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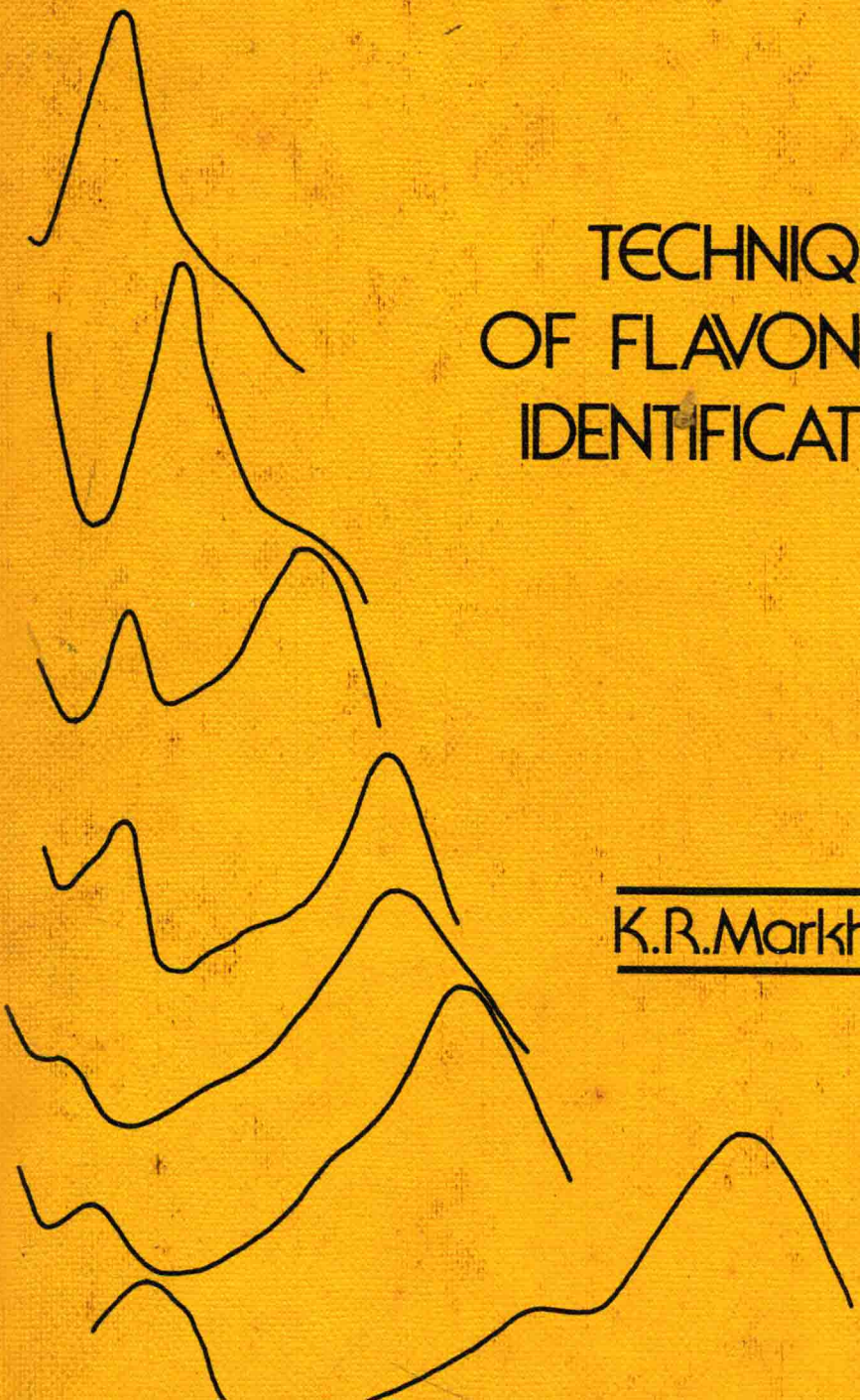

TECHNIQUES OF FLAVONOID IDENTIFICATION

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Techniques of Flavonoid Identification

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To the girls in my life, my wife Pauline
and my daughters, Pamela and Carolyn

Preface

A great deal has already been written on flavonoids, but with few exceptions these are review articles for the specialist which do not discuss practical aspects of techniques in any detail, and so are of limited usefulness to scientists from other disciplines. In the present volume I have attempted to provide the non-specialist with an introduction to, and practical details of, the techniques commonly used to isolate and identify flavonoids from natural sources. However, I am hopeful that much of the information contained herein will also be of value to active researchers in the field, especially if their major discipline is biological rather than chemical. With this in view I have included liberal referencing to alternative techniques and to sources of additional data, which I hope will extend the usefulness of the book as a primary source of information.

