	0	105

a Calculated from relevant ε values of flavonoid A- and B- moieties from Table 9.

with *ortho*-dihydroxyl substitution give higher ε values suggests that steric interactions play a role in further electron transfer.

Unlike the UV chromophores of the flavonoid A- and B-moieties which are approximately additive,[107] the F-C ε values for the reference flavonoids are greater than expected (Table 10). The fact that 3-hydroxyflavone, with no phenolic substituents, produces some colour indicates the involvement of the aliphatic hydroxyl groups. This is attributed to the enol reaction involving the substitution pattern of C-2, -3 and -4 in the flavonol series.[105] The flavonols, therefore, give higher ε values than their flavanol counterparts.

The ε values were calculated from the specific extinction coefficients a of the respective analytical calibration curves.[130] The a value of 0,1204 for wattle (Fig. 15) lay between that of (+)-leucorobinetinidin,

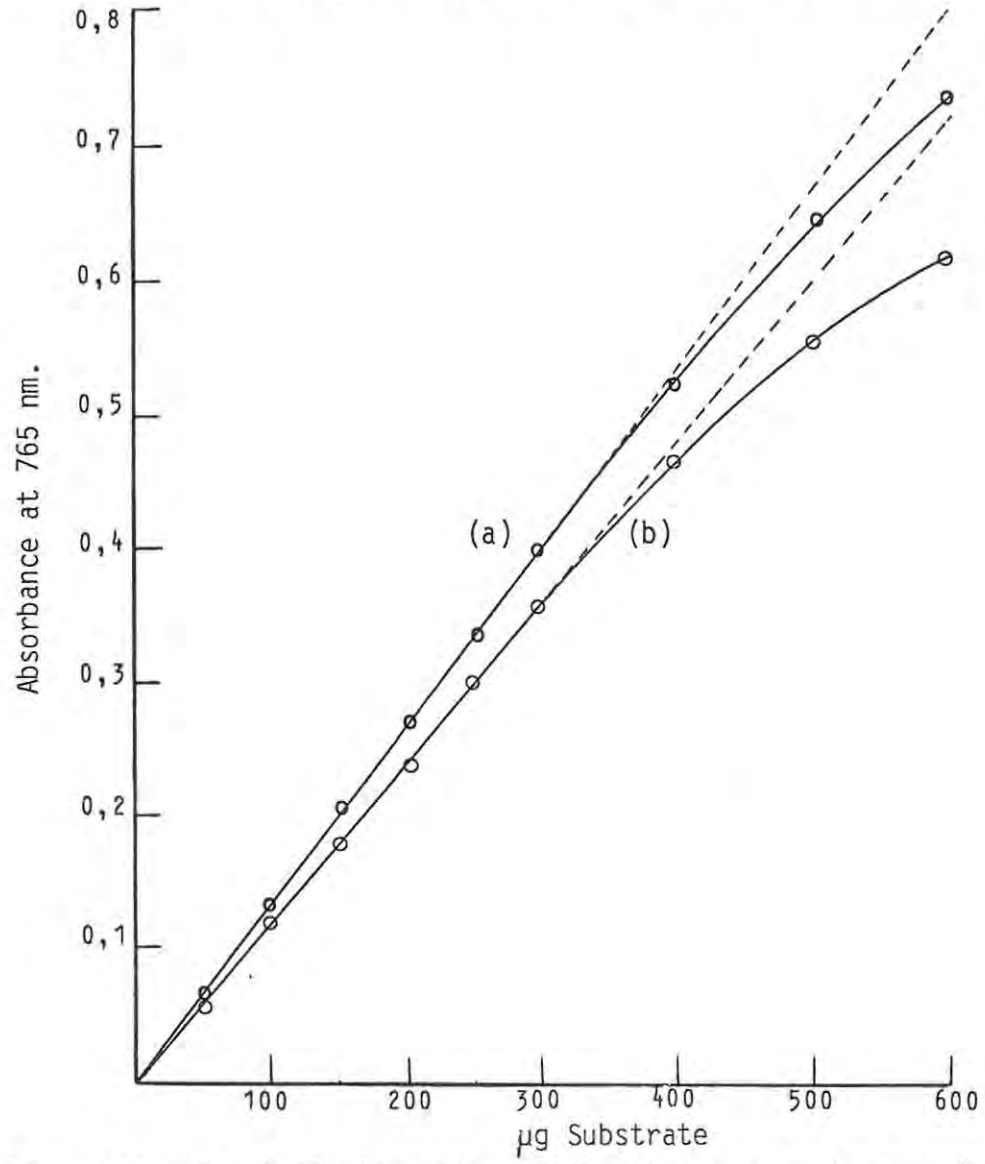


Figure 15. The Folin-Ciocalteu calibration curves for (a) (+)-catechin and (b) wattle polyphenols.

(0,1156) and (+)-catechin (0,1334). Deviation from Beer's Law occurred above an absorbance of 0,3 and linear regressions were performed up to this absorbance value and forced through the origin.[131] The F-C calibration curves for wattle polyphenols showed a greater deviation from linearity than catechin above an absorbance of 0,3 (Fig. 15). This deviation can be attributed to the slower rate of electron transfer of the inaccessible active hydroxyl substituents of the large oligomers. Although only the blue heteropoly anions survive the alkali test conditions after the first minute of the reaction, as shown by the rapid disappearance of the yellow anion in the blank, further colour does generally develop in the next 2 h. This is due to the blue anions accepting more electrons.[125] The linear relationship between absorbance and number of electrons transferred, is true for up to 4 electrons for tungsten and 6 for molybdenum. Thereafter, the d-orbitals of these metals start to overlap[125] and characteristics change, with decrease in blue colour formation, due to probable formation of $M_3^{4+}O_{13}$ clusters within the 2:18 anionic complex.[132] If there are limited blue anions after the first minute of reaction, due to slower oxidation of the substrate, these blue 2:18 anions may subsequently accept an excess of electrons to adversely affect linearity. While one electron 'blues' have a λ_{max} at *ca.* 850 nm, the addition of more electrons result in a hypsochromic shift. The λ_{max} for this method occurs at 765 nm, indicative of a 4-electron heteropoly blue.[125] This wavelength decreased further when deviation from linearity occurred, indicating the formation of greater than 4-electron 'blues'.

In comparison to the UV method, the F-C method is about 10 times more sensitive, as shown by the a values in Table 11.

The average relative standard deviations of about 1,7% for the a values of catechin and wattle extract and the high correlation coefficients for both analytical calibration curves (Fig. 15) indicate high precision, even though the linear regression was forced through zero. This procedure was adopted as it has been shown [131] to increase accuracy for low concentrations of analyte. The limit of detection is in the same range as found by APHA,[88] *viz.* 0,1 - 0,3 mg/ℓ for typical phenolic compounds.

Table 11. a Values and limit of detection for F-C determinations.

Substrate	a[a]	Correlation Coefficient (r)[c]	Limit of detection (mg/ℓ)[d]
Catechin	0,1334 (0,01309)[b]	0,9995	0,23
Wattle extract	0,1204 (0,01283)[b]	0,9991	0,26

a (+)-Catechin and wattle extract F-C a values had relative standard deviations of 1,6 and 1,8%, respectively, for n = 5 analytical calibration curves.

b UV a values in parenthesis, for comparison.

c Average r value for n = 5 analytical calibration curves, each determined with 6 points and drawn through the origin.

d Calculated[130] with reagent blanks from $C_L = kS_b/a$, where:
C_L = limit of concentration; S_b = standard deviation of blank absorbance measured against water = 0,000831 (n = 5);
a = sensitivity = specific extinction coefficient; k = confidence factor = 3.

No interference was found for the carbohydrates dextrose, fructose, sucrose, galactose, arabinose and dextrin (up to 500 μg) or the amino acids alanine, glycine, arginine, aspartic acid, glutamic acid or the imino acids proline and hydroxy-proline (up to 500 μg). This indicates that the non-phenolic, nitrogenous and carbohydrate portions of wattle extract[22] should not interfere. Reducing agents, like iron (II), sodium sulphite, aromatic amines and ascorbic acid do, however, produce colour with F-C reagent,[105] and would have to be corrected for in wastewaters.

