

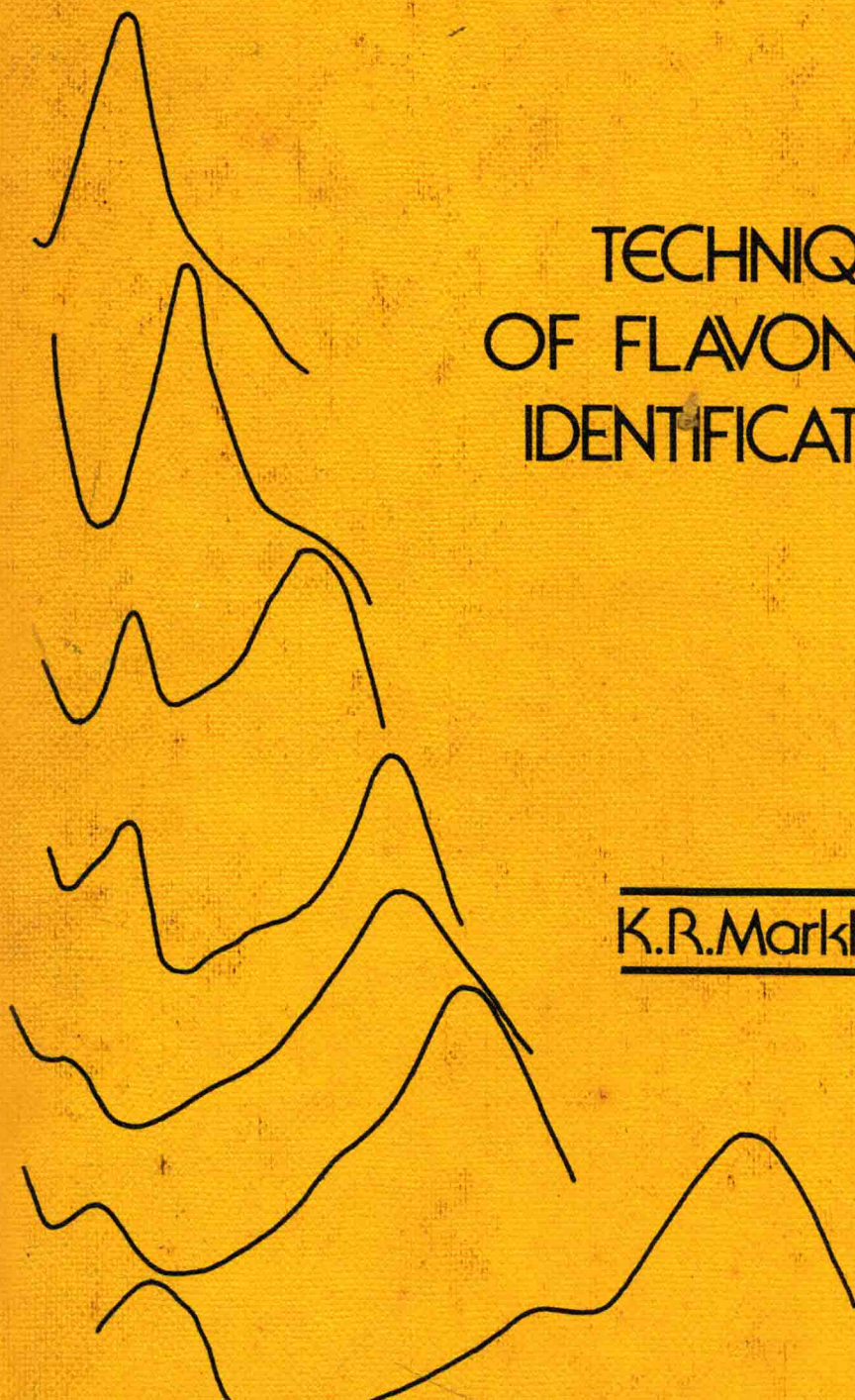


Academic
Press

Biological
Techniques
Series

TECHNIQUES
OF FLAVONOID
IDENTIFICATION

K.R. Markham



0041362

Techniques of Flavonoid Identification

K. R. Markham

Chemistry Division
Department of Scientific and Industrial Research
Petone, New Zealand