The chapters are ordered in a sequence which I feel is best followed when first approaching the problem of flavonoid identification. Thus, isolation and purification of the flavonoid must be achieved first. Information gained from chromatographic behaviour can then be used in conjunction with u.v.-visible absorption data to help identify possible structures. Various forms of hydrolysis may then be appropriate, followed by analysis of the products. The more intractable problems may require additionally the use of more sophisticated techniques such as chemical manipulation, n.m.r. or m.s. Finally, direct comparison with an authentic sample (if possible) is always desirable for confirmation of the proposed structure.

The information presented in this book represents knowledge accumulated over many years of experience in this field, and I am particularly indebted to Professors Tom Mabry (Botany Department, University of Texas) and Jeffrey Harborne (Botany Department, University of Reading) in whose laboratories I gained much of this experience. I am also grateful to my friend and colleague, Dr Lawrence Porter (Chemistry Division, DSIR, New Zealand) whose cooperation and enthusiasm I have enjoyed over the past decade. The support and encouragement I have received from both the administration and staff of the Chemistry Division, DSIR, is also gratefully acknowledged.

The study of flavonoid chemistry and its application to such diverse fields as plant taxonomy and evolution, plant dispersal, plant breeding and fruit and vegetable preservation and processing etc., has proved both challenging and rewarding to me and has added immeasurably to the enjoyment of my

scientific career to date. It is my earnest hope that application of the information contained in this book will assist others to gain similarly from this fascinating field of endeavour.

October 1981 K. R. MARKHAM

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Introduction to the Flavonoids

It is estimated that about 2% of all carbon photosynthesized by plants (or about 1×10^9 tons per annum) is converted into flavonoids or closely related compounds (Smith, 1972). Most tannins too are flavonoid derived. Flavonoids thus constitute one of the largest groups of naturally occurring phenols. They are virtually ubiquitous in green plants and as such are likely to be encountered in any work involving plant extracts. For this reason it is important that chemists, biochemists, plant physiologists and biologists generally, know how to recognize, isolate and identify these natural products in all their many forms. The following discussion is designed to provide a basic introduction to the flavonoids for newcomers to this field.

1.1 Flavonoid structure variation—general

In plants, flavonoid aglycones (i.e. flavonoids without attached sugars) occur in a variety of structural forms. All contain fifteen carbon atoms in their basic nucleus and these are arranged in a C_6–C_3–C_6 configuration, that is, two aromatic rings linked by a three carbon unit which may or may not form a third ring. For convenience the rings are labelled A, B and C and the individual carbon atoms are referred to by a numbering system which utilizes ordinary numerals for the A- and C-rings and "primed" numerals for the B-ring (see (**1**) but note modified numbering systems used for chalcones, Fig. 1.1).

The flavonoid variants are all related by a common biosynthetic pathway which incorporates precursors from both the "Shikimate" and "Acetate-Malonate" pathways (Hahlbrock and Grisebach, 1975; Wong, 1976), the first flavonoid being produced immediately following confluence of the two pathways (Fig. 1.1). The flavonoid initially formed in the biosynthesis is now