3.2.3 Application of spectrophotometric methods to wattle-based wastewaters

The tannin contents of two different wattle extracts determined by the UV method correlated well with the official hide-powder method (Table 12). The results are within the 2% absolute error margin that is required of duplicate hide-powder determinations.[7]

Both F-C and UV methods for monitoring the removal of polyphenols by hide-powder treatment gave close agreement. They indicated that, for the methanolic extract, an average of 4,3% of the polyphenols were non-tannins. This is in fair agreement with the 6,2% value calculated from HPLC (Section 3.1.10, p. 46), considering the precision (relative standard deviation of about 5%) of the comparative wattle/catechin

Table 12. Tannin analyses and the determination of polyphenols removed by hide-powder.

Extract type	Hide-powder tannins (%)[a]	UV tannins (%)[b]	Polyphenols removed (%)	
			F-C method	UV method
Mimosa ME	66,2	65,8	95,3	94,8
Wattle extract[c]	74,6	74,1	95,8	95,6

a SLTC method.[7]

b UV method.[94]

c Methanolic extract of fresh bark.

integrations.

The influent and effluent from the activated sludge treatment of domestic sewage containing wattle extract (*cf.* p.48) as well as from the aluminium sulphate treatment of a LIRITAN process wastewater and of 1% (m/v) solutions of wattle and Mimosa ME extracts (*cf.* p. 47), were also monitored by the UV and F-C methods (Table 13).

The percentages of polyphenols removed were close for both UV and F-C methods except for the activated sludge effluent, which had a low F-C value due to the oxidized nature of the polyphenols after the aerobic activated sludge treatment. This underestimation of oxidized phenols in the F-C method is similar to the quinhydrone (49) example (Table 9), where the ε value was found to be similar to that of hydroquinone (48), due to the F-C induced oxidation not preceeding beyond the *para*-quinone stage. The UV method is also not reliable for oxidized wattle solutions,[17] as was evidenced by a 6% rise in absorbance at 280 nm after a 1 000 mg/ℓ solution of wattle extract had been aerated (pH of 5,3) for 24 h.

A useful determinant for comparing the UV and F-C methods is the individual UV tannin : F-C catechin equivalent ratio. This should equal the ratio of the catechin a value : wattle a value of 1,110 (Table 11) because the standard for the wattle, used in the F-C analytical calibration curve (Fig. 14),was determined by the UV method. Again, only the ratio for the activated sludge effluent was different (Table 13); the other 7 unoxidized samples with an average ratio of 1,113 (relative standard deviation = 3,1%, n = 7), agree closely with the above value.

Table 13. Comparison of UV and F-C methods for monitoring of wastewater treatments.

Treatment	UV method (mg/ℓ) tannin			F-C method (mg/ℓ catechin equivilent)		
	Influent	Effluent	Polyphenol removed (%)	Influent	Effluent	Polyphenol removed (%)
$Al_2(SO_4)_3$						
1. Liritan colour vat liquor	24 950	1 711	95,3	22 710 (1,099)[a]	1 053 (1,112)	95,4
2. 1% (m/v) methanolic wattle	7 436	253	96,6	6 629 (1,122)	239 (1,059)	96,4
3. 1% (m/v) Mimosa ME extract	6 597	273	96,4	6 122 (1,173)	194 (1,124)	96,8
Activated sludge						
4. Sewage containing wattle extract	660	204[b]	69,1	599 (1,102)	86[b] (2,37)	83,8

a Values in parenthesis refer to the UV tannin : F-C catechin equivalent ratio.

b Corrected for sewage from control activated sludge unit.

Therefore, although the UV and F-C methods are suitable for monitoring unoxidized polyphenols, neither is reliable for oxidized wastewaters. The UV method will overestimate the oxidized polyphenols and is also open to interference by other organic matter which absorb at 280 nm. The F-C method, on the other hand, will underestimate polyphenols. This combination of over- and underestimation would explain why the F-C method gave results[95] which were less than half the value determined by the UV method for aerobically treated vegetable tannery wastewaters. The F-C method could, however, be a useful indirect method for determining the amount of oxidized polyphenols, provided an alternative method is available for the total polyphenols.

3.3 Carbonaceous Oxygen Demand Methods

3.3.1 Chemical methods, chemical oxygen demand (COD) and permanganate value (PV)

The theoretical oxidation of an organic compound to CO_2 and H_2O is calculated as the total oxygen demand (TOD), which should equal the experimental COD by acidic dichromate. For example, if the average wattle flavanoid is considered to have molecular mass 288 (*i.e.* $C_{15}H_{12}O_6$),[107] the TOD can be calculated from reaction (3).

$$C_{15}H_{12}O_6 + 15O_2 \longrightarrow 15CO_2 + 6H_2O \qquad (3)$$

therefore, TOD = (15 x 31,98 mg O_2)/288 mg wattle polyphenol

= 1,666 mg O_2 /mg wattle polyphenol

While straight-chain aliphatic compounds and pyridine are not oxidized under acidic dichromate conditions, most organic compounds give COD values of 95 - 100% of the TOD.[90] The results for standard phenols and the two wattle extracts confirm this (Table 14).

Although the PV method with acidic potassium permanganate has milder oxidation conditions than the COD method, as shown by the PV:COD ratios of less than 1 and a value of 0 for glucose, phenols are more sensitive

Table 14. Carbonaceous oxygen demand results.

Compound	TOD[a]	COD[a]	PV[a]	PV:COD
Hydroquinone (48)	1,888	1,842	1,494	0,811
Quinhydrone (49)	1,832	1,800	1,419	0,789
p-Quinone (50)	1,775	1,753	1,353	0,771
Resorcinol (40)	1,888	1,845	1,587	0,860
Phloroglucinol (41)	1,522	1,486	1,235	0,831
Catechol (44)	1,888	1,809	1,231	0,680
Pyrogallol (45)	1,522	1,474	1,107	0,751
(±)Dihydroquercetin (9)	1,525	1,476	1,083	0,710
(+)-Catechin (3)	1,708	1,626	1,033	0,635
Methanolic wattle extract	NA	1,480	0,809	0,547
Mimosa ME extract	NA	1,433	0,728	0,508
Glucose	1,066	1,031	0	0

a Results expressed as mg O_2/mg compound.

to permanganate oxidation than most organic compounds. A variety of permanganate oxidation methods have been developed for both phenolic determinations [133] and wastewater monitoring.[134]

Since the Mimosa ME and methanolic wattle extracts contained 66,0 and 74,3% of polyphenols, respectively, the COD and PV values can be calculated for the polyphenols and the non-polyphenolic constituents from the following simultaneous equations:

COD equations :

For Mimosa ME	0,340X	+	0,660Y	=	1,433	(4)
For Methanolic wattle	0,257X	+	0,743Y	=	1,480	(5)

where :

X = an average of the COD values for the non-phenols, and

Y = an average of the COD values for the polyphenols.

therefore X = 1,056 mg COD/mg non-phenol,

Y = 1,627 mg COD/mg wattle polyphenol.

This COD value for the wattle polyphenols agrees with the TOD value calculated from reaction (3).

PV equations :

For Mimosa ME	0,340U	+	0,660V	=	0,728	(6)
For Methanolic wattle	0,257U	+	0,743V	=	0,809	(7)

where :

U = an average of the PV values for the non-phenols, and

V = an average of the PV values for the polyphenols.

therefore U = 1,061 mg PV/mg wattle polyphenol,

V = 0,081 mg PV/mg non-phenol.

These results give PV:COD ratios of 0,652 and 0,077 for the polyphenolic and non-phenolic constituents, respectively. Both the individual COD and PV values as well as the PV:COD ratio for the polyphenols and non-phenolic constituents are close to the respective values of catechin (3) and glucose, which is expected since the phenolic constituents are mainly flavanoids and non-phenols are mainly sugars and gums(*viz.* carbohydrates).

The COD and PV values, however, do not validate the biodegradability of phenols, which can only be ascertained on comparison of BOD and COD results.

3.3.2 The biological oxygen demand (BOD) method

Before proceeding with routine BOD analyses, interference due to abiotic removal of DO[13] was investigated. Earlier studies[93,135] have shown that even in darkness, the phenolic compounds tannic acid, pyrogallol (45) and hydroquinone (48) consumed DO at the rate of about 0,6 mg/ℓ.h for 100 mg/ℓ solutions containing 6 mg/ℓ of Fe (III). Tannic acid gave appreciable oxygen consumption in the dark without Fe (III).[93] This indicates that a 10 mg/ℓ solution of such phenolic compounds would consume the available oxygen from a saturated solution in about 6 days. Parallel BOD determinations were performed on phenolic compounds with and without Hg(II) (1 mℓ saturated $HgCl_2$ / determination to prevent biodegradation) but no appreciable abiotic DO loss occurred with Hg(II). At low concentrations of phenols (<10 mg/ℓ), no interference occurred with the Winkler iodometric titration, as found in an earlier study[93] and it was not necessary to use the Rideal-Stewart (permanganate) modification.[136]

As the BOD method is limited by the maximum oxygen saturation value of water (9,0 mg/ℓ O_2 at 20°C and atm. pres.),[88] BOD solutions were diluted to COD values of about 10 mg/ℓ to ensure that the concentration of DO did not fall below the rate-limiting value of 0,5 mg/ℓ.[87] Although this COD concentration ensured acceptable DO consumption for easily degraded phenols, the more intractable phenolic material gave lower DO consumption. While a higher COD concentration could have been used in these cases, it was expedient to work at similar COD's to enable realistic comparisons. Furthermore, the toxicity of tannins to aquatic microfauna[9] could have adversely affected the bacteria in the BOD determinations and, therefore, low concentrations were adhered to.

The DO consumption for various standards and wattle extracts was monitored over a period of 20 days and the results expressed as the ratio of BOD_t:COD (Table 19, Appendix II, p. 80). This can be interpreted as the degree of biodegradability of a dissolved substance at a particular time. Graphical comparison of these results (Fig. 16) shows a variation of biodegradabilities. The easily degraded glucose/glutamic

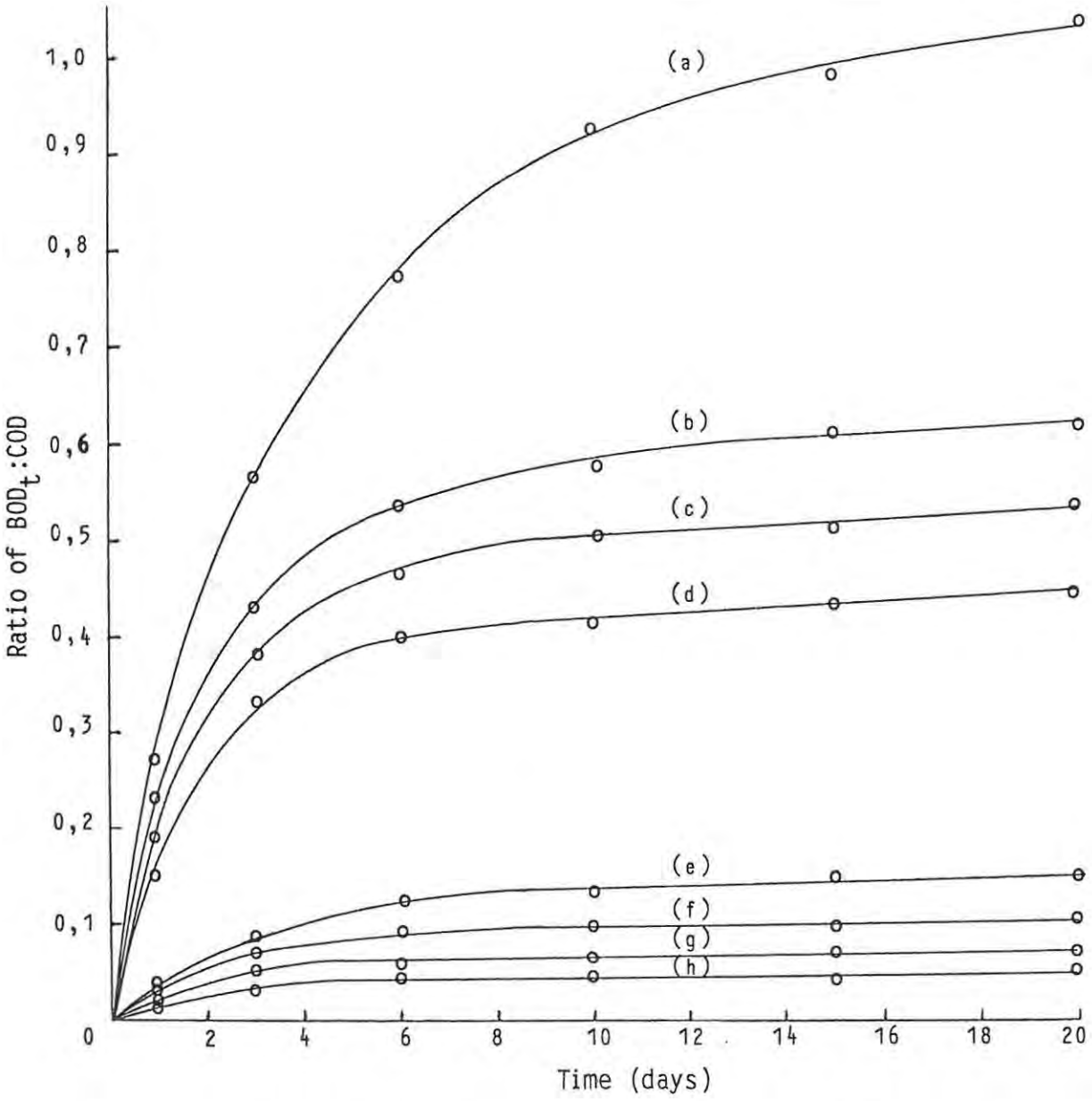


Figure 16. Biodegradability curves for wattle-type material.

(a) Glucose/glutamic acid mixture.

(b) (+)-Catechin.

(c) [*4,8*]-*R-trans-C*.

(d) Phenolic non-tannins.*

(e) Mimosa ME extract.

(f) Methanolic wattle extract.

(g) Ethyl acetate extract (*post* ether extraction).

(h) Activated sludge treated wattle extract.

*Phenolic non-tannins extracted with ethyl acetate from a 1% (m/v) solution of wattle extract after the solution had been treated with hide-powder.

acid mixture was used as a standard to ensure that a particular dilution water was biologically active.[88] Curve (a) in Fig. 16 indicates that by day 20, this mixture was totally degraded (*i.e.* a BOD:COD ratio close to 1). The results also indicate that, in general, the mono- and bi-flavanoids and the wattle extracts are approximately 50 and 10% biodegradable, respectively. This suggests that while the low molecular mass flavanoids are partially biodegradable, the high molecular mass tannins are not.

In the absence of toxic material, the complete bio-oxidation of dissolved organic matter occurs in two distinct stages.[137] The first, due to the oxidation of carbonaceous matter, extends over a period of 15 - 20 days. Towards the end of this period, sufficient nitrifying organisms have developed to initiate the second stage, nitrification of ammonia which was initially present in the dilution water, together with that produced by the decomposition of any nitrogenous organic matter. This nitrification process explains the high BOD_{20}:COD ratio of 1,04 for the glucose/glutamic acid mixture at day 20.

Kinetic studies[137] on sewage biodegradation have shown that if it is assumed that, by day 20, all of the biodegradable matter has been completely decomposed or denatured to inert non-biodegradable matter, the rate of reaction is second-order, thus,

$$dx/dt = k_2 (a - x)(b - x) \qquad (8)$$

where :

t = time (days),

a = initial DO,

b = BOD_{20},

x = BOD_t,

(a - x) = residual DO at t,

(b - x) = unsatisfied BOD, and

k_2 = second-order velocity coefficient.

Therefore, the rate of oxidation of the carbonaceous matter at any time during the first stage is proportional to both the residual DO concentration and the undegraded portion of the degradable matter (*i.e.* unsatisfied BOD).

$$\text{By integration,} \quad k_2 = \frac{2,303}{t(a - b)} \log \frac{b(a - x)}{a(b - x)} \qquad (9)$$

Equation (9) was applied to the BOD results in Table 19. As previously found for sewage,[137] during first stage bio-oxidation, second-order kinetics also applied (Table 15).

Table 15. Velocity coefficients, k_2 for the second-order biodegradation reactions.