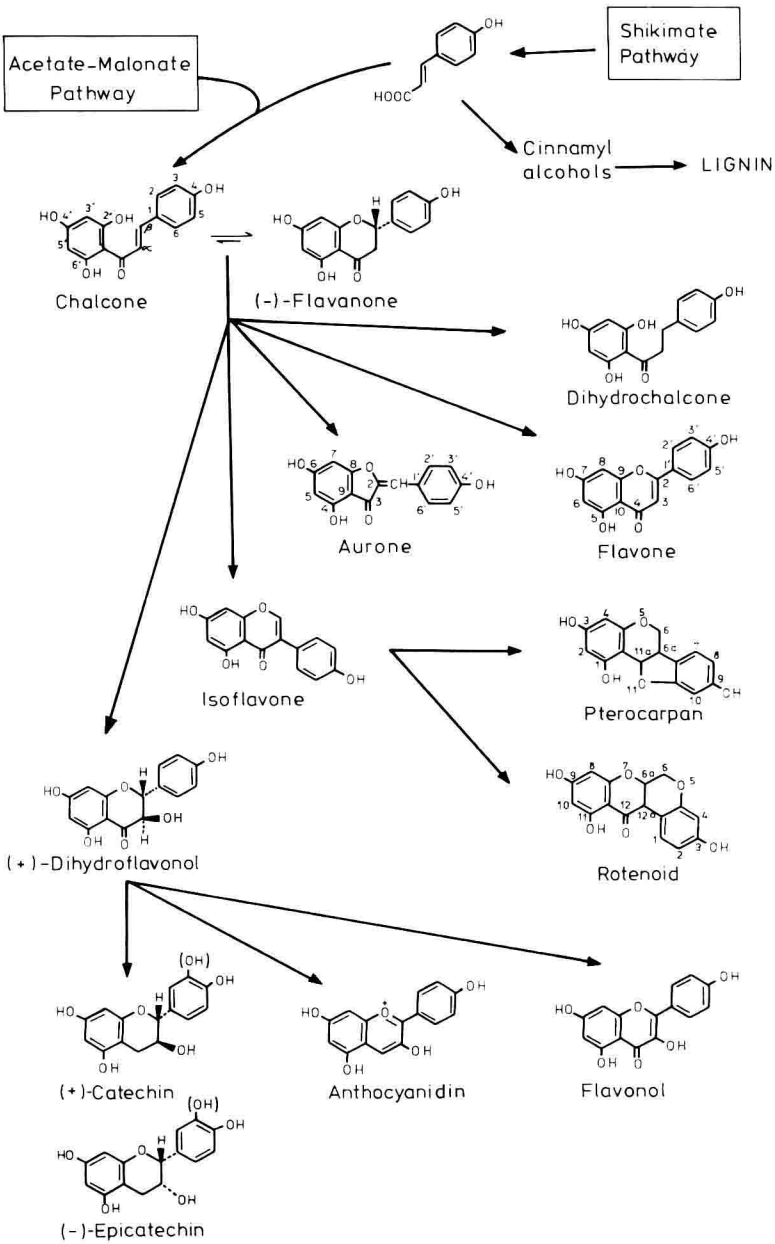
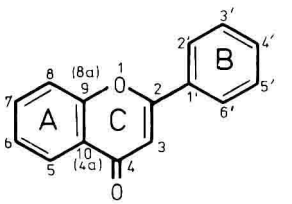


Fig. 1.1. Currently proposed interrelationships between flavonoid monomer types (supported by varying levels of experimental evidence, see Wong, 1976).

thought to be the chalcone (Hahlbrock, 1980) and all other forms are derived from this by a variety of routes (Fig. 1.1). Further modification of the flavonoid may occur at various stages resulting in: additional (or reduced) hydroxylation; methylation of hydroxyl groups or of the flavonoid nucleus;



(1)

isoprenylation of hydroxyl groups or of the flavonoid nucleus; methylenation of *ortho*-dihydroxyl groups; dimerization (to produce biflavonoids); bisuphate formation; and most importantly, glycosylation of hydroxyl groups (to produce *flavonoid O-glycosides*) or of the flavonoid nucleus (to produce *flavonoid C-glycosides*). The range of known flavonoids is thus vast and lists of known variants have been published (Harborne *et al.*, 1975) and recently updated (Harborne and Mabry, 1982). For the reader unfamiliar with the myriad of "trivial" names given to many flavonoids, an excellent summary has been compiled by Swain (1976) which relates name, structure and primary source and another by Wollenweber and Dietz (1981). A selection of these from Swain (1976) and Geissman (1962) is included here in Table 1.1 as an aid to understanding later chapters in this book in which trivial names are used for both convenience and conciseness.