Time (days)	1	3	6	10	15	Average
(a) Glucose/glutamic acid	0,0474	0,0418	0,0416	0,0470	0,0433	0,0442 ± 6,3%
(b) (+)-Catechin (3)	0,0850	0,0839	0,0941	0,0870	0,0941	0,0888 ± 5,6%
(c) [4,8]-*R-trans-C* (29)	0,0777	0,0902	0,0780	0,0768	0,0619	0,0769 ± 13,1%
(d) Phenolic non-tannins	0,0674	0,0828	0,0727	0,0734	0,0585	0,0710 ± 12,5%
(e) Mimosa ME extract	0,0397	0,0496	0,0346	0,0368	0,0340	0,0389 ± 16,3%
(f) Methanolic wattle extract	0,0549	0,0548	0,0441	0,0597	0,0471	0,0521 ± 12,2%
(g) Ethyl acetate extract	0,0553	0,0597	0,0506	0,0424	0,0444	0,0505 ± 14,4%
(h) Activated sludge treated wattle extract	0,0596	0,0447	0,0303	0,0226	0,0177	-

The scatter of the individual k_2 values with an average relative standard deviation of 11,5% reflects the precision of the BOD method, which in an extensive study[88] was found to have a relative standard deviation of 18%.

While the glucose/glutamic acid curve (a) in Fig. 16 shows complete degradation and the k_2 of 0,0442 is close to 0,0401 of a sewage study,[137] the phenolic material differed. For catechin, while k_2 is twice that of glucose/glutamic acid, curve (b) in Fig. 16 indicates that catechin fragmented to inert byproducts, which were not further degraded since the BOD_{20} only accounted for 62% of the total COD. [4,8]-*R-trans-C* (29) and the phenolic non-tannins, had similar k_2 values and were degraded 54 and 45%, respectively, indicating that inert byproducts were also formed.

Fig. 16 also shows that the wattle extracts, and notably the ethyl acetate extract, were only slightly biodegradable. The marginally improved results for Mimosa ME relative to methanolic wattle extract is probably due to the

larger carbohydrate fraction of the former. The ethyl acetate extract was least degraded since it contained no carbohydrates and fewer simple phenols due to the prior ether extraction.

Although empirical measurement of the amount of DO consumed in 5 days at 20°C, constitutes the official BOD_5 method, several weeks or even months are required for complete destruction of more intractable compounds. Direct total determination of organic compounds by the BOD method is therefore impractical.

Table 16. BOD_5 data for standard compounds and phenolic material.

Compound	BOD_5 (mg O_2/mg compound)	BOD_5:COD
(a) Glucose/Glutamic acid	0,594	0,723
(b) (+)-Catechin (3)	0,317	0,516
(c) [4,8]-*R-trans-C* (29)	0,277	0,451
(d) Phenolic non-tannins	0,238	0,388
(e) Mimosa ME extract	0,079	0,114
(f) Methanolic wattle extract	0,057	0,084
(g) Ethyl acetate extract	0,039	0,064
(h) Activated sludge treated wattle extract	0,080[a]	0,043
Resorcinol (40)	0,248	0,457
Phloroglucinol (41)	0,029	0,043
Catechol (44)	0,279	0,505
Pyrogallol (45)	< 0,007	<0,010

a Calculated from BOD_5 = 11 mg/ℓ, PV = 145 mg/ℓ and 1,061 PV/mg wattle polyphenol.

The comparison of BOD_5 values with those of mononuclear phenols (Table 16) shows that phloroglucinol (41) and pyrogallol (45) are relatively non-biodegradable. The formation of such compounds from primary degradation of flavanoids would explain why catechin (3), [4,8]-*R-trans-C* (29) and the phenolic non-tannins are only semi-biodegradable.

3.3.3 Application of the carbonaceous oxygen demand methods to wattle-based wastewaters

The COD, PV and BOD_5 values enable the wastewaters discussed in Section 3.2.3 to be typecaste in terms of their phenolic content and their biodegradability (Table 17).

Table 17. Carbonaceous oxygen demand results for treated and untreated wattle-based wastewaters.

Description	COD[a]	PV[a]	PV:COD	BOD_5[a]	BOD_5:COD
1. Liritan colour vat liquor	66 600	26 580	0,399	17 400	0,261
2. Aluminium sulphate treated LIRITAN liquor	27 700	1 360	0,049	19 200	0,693
3. 1% (m/v) Methanolic wattle extract	14 800	8 100	0,547	1 250	0,084
4. Total non-tannins[b]	4 210	429	0,102	2 370	0,563
5. Phenolic non-tannins[b]	518	348	0,672	201	0,388
6. Activated sludge influent containing Mimosa ME extract	1 360	690	0,508	155	0,114
7. Activated sludge effluent containing[c] oxidized polyphenols	252	145	0,575	11	0,044

a Results expressed as mg/ℓ O_2.

b From 1% (m/v) wattle extract.

c. Corrected for sewage control.

The COD and PV results of the average wattle polyphenols and non-phenolic material (see Section 3.3.1) can be used to estimate their concentrations in wastewaters. For example, in the untreated LIRITAN liquor, the PV of 26 580 mg/ℓ indicates 2,51% (m/v) (calculated from 1,061 mg PV/mg wattle polyphenol) polyphenols are present. This 2,51% of polyphenols would be responsible for 40 800 mg/ℓ COD (calculated from 1,627 mg COD/mg wattle polyphenol) leaving 25 800 mg/ℓ COD due to the accumulated non-tannins consisting of carbohydrates and nitrogenous compounds, which gives 2,44% (m/v) of non-phenolic material (calculated from 1,056 mg COD/mg non-phenol). This spent liquor, therefore, contains extract which comprises of about 51% polyphenols on an organic material basis,

whereas wattle extract contains a higher proportion of polyphenols. The BOD_5:COD value of 0,261 for this liquor (1) is greater than the value of 0,114 for Mimosa ME extract (with which the tanning liquor was originally prepared) indicating that the spent liquor is more biodegradable due to accumulation of carbohydrates during tanning.

BOD_5:COD ratios of typical domestic sewage and mixed industrial wastewaters have been reported as 0,541 and 0,372, respectively.[138] The total non-tannins after hide-powder treatment (4) and especially the aluminium sulphate treated LIRITAN liquor (2), with a BOD_5:COD values close to that of 0,723 for the glucose/glutamic acid standard (*c.f.* Table 16), indicate easier biodegradation since both contained high proportions of carbohydrates.

The phenolic non-tannins (5) had a higher BOD_5:COD value than that of the total wattle extract (3), probably due to the fact that the phenolic non-tannins are semi-biodegradable whereas the tannins in wattle extract are not. The value for (5), of 0,388, is similar to that of mixed industrial wastewaters, of 0,372.

The PV results for the aluminium sulphate treatment of LIRITAN liquor (*i.e.* (1) $\longrightarrow$ (2)) gives a removal of 94,9% while hide-powder treatment of the methanolic wattle extract solution (*i.e.* (3) $\longrightarrow$ (4)) gives 94,7% removal (Table 17). These results compare favourably with the F-C and UV results obtained in Section 3.2.3 (*cf.* Tables 12 and 13) and show consistancy for the three methods. However, the PV method as a determinant for wattle polyphenols can be expected to give slightly lower values for oxidized samples, as shown by the decrease in PV values on progressive oxidation from hydroquinone to *p*-quinone (Table 14). While the UV and F-C methods for the activated sludge treatment gave absolute values of 204 and 95 mg/ℓ wattle polyphenol (=86 mg/ℓ cat. equiv. in Table 13), respectively, the PV value gives 137 mg/ℓ (calculated from 1,061 mg PV/mg polyphenol and Table 17). This dilemma may be resolved by adopting the PV method, which gives intermediate values, and expressing results for oxidized samples in empirical terms of 'oxidized wattle polyphenols'. This PV method could also be utilized as an alternative to the UV, F-C or hide-powder methods to determine the polyphenol content of wattle extracts.

The final effluent after the activated sludge treatment of Mimosa ME extract (7) contained intractable residual polyphenols as shown by its low BOD_5:COD value.

In general, the PV:COD and BOD_5:COD values were found to be empirical relationships that characterize a wastewater. The interpretation, based on the prototype compounds and extracts investigated above, however, may not hold for mixed industrial wastewaters, where other compounds could interfere.

In conclusion, the level of sophistication required for the polyphenolic analysis would dictate the choice of method. While the gel chromatographic/HPLC procedure enables identification and quantitation of the low molecular mass constituents, up to the biflavanoid level, the scope of this technique is narrow, accounting for separations of less than 30% of the total polyphenols. In addition, the complexity of this technique disqualifies it from routine detailed analysis of wattle constituents, although HPLC alone does give meaningful comparative fingerprints. HPLC alone is successful for the analysis of unoxidized treated wastewaters, where the removal of the tannins results in simpler chromatograms. However, failure to record oxidized polyphenols, possibly due to irreversible adsorption of such material on the μBondapak C_{18} column, requires other less specific methods for their determination.

The less sophisticated spectrophotometric methods at least give a direct overall measure of the polyphenolic compounds, albeit on an average basis. In a mixed industrial wastewater, the F-C method would be more specific than the UV method, since only strong reducing agents will interfere with the former while many potential organic pollutants have some absorbance at 280 nm. In addition, the high sensitivity of the F-C method is ideal for low concentrations (> 0,3 mg/ℓ) of wattle polyphenols. With oxidized polyphenols, however, overestimation by the UV method and underestimation by the F-C method detract from their usefulness.

The classical carbonaceous oxygen demand methods are general and, therefore, open to interference, especially for COD, which measures most organic compounds in mixed industrial wastewaters. The BOD_t

values, however empirical, give a measure of biodegradability when related to COD. The PV value is the most useful classical determinant available since oxidation does not affect this method to the same extent as for spectrophotometric determinations.

4. SUMMARY

Analytical procedures for the determination of wattle polyphenols in wastewaters are made difficult by the complexity of wattle extract and the concomitant formation of oxidation-degradation products in the wastewater. The recently developed method, high-performance liquid chromatography (HPLC) was at first considered to be well suited for this purpose. However, since no HPLC study on wattle extract had been performed, a preliminary HPLC investigation was initially carried out prior to assessing HPLC as a method for analysing wattle-based wastewaters.

Initial HPLC of the total extract gave a complicated unresolved chromatogram. To simplify these separations, wattle extract was then first subjected to gel chromatography, where separation occurred due to adsorption, with the more highly adsorbed components (*viz.* the higher molecular mass flavanoids) being eluted later. A total wattle extract, as well as three solvent partitioned extracts, from the consecutive extractions of an aqueous solution of wattle extract with ether and ethyl acetate, were subjected to gel chromatography on Sephadex LH-20 using ethanol as eluent. Fractions from these separations were in turn analysed by HPLC. The combined separations from both gel chromatography and HPLC were enumerated on a two-dimensional chromatogram to give 77 components from the gel chromatographic fractions collected in the first 50 hours, during which time 27% of the wattle polyphenols were eluted. Most of these components were minor constituents which concentrated in the ether extract.

Major components were identified with available reference compounds and information from two-dimensional paper chromatography; and quantified in catechin equivalents by integration of peaks in HPLC. The monoflavanoids identified were, (–)-fisetinidol (0,23%), (–)-robinetinidol (0,74%), (+)-catechin (1,20%), (+)-gallocatechin (0,26%), (+)-fustin (trace) and (+)-dihydrorobinetin (0,40%). Traces of (+)-leucofisetinidin and (+)-leucorobinetinidin were observed on two-dimensional paper chromatograms. Both flavonols, robinetin and fisetin were identified by HPLC from gel chromatographic fractions of the enriched ether extract.

Biflavanoids that were identified included the two-dimensional paper

chromatographic spot B, [4,8]-all-*trans*-(–)-robinetinidol-(+)-catechin (5,26%) and [4,8]-all-*trans*-(–)-robinetinidol-(+)-gallocatechin (>1,65%). A further six components were detected in the F/A region. Tentative identifications of four of these components were made, including [4,8]-3,4-*cis*-(–)-fisetinidol-(+)-catechin (1,77%), [4,8]-3,4-*trans*-(–)-fisetinidol-(+)-catechin (0,80%) and [4,8]-3,4-*cis*-(–)-robinetinidol-(+)-gallocatechin (1,02%). The further three components were candidates for [4,8]-3,4-*cis*-(–)-robinetinidol-(+)-catechin. A further two components which had similar R_f co-ordinates to (+)-catechin showed UV spectrophotometric properties of the [4,6]-(–)-fisetinidol-(+)-leucofisetinidin class of biflavanoids.

No higher oligomers were identified although trimers from two-dimensional paper chromatographic spots C and E had similar HPLC retentions to both mono- and biflavanoids which accounts for the complexity of the total wattle HPLC chromatogram.

HPLC of a 1% wattle extract solution that had been treated with hide-powder gave a simpler chromatogram which showed that, besides the removal of tri- and oligomeric flavanoids (the tannins), about 80% of the mono- and biflavanoids (the so-called phenolic non-tannins) were also adsorbed by hide-powder. Similar chromatograms resulted from a fresh wattle solution and a spent LIRITAN colour vat liquor that had both been treated with aluminium sulphate. These chromatograms showed major components with low retentions, similar to standard mononuclear phenols.

HPLC of wattle extract that had been subjected to biological activated sludge treatment, showed no component peaks at all, although spectrophotometric methods indicated at least 30% of the polyphenols had not been removed from the effluent. This was probably due to oxidative polymerization of the phenolic components to products not recorded by HPLC.

Both the ultra-violet (UV)and Folin-Ciocalteu (F-C) methods were applied to standard mononuclear phenols, flavanoids and wattle extracts. Results showed that both methods were suitable for unoxidized samples, with the F-C method being ten times more sensitive than the UV method. In addition, the F-C method would be expected to be more specific in mixed industrial wastewaters, although it is open to interference from strong reducing agents. Both spectrophotometric methods indicated a

95% removal of polyphenols by hide-powder treatment of wattle solutions, and also correlated well when used to monitor aluminium sulphate treatment of wattle-based wastewaters. Wattle solution that had been oxidized in biological activated sludge treatment, however, gave F-C results about half those of the UV method, due to underestimation of oxidized polyphenols in the F-C method and overestimation in the UV method.

The general carbonaceous oxygen demand methods chemical oxygen demand (COD), permanganate value (PV) and the biological demand method (BOD) proved useful in typecasting wattle-based wastewaters. The BOD_t:COD ratios indicated that mono- and biflavanoids and non-tannins of wattle extract are as biodegradable as sewage and mixed industrial wastewaters. However, the higher molecular mass tannins, and oxidized wattle components are sparingly biodegradable. While the COD method is non-specific and measures most organic compounds, the PV method was more specific, with 1,06 mg PV/mg wattle polyphenol. Results from the PV method, on monitoring aluminium sulphate treated wattle liquors, agreed with those from the UV and F-C methods. The PV method was also sensitive to biological activated sludge treated wastewaters and results, between those of the overestimated UV and underestimated F-C results, were obtained.

The PV:COD and BOD_5:COD ratios gave empirical values that are useful for characterizing a wattle-based wastewater. Interpretation, based on the prototype compounds and wattle extracts investigated here-in, would propably not hold for mixed industrial wastewaters, where other dissolved organic substances could interfere.

5. APPENDIX I

General Liquid Chromatographic Terms and Symbols

Liquid chromatography has developed more or less empirically. Prior to HPLC there was little theory and, therefore, no need to standardize terms, definitions and symbols. The variants of liquid chromatography have developed as separate techniques, resulting in varied nomenclatures. To avoid ambiguity, the following definitions, terms and symbols were adopted, in accordance with a recent review.[107]

Retention time is the most direct method for measuring the retention of a solute.

Total retention time, t_R, is defined as the time between the start of elution and the emergence of the peak maximum.

Mobile phase hold-up time, t_M, is the observed elution time of a non-retained solute.

Adjusted retention time, t'_R, is given by

$$t'_R = t_R - t_M$$

The analogous retention volumes, V_R, V_M and V'_R are the product of the retention times and the flow rate, F. V_o is used in preference to V_M for gel chromatography.

Capacity factor, k. This expresses the following ratio for a given solute at any point in the column at equilibrium:

$$k = \frac{\text{amount of solute on the stationary phase}}{\text{amount of solute in the mobile phase}} = \frac{1 - R}{R}$$

$$= t'_R / t_M \qquad \text{(optimum value, } 1 < k < 10\text{)}$$

The R value is the fraction of solute in the mobile phase.

$$R = \frac{1}{k + 1}$$

R is the thermodynamic reflection of the distribution co-efficient, K, and can be related to fundamental values in all variants of chromatography.