Table 1.1
A selection of frequently encountered flavonoid aglycones, their trivial names, structures and primary sources

Flavonoid aglycones	Structure	Source
Flavones		
Chrysin	5,7-OH	*Populus*
Baicalein	5,6,7-OH	*Scutellaria*
Apigenin	5,7,4′-OH	*Petroselinum*
Acacetin	4′-Me apigenin	*Robinia*
Scutellarein	5,6,7,4′-OH	*Scutellaria*
Hispidulin	6-Me scutellarein	*Ambrosia*
Luteolin	5,7,3′,4′-OH	*Reseda*
Chrysoeriol	3′-Me luteolin	*Eriodictyon*
Diosmetin	4′-Me luteolin	*Diosma*
Tricetin	5,7,3′,4′,5′-OH	*Lathyrus*
Tricin	3′,5′,-Me tricetin	*Triticum*

Table 1.1 (contd)

Flavonoid aglycones	Structure	Source
Flavonols		
Galangin	3,5,7-OH	*Alpinia*
Fisetin	3,7,3′,4′-OH	*Rhus*
Kaempferol	3,5,7,4′-OH	*Delphinium*
Kaempferide	4′-Me kaempferol	*Alpina*
Robinetin	3,7,3′, 4′, 5′-OH	*Robina*
Herbacetin	3,5,7,8,4′-OH	*Gossypium*
Quercetin	3,5,7,3′,4′-OH	*Quercus*
Rhamnetin	7-Me quercetin	*Rhamnus*
Isorhamnetin	3′-Me quercetin	*Cheiranthus*
Myricetin	3,5,7,3′,4′,5′-OH	*Myrica*
Quercetagetin	3,5,6,7,3′,4′-OH	*Tagetes*
Gossypetin	3,5,7,8,3′,4′-OH	*Gossypium*
Anthocyanidins		
Apigenidin	5,7,4′-OH	*Rechsteineria*
Luteolinidin	5,7,3′,4′-OH	*Rechsteineria*
Pelargonidin	3,5,7,4′-OH	*Pelargonium*
Cyanidin	3,5,7,3′,4′-OH	*Centaurea*
Peonidin	3′-Me cyanidin	*Paeonia*
Delphinidin	3,5,7,3′,4′,5′-OH	*Delphinium*
Petunidin	3′-Me delphinidin	*Petunia*
Malvidin	3′,5′-Me delphinidin	*Malva*
Isoflavones		
Daidzein	7,4′-OH	*Pueraria*
Formononetin	4′-Me daidzein	*Ononis*
Genistein	5,7,4′-OH	*Genista*
Biochanin-A	4′-Me genistein	*Cicer*
Orobol	5,7,3′,4′-OH	*Orobus*
Tectorigenin	5,7,4′-OH 6-OMe	*Iris*
Baptigenin	5,7,3′,4′,5′-OH	*Baptisia*
Flavanones		
Pinocembrin	5,7-OH	*Pinus*
Liquiritigenin	7,4′-OH	*Glycyrrhiza*
Naringenin	5,7,4′-OH	*Prunus*
Sakuranetin	7-Me naringenin	*Prunus*
Eriodictyol	5,7,3′,4′,-OH	*Eriodictyon*
Hesperetin	4′-Me eriodictyol	*Prunus*

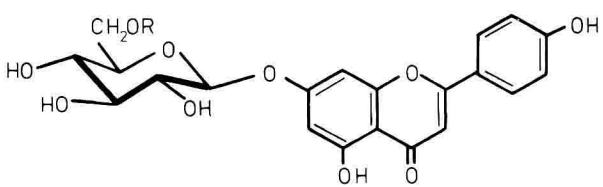
Flavonoid aglycones	Structure	Source
Dihydroflavonols		
Pinobanksin	3,5,7-OH	*Pinus*
Aromadendron	3,5,7,4′-OH	*Eucalyptus*
Fustin	3,7,3′,4′-OH	*Rhus*
Taxifolin	3,5,7,3′,4′-OH	*Pseudotsuga*
Biflavonoids		
Agathisflavone	6,8″-biapigenin	*Agathis*
Cupressuflavone	8,8″-biapigenin	*Cupressus*
Amentoflavone	3′,8″-biapigenin	*Cupressus*
Ginkgetin	amentoflavone 7,4′-dimethyl ether	*Ginkgo*
Sciadopitysin	amentoflavone 7,4′,4‴ trimethyl ether	*Ginkgo*
Robustaflavone	6,3‴-biapigenin	*Agathis*
Hinokiflavone	6,4‴-bi-O-apigenin	*Cupressus*
Ochnaflavone	3′,4‴-bi-O-apigenin	*Ochna*
Chalcones[a]		
Isoliquiritigenin	2′,4′,4-OH	*Acacia*
Chalconaringenin	2′,4′,6′,4-OH	*Salix* (as 2′-*O*-glucoside)
Butein	2′,4′,3,4-OH	*Acacia*
Okanin	2′,3′,4′,3,4,-OH	*Acacia*
Aurones[a]		
Sulphuretin	6,3′,4′-OH	*Bidens*
Aureusidin	4,6,3′,4′-OH	*Antirrhinum*
Maritimetin	6,7,3′,4′,-OH	*Bidens*
Leptosidin	6,3′,4′,-OH,7-OMe	*Coreopsis*