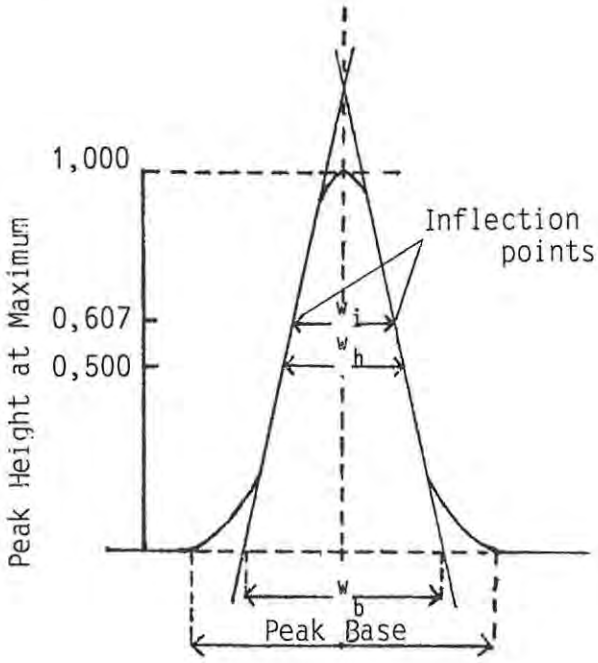
$$K = k\beta$$

$$\text{where } \beta = \frac{\text{volume of mobile phase in column}}{\text{volume of stationary phase in column}}$$

In the ideal case, R = Retention factor of planar chromatography (*i.e.*, Rf)

R_f = ratio of zone velocity to the mobile phase velocity.

Column efficiency is reflected in the band spread throughout the chromatographic system. This band spread varies for difference substrates.



Peak widths:

at base w_b

at half-height w_h

at inflection points w_i

The peak width at base is the segment of baseline intercepted by tangents drawn to the inflection points; in the Gaussian peak case, the inflection points are at 0,607 of maximum peak height.

Theoretical plate number,

$$n = 16(t_R/w_b)^2$$

$$= 5{,}545\ (t_R/w_h)^2$$

$$= 4\ (t_R/w_i)^2$$

(usually $3000 < n < 8000$)

Effective plate number,

$$N = 16\ (t'_R/w_b)^2$$

$$= 5{,}545\ (t'_R/w_h)^2$$

$$= 4\ (t'_R/w_i)^2$$

Height equivalent to one theoretical plate (HETP), $h = L/n$ (L in mm with h usually approximately 14 - can vary by a factor of 2)

Height equivalent to one effective plate (HEEP), $H = L/N$

Resolution, R_s, is the measure of separation of two peaks.

$$R_s = 2\ (t_{R2} - t_{R1})/(w_{b1} + w_{b2})$$

where $t_{R2} > t_{R1}$

For baseline resolution $R_s \geq 1{,}5$.

Separation factor, α, is used to designate the relative retention of two consecutive peaks.

$$= t'_{R2}/t'_{R1} = K_2/K_1 = k_2/k_1 \neq t_{R2}/t_{R1}$$

where $t'_{R2} > t'_{R1}$ (i.e. $\alpha > 1$)

Relative retention, $r_{i,s}$, can be larger, equal to, or smaller than unity. i refers to the solute of interest and s to the standard.

$$r_{i,s} = t_{Ri}/t_{Rs}$$

Throughout this work (+)-catechin was used as the standard. To minimize long subscripts, the symbol r_{Cat} was used.

Peak ratios. With a dual absorbance detector system, peak ratios provides a useful diagnostic which, when used in conjunction with the retention time, t_R, or relative retention, r_{Cat}, gives reliable substrate identifications.[50] The 280/254 ratio was used as most of the wattle components have a higher absorbance at 280nm than at 254nm. While the height ratio (h_r) is easier to measure, the area ratio (a_r) gives more accurate results especially where peaks are not sharp and band spreading occurs.

$$a_r = \frac{\text{peak area at 280nm}}{\text{peak area at 254nm}} \qquad h_r = \frac{\text{peak height at 280nm}}{\text{peak height at 254nm}}$$

Table 18. Retention times, capacity factors,[a] and log k values of the biflavanoids and reference standards on the μBondapak C_{18} column with increasing methanol concentration in the mobile phase

Methanol Concentration	10%			14%			16%			18%			20%		
Components	t_R	k	log k	t_R	k	log k	t_R	k	log k	t_R	k	log k	t_R	k	log k
Acetophenone	51,7	33,5	1,53	34,8	22,2	1,35	27,9	17,6	1,25	23,7	14,8	1,17	19,1	11,7	1,07
Dihydroquercetin	41,7	26,8	1,43	26,8	16,9	1,23	21,5	13,3	1,12	17,8	10,9	1,03	14,1	8,40	0,924
14d	78,6	51,4	1,71	37,2	23,8	1,38	25,6	16,1	1,21	18,1	11,1	1,04	12,9	7,60	0,881
14c	57,2	37,2	1,57	28,7	18,1	1,26	20,0	12,3	1,09	14,3	8,53	0,931	10,6	6,07	0,783
13b	56,7	36,8	1,56	27,8	17,5	1,25	19,4	11,9	1,08	13,7	8,13	0,910	10,4	5,93	0,773
9c	46,7	30,1	1,48	23,8	14,9	1,17	16,8	10,2	1,01	12,6	7,40	0,869	9,00	5,00	0,699
15b	29,5	18,7	1,28	16,3	9,87	0,994	12,1	7,07	0,849	9,10	5,07	0,705	6,80	3,53	0,548
12a	29,4	18,6	1,27	16,2	9,80	0,991	11,8	6,87	0,837	9,00	5,00	0,699	6,80	3,53	0,548
14b	22,4	14,0	1,15	12,20	7,13	0,853	8,95	4,97	0,696	6,93	3,62	0,559	5,30	2,53	0,404
11a	16,9	10,3	1,01	9,54	5,36	0,729	7,21	3,80	0,580	5,90	2,93	0,467	4,50	2,00	0,301
10a	15,5	9,31	0,969	8,78	4,85	0,686	6,59	3,40	0,531	5,27	2,51	0,400	4,20	1,80	0,255
(+)-Catechin	10,4	5,96	0,775	7,10	3,73	0,572	5,81	2,87	0,458	4,90	2,27	0,356	4,20	1,80	0,255
15a	8,90	4,93	0,693	5,70	2,80	0,447	4,71	2,14	0,330	3,93	1,62	0,209	3,30	1,20	0,079
13a	8,05	4,37	0,640	5,10	2,40	0,380	4,29	1,86	0,269	3,51	1,34	0,128	3,00	1,00	0,000

a. Calculated with t_M = 1,50

Table 19. Biochemical oxidation of wattle-related phenolic compounds.

Description	Time, t (days)	Residual DO [a]	BOD_t [a]	BOD_t:COD
Seeded dilution water control. Initial DO = 8,043	1	7,997	0,046	NA
	3	7,949	0,094	NA
	6	7,905	0,138	NA
	10	7,880	0,163	NA
	15	7,449	0,194	NA
	20	7,822	0,221	NA
Glucose + glutamic acid (3 mg/ℓ each) COD = 4,927 Initial DO = 7,931	1	6,412	1,473	0,299
	3	5,043	2,794	0,567
	6	3,984	3,809	0,773
	10	3,220	4,548	0,923
	15	2,928	4,809	0,976
	20	2,571	5,139	1,043
(+)-Catechin (3) COD = 9,786 Initial DO = 7,454	1	4,958	2,450	0,250
	3	3,147	4,213	0,431
	6	2,051	5,265	0,538
	10	1,649	5,642	0,576
	15	1,349	5,911	0,604
	20	1,136	6,097	0,623
[4,8]-*R-trans-C* (29) COD = 9,985 Initial DO = 7,539	1	5,397	2,096	0,210
	3	3,491	3,954	0,396
	6	2,778	4,623	0,463
	10	2,334	5,042	0,505
	15	2,193	5,152	0,516
	20	1,936	5,382	0,539
Phenolic Non-tannins COD = 9,561 Initial DO = 7,164	1	6,000	1,568	0,164
	3	4,317	3,203	0,335
	6	3,690	3,786	0,396
	10	3,330	4,121	0,431
	15	3,232	4,188	0,438
	20	3,100	4,293	0,449

Table 19 continued.