[a] Note different numbering systems used (Fig. 1.1).

1.2 Flavonoid *O*-glycosides

Flavonoids commonly occur as flavonoid *O*-glycosides in which one or more of the flavonoid hydroxyl groups is bound to a sugar or sugars by an acid-labile hemiacetal bond (e.g. (**2**)). The effect of glycosylation is to render the flavonoid less reactive and more water (sap) soluble, the latter property permitting storage of the flavonoids in the cell vacuole (where they are commonly found). Although hydroxyl groups in any position on the flavonoid nucleus may be glycosylated, in fact hydroxyls in certain sites have a much higher probability of being so than others, e.g. the 7-hydroxyl in flavones, isoflavones and dihydroflavones, the 3- (and 7-) hydroxyl in flavonols and

dihydroflavonols, and the 3- (and 5-) hydroxyl in anthocyanidins. Glucose is the sugar most commonly involved, although galactose, rhamnose, xylose and arabinose are not uncommon. Other sugars occasionally encountered include allose, mannose, fructose, apiose and glucuronic and galacturonic acids. Disaccharides are also often found in association with flavonoids, e.g. sophorose (2-*O*-*β*-D-glucosyl-D-glucose), gentiobiose (6-*O*-*β*-D-glucosyl-D-glucose), rutinose (6-*O*-*α*-L-rhamnosyl-D-glucose) and neohesperidose (2-*O*-*α*-L-rhamnosyl-D-glucose), and occasionally tri- and even tetra-saccharides. It is accepted that in plants, *O*-glycosylation (and methylation) occurs as one of the last stages in the biosynthesis and is catalysed by enzymes of high specificity. Glycosides occasionally exhibit one further modification, that of acylation. Acylated glycosides have one (or more) of their sugar hydroxyls derivatized with an acid such as acetic or ferulic. The bond in this case is an ester bond, the acid effectively being esterified by the sugar, as for example in (**3**). The range of flavonoid *O*-glycosides found in nature has been summarized (Harborne *et al.*, 1975) and recently updated (Harborne and Mabry, 1982).



(2) (R = H) Apigenin 7-*O*-*β*-D-glucopyranoside
(3) (R = $OCOCH_3$) Apigenin 7-*O*-*β*-D-(6″-*O*-acetyl)glucopyranoside

1.3 Flavonoid *C*-glycosides

Sugars may also be *C*-linked to the flavonoid and in this case they are attached directly to the benzene nucleus by a carbon-carbon bond (e.g. (**4**)) which is acid resistant (c.f. *O*-glycosides). Such glycosides are referred to as flavonoid *C*-glycosides. To date *C*-linked sugars have been found only at the 6- and 8-positions on flavonoid nuclei. The range of sugars involved is apparently very much smaller than in *O*-glycosides and includes glucose most commonly (e.g. vitexin, orientin), and also galactose (e.g. apigenin 8-*C*-galactoside), rhamnose (e.g. violanthin), xylose (e.g. vicenin-1) and arabinose (e.g. schaftoside). The range of flavonoid aglycone types involved is also very restricted. Thus, although isoflavones, flavanones and flavonols occur occasionally in *C*-glycosylated form, flavone *C*-glycosides are by far the most prevalent. As with *O*-glycosides, *C*-glycosides are often found