Description	Time, t (Days)	Residual DO[a]	BOD_t[a]	BOD_t:COD
Mimosa ME extract COD = 10,01 Initial DO = 7,497	1	7,061	0,390	0,039
	3	6,602	0,801	0,080
	6	6,188	1,171	0,117
	10	5,923	1,411	0,141
	15	5,811	1,492	0,149
	20	5,724	1,552	0,155
Methanolic wattle extract COD = 9,991 Initial DO = 7,408	1	7,022	0,340	0,034
	3	6,605	0,709	0,071
	6	6,401	0,869	0,087
	10	6,226	1,019	0,102
	15	6,185	1,029	0,103
	20	6,148	1,039	0,104
Ethyl acetate wattle extract COD = 10,16 Initial DO = 7,387	1	7,071	0,250	0,0245
	3	6,750	0,543	0,0535
	6	6,584	0,665	0,0655
	10	6,508	0,716	0,0705
	15	6,472	0,721	0,0710
	20	6,409	0,757	0,0745
Activated sludge treated wattle extract COD = 10,04 Initial DO = 7,852	1	7,602	0,204	0,0203
	3	7,404	0,354	0,0353
	6	7,301	0,413	0,0411
	10	7,237	0,452	0,0450
	15	7,181	0,477	0,0475
	20	7,079	0,552	0,0550

a Results in mg/ℓ O_2.

6. REFERENCES

1. African Territories Wattle Industry Fund Limited, *Mimosa - Properties, Composition, Reactions and Industrial Applications of Mimosa Extract*, London, 1980.

2. Gustavson K.H., *The Chemistry of Tanning Processes*, Academic Press, New York, 1956.

3. Roux D.G., and Paulus E., *Biochem. J.*, 82 (1962) 320.

4. Roux D.G., Ferreira D., and Botha J.J., *J. Agric. Food Chem.*, 28 (1980) 216.

5. Haslam E., *Chemistry of Vegetable Tannins*, Academic Press, London, 1966.

6. Okadu T., Mori K., and Aoi K., *Yakugaku Zasshi*, 97 (1977) 1267.

7. Society of Leather Trades' Chemists, *Official Methods of Analysis*, Section 5, 1965.

8. Garbutt D.C.F., and Noble M.A., *Report Wattle Research Institute for 1982 - 1983*, (in press).

9. Grant W.D., *Science*, 193 (1976) 1137.

10. Mitchell R., (Ed.), *Water Pollution Microbiology*, Wiley-Interscience, New York (1972).

11. Zillick A.Z., *J. Wat. Pollut. Control Fed.*, 44 (1972) 212.

12. Onodera S., Tabata M., Suzuki S., and Ishikura S., *J. Chromatogr.*, 200 (1980) 137.

13. Larson R.A., and Hufnal J.M., *Limnol. Oceanogr.*, 25 (1980) 505.

14. Stone T., *Wat. Pollut. Control*, 80 (1981) 659.

15. Howe R.H.L., Howe R.C., and Howe J.M., *Chem. Eng.*, 59 (1981) 99.

16. Harborne J.B., *Comparative Biochemistry of the Flavonoids*, Academic Press, London, 1967.

17. Leather Industries' Research Institute, *Wattle Tannin and Mimosa Extract*, Grahamstown, South Africa, 1955.

18. Roux D.G., and Evelyn S.R., *J. Chromatogr.*, 1 (1958) 537.

19. Roux D.G., and Maihs A.E., *J. Chromatogr.*, 4 (1960) 65.

20. Roux D.G., Maihs A.E., and Paulus E., *J. Chromatogr.*, 5 (1961) 9.

21. Roux D.G., Drewes S.E., and Saayman H.M., *J. Amer. Leather Chem. Assoc.*, 59 (1965) 416.

22. Saayman H.M., and Roux D.G., *Biochem. J.*, 97 (1965) 794.

23. Roux D.G., *J. Amer. Leather Chem. Assoc.*, 55 (1960) 272.

24. Roux D.G., and Maihs E.A., *Biochem.J.*, 74 (1960) 44.

25. Drewes S.E., and Roux D.G., *Biochem. J.*, 87 (1963) 167.

26. Roux D.G., and Ferreira D., in *Progress in the Chemistry of Organic Natural Products*, 41, Herz W., Griseback H., and Kirby G.W., (Eds.), Springer-Verlag, New York, 1982.

27. Botha J.J., Young D.A., Ferreira D., and Roux D.G., *J. Chem. Soc., Perkin Trans. 1*, (1981) 1213.

28. van der Westhuizen J.H., Ferreira D., and Roux D.G., *J. Chem. Soc., Perkin Trans. 1*, (1981) 1220.

29. Hundt H.K.L., and Roux D.G., *J. Chem. Soc., Perkin Trans. 1*, (1981), 1227.

30. Botha J.J., Ferreira D., and Roux D.G., *J. Chem. Soc., Perkin Trans. 1*, (1981) 1235.

31. Botha J.J., Viviers P.M., Young D.A., du Preez I.C., Ferreira, D., Roux D.G., and Hull W.E., *J. Chem. Soc. , Perkin Trans 1*, (1982) 527.

32. Viviers P.M., Young D.A., Botha J.J., Ferreira D., Roux D.G., and Hull W.E., *J. Chem. Soc., Perkin Trans. 1*, (1982) 535.

33. Viviers P.M., Botha J.J., Ferreira D., Roux D.G., and Saayman H.M., *J. Chem. Soc., Perkin Trans. 1*, (1983) 17.

34. Steenkamp J.A., Ferreira D., Roux D.G., and Hull W.E., *J. Chem. Soc., Perkin Trans. 1*, (1983) 23.

35. Botha J.J., Ferreira D., and Roux D.G., *J. Chem. Soc., Chem. Commun.*, (1978) 700.

36. Botha J.J., Ferreira D., and Roux D.G., *J. Chem. Soc., Chem. Commun.*, (1979) 510.

37. Roux D.G., and Evelyn S.R., *Biochem. J.*, 76 (1960) 17.

38. Pharmacia Fine Chemicals, *Sephadex - Gel Filtration in Theory and Practice*, Uppsala, Sweden, 1966.

39. Woof J.B., and Pierce J.S., *J. Chromatogr.*, 28 (1967) 94.

40. Harborne J.B., Mabry T.J., and Mabry H., (Eds.), *The Flavonoids*, Chapman and Hall, London, 1975.

41. Johnston K.M., Stern D.J., and Waiss A.C. Jr., *J. Chromatogr.*, 33 (1968) 539.

42. Thompson R.S., Jacques D., Haslam E., and Tanner R.J.N., *J. Chem. Soc., Perkin Trans. 1*, (1972) 1387.

43. Lamparski L.L., and Nestrick T.J., *J. Chromatogr.*, 156 (1978) 45.

44. Katagi T., Horii A., Oomura Y., Miyakawa H., Kyu T., Ikeda Y., Isoi K., and Masami M., *J. Chromatogr.*, 79 (1973) 45.

45. Kusy V., *J. Chromatogr.*, 57 (1971) 132.

46. Collier P.D., and Mallous R., *J. Chromatogr.*,57 (1971) 29.

47. Horvāth C., *Trends in Anal. Chem.*, 1 (1981) 6.

48. Karger B.L., and Giese R.W., *Anal. Chem.*, 50 (1978) 1048 A.

49. Anderson J.L., Weishaar D.E., and Tallman D.E., *Anal. Chem.*, 53 (1981) 906.

50. Baker J.K., Shelton R.E., and Cheng-yu M., *J. Chromatogr.*, 168 (1979) 417.

51. Erni F., Keller H.P., Morin C., and Schmitt M., *J. Chromatogr.*, 204 (1981) 65.

52. Rhaghavan R.V., *J. Chromatogr.*, 168 (1979) 523.

53. Adamovics J., and Stermitz F., *J. Chromatogr.*, 129 (1976) 464.

54. Wulf L.W., and Nagel C.W., *J. Chromatogr.*, 116 (1976) 271.

55. Villeneuve F., Abravanel G., Moutounet M., and Alibert G., *J. Chromatogr.*, 234 (1982) 131.

56. Hoefler A.C., and Coggon P., *J. Chromatogr.*, 129 (1976) 460.

57. Qureshi A.A., Prentice N., and Burger W.C., *J. Chromatogr.*, 170 (1979) 343.

58. Lea A.G.H., *J. Chromatogr.*, 194 (1980) 62.

59. Lea A.G.H., *J. Chromatogr.*, 238 (1982) 253.

60. Bankova V.S., Popov S.S., and Marekov N.L., *J. Chromatogr.*, 242 (1982) 135.

61. Okadu T., Kazuko M., Seno K., and Hatano T., *J. Chromatogr.*, 171 (1979) 313.

62. Vande Casteele K., Geiger H., and van Sumere C.F., *J. Chromatogr.*, 240 (1982) 81.

63. Daigle D.J., and Conkerton E.J., *J. Chromatogr.*, 240 (1982) 202.

64. Jerumanis J., *Proc. Congr. - Eur. Brew. Conv. 17th*, (Fr.), (1979) 309.

65. Rousseff R.L., and Ting S.V., *J. Chromatogr.*, 176 (1979) 75.

66. Rohrschneider L., *Anal. Chem.*, 45 (1973) 1241.

67. Snyder L.R., *J. Chromatogr. Sci.*, 16 (1978) 223.

68. Glajck J.L., Kirkland J.J., and Squire K.M., *J. Chromatogr.*, 199 (1980) 57.

69. Smith R.M., *J. Chromatogr.*, 236 (1982) 313.

70. Halāsz I., *Anal. Chem.*, 52 (1980) 1393 A.

71. Smith R.M., *J. Chromatogr.*, 236 (1982) 321.

72. Fourie T.G., Ferreira D., and Roux D.G., *J. Chem. Soc., Perkin Trans. 1*, (1977) 125.

73. Roux D.G., *J. Amer. Leather Chem. Assoc.*, 53 (1958) 384.

74. Farkas L., Gābor M., and Kallay F., (Eds.), *Topics in Flavonoid Chemistry and Biochemistry*, Elsevier, Amsterdam, 1975.

75. Griffiths L.A., in *ref. 74*, p. 201.

76. Takācs Ö., and Gābor M., in *ref. 74*, p. 227.

77. Schultz E., Engle F.E., and Wood J.M., *Biochem.*, 13 (1974) 1768.

78. Barz W., and Hösel W., in *ref. 40*, p. 916.

79. Mythirayee C., Krishnamurthy H., and Madhavakrisha W., *Leather Sci.*, 26 (1979) 198.

80. Atluru M.D., *Ph.D. Thesis*, West Virginia Univ., Morgantown, 1972.

81. Pollack S.S., Lentz H., and Ziechmann W., *Soil Sci.*, 112 (1971) 318.

82. Dufek P., Černohorský I., and Pacākova V., *J. Chromatogr.*, 241 (1982) 19.

83. Somers T.C., *Phytochem.*, 10 (1971) 2175.

84. La Noce T., *Cuoio, pelli, mat. concianti*, 50 (1974) 75.

85. Aziz F.A., and Tebbutt T.H.Y., *Wat. Res.*, 14 (1980) 319.

86. Cooper J.E., Bitcover E.H., Filachione E.M., Harlan J.W., Bailey D.G., and Feairheller S.H., *J. Amer. Leather Chem. Assoc.*, 75 (1980) 331.

87. Green M., Shelef G., and Moraine R., *Wat. Pollut. Control*, 80 (1981) 655.

88. Am. Pub. Health Assoc., *Standard Methods for the Examination of Water and Wastewater* 15th Ed., Washington D.C., 1980.

89. *South African Gov. Gaz.*, No. 2512, 29 August 1969, p. 10.

90. Moore W.A., Kroner R.C., and Ruckhoft C.C., *Anal. Chem.*, 8 (1949) 953.

91. Phelps E.B., *Stream Sanitation*, John Wiley and Sons, New York, (1944).

92. Arthur R.M., *Wat. Sewage Wks.*, 121 (1974) 100.

93. Miles C.F., and Brezonik P.L., *Env. Sci. Tech.*, 15 (1981) 1089.

94. Roux D.G., *J. Soc. Leather Trades Chem.*, 35 (1951) 322.

95. Corning D.R., *Report LR111*, Brit. Leather Man. Res. Assoc., September 1981.

96. Mabry T.J., Markham K.R., and Thomas M.B., *The Systematic Identification of Flavonoids*, Springer-Verlag, New York, 1970.

97. Gordon-Gray C.G., *J. Soc. Leather Trades Chem.*, 41 (1957) 269.

98. Roux D.G., *J. Soc. Leather Trades Chem.*, 41 (1957) 275.

99. Slabbert N.P., *L.I.R.I. Res. Bull.* 632 (1973).

100. Folin O., and Ciocalteu V., *J. Biol. Chem.*, 73 (1927) 239.

101. Wu H., *J. Biol. Chem.*, 43 (1920) 189.

102. Dawson B., *Acta. Cryst.* 6 (1953) 113.

103. So H., and Pope M.T., *Inorg. Chem.*, 11 (1972) 1441.

104. Tsigdinos G.A., *Topics in Current Chem.*, 76 (1978) 1.

105. Singleton V.L., *Adv. in Chem.*, 137 (1974) 184.

106. Del Pezzo L., and Grasso G., *Cuoio, pelli, mat. concianti*, 54 (1978) 607.

107. Slabbert N.P., *Ph.D. Thesis*, Rhodes University, 1972.

108. Ettre L.S., *J. Chromatogr.*, 220 (1981) 29.

109. Ilsley A.H.,M.Sc. Thesis, Rhodes University, 1969.

110. Drewes S.E.,Ph.D. Thesis, Rhodes University, 1963.

111. Maihs E.A., Ph.D. Thesis, Rhodes University, 1961.

112. Roux D.G., *J. Soc. Leather Trades Chem.*, 37 (1953) 229.

113. Weinges K., *Ann.*, 615 (1958) 203.

114. Roberts E.A.H., *Chem. and Ind. (London)*, (1956) 737.

115. Baker J.K., *Anal. Chem.*, 49 (1977) 737.

116. Drewes S.E., Roux D.G., Eggers S.H., and Feeney J., *J. Chem. Soc. C.*, (1967) 1217.

117. Roux D.G., *Phytochem.*, 11 (1972) 1219.

118. Katyal M., and Prakash S., *Talanta*, 24 (1977) 367.

119. Degrémont, *Water Treatment Handbook*, Stephen Austin & Sons, Caxton Hill, 1973.

120. Aloy M., Folachier A., and Vulliermet B., *Pollution and Tannery*, Centre Tecnnique Du Cuir, Lyon, 1976.

121. Symons J.M., McKinney R.E., and Hassis H.H., *J. Wat. Pollut. Centr. Fed.*, 32 (1960) 841.

122. Roux D.G., *J. Soc. Leather Trade's Chem.*, 41 (1957) 287.

123. Sparrow N.A., Ph.D. Thesis, Rhodes University, 1982.

124. Roux D.G., and Paulus E., *Biochem. J.*, 82 (1962) 324.

125. Papaconstantinou E., and Pope M.T., *Inorg. Chem.*, 9 (1970) 667.

126. Papaconstantinou E., and Pope M.T., *Inorg. Chem.*, 6 (1967) 1152.

127. Varga Jr. G.M., Papaconstantinou E., and Pope M.T., *Inorg. Chem.*, 9 (1970) 662.

128. Slabbert N.P., *Tetrahedron.*, 33 (1977) 821.

129. Albert A., and Serjeant E.P., *The Determination of Ionization Constants*, Chapman and Hall, London, 1971.

130. International Union of Pure and Applied Chemistry, *Anal. Chem.*, 48 (1976) 2294.

131. Strong III F.C., *Anal. Chem.*, 51 (1979) 298.

132. Jeanin Y., Launay J.P., and Seid Sedjadi M.E., *Inorg. Chem.*, 19 (1980) 2933.

133. Assoc. Offic. Agr.Chem., *Official Methods of Analysis*, 7th Ed., Washington D.C., 1950.

134. Am Pub. Health Assoc. and Am Water Works Assoc., *Standard Methods for the Examination of Water and Sewage*, 9th Ed., New York, 1946.

135. Theis T.L. and Singer P.C., *Environ. Sci. Technol.*, 8 (1974) 569.

136. Theriault E.J., and McNamee P.D., *Ind. Eng. Chem.*, Anal. Ed., 4 (1932) 59.

137. Stones T., *Wat. Pollut. Control*, 80 (1981) 513.

138. Stones T., *Wat. Pollut. Control*, 80 (1981) 659.