1982



ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

*London New York
Paris San Diego San Francisco
São Paulo Sydney Tokyo Toronto*

0041365
Techniques of
Flavonoid
ACADEMIC PRESS INC. (LONDON) LTD
24-28 Oval Road,
London NW1

U.S. Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

Copyright © 1982 by ACADEMIC PRESS INC. (LONDON) LTD

All Rights Reserved

No part of this book may be reproduced in any form by photostat, microfilm, or
any other means, without written permission from the publishers

British Library Cataloguing in Publication Data

Markham, K.R.

Techniques of flavonoid identification. — (Biological
techniques series)

1. Flavonoids

I. Title

547.7 QP925.F5

ISBN 0-12-472680-1

LCCCN 81-68987

Typeset by Latimer Trend & Company Ltd, Plymouth
Printed in Great Britain by
St Edmundsbury Press, Bury St Edmunds, Suffolk

0041362

Techniques of Flavonoid Identification

K. R. Markham

Techniques of Flavonoid Identification. K. R. Markham 1982
R. D. Purves 1981
Microelectrode Methods for Intracellular Recording and Ion-transport
R. A. McCulloch and C. M. Purves 1981
Whole-body Autoradiography. C. C. Cairns, S. A. M. K. Markham
Microclimate Measurement for Ecology. D. M. T. in 1980
Proteins. R. J. Mayer and J. H. H. in 1980
Immunochromatological Methods in the Biological Sciences. Enzymes and
Time-lapse Cinematography. P. M. Riddle 1979
Ion-sensitive Intracellular Microelectrodes. R. G. Thomas 1978

Red Cell Membranes - A Membrane - A Membrane - A Membrane
J. D. Young



ACADEMIC PRESS

1221 Avenue of the Americas, New York, N.Y. 10020, U.S.A.

London New York

San Diego San Francisco

Singapore Sydney Tokyo Toronto

Biological Techniques Series

J. E. TREHERNE

*Department of Zoology
University of Cambridge
England*

P. H. RUBERY

*Department of Biochemistry
University of Cambridge
England*

Ion-sensitive Intracellular Microelectrodes, *R. C. Thomas* 1978

Time-lapse Cinemicroscopy, *P. N. Riddle* 1979

Immunochemical Methods in the Biological Sciences: Enzymes and
Proteins, *R. J. Mayer* and *J. H. Waller* 1980

Microclimate Measurement for Ecologists, *D. M. Unwin* 1980

Whole-body Autoradiography, *C. G. Curtis*, *S. A. M. Cross*,
R. J. McCulloch and *G. M. Powell* 1981

Microelectrode Methods for Intracellular Recording and Ionophoresis,
R. D. Purves 1981

Techniques of Flavonoid Identification. *K. R. Markham* 1982

In preparation

Red Cell Membranes—A Methodological Approach, *J. C. Ellory* and
J. D. Young

*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

Contents

Preface	vii
1	
Introduction to the Flavonoids	
1.1 Flavonoid structure variation—general	1
1.2 Flavonoid <i>O</i> -glycosides	5
1.3 Flavonoid <i>C</i> -glycosides	6
1.4 Flavonoid sulphates	8
1.5 Biflavonoids	8
1.6 Optically active flavonoid aglycones	9
1.7 Guide to the distribution of flavonoid types in nature	10
1.8 Suggested stepwise procedure for flavonoid structure determination	12
2	
Isolation and Analytical Techniques	
2.1 Solubility characteristics of flavonoids	15
2.2 Selection, preparation and extraction of plant material	15
2.3 Paper chromatography and the recognition of flavonoids	16
2.3.1 General technique	17
2.3.2 Flavonoid structure information from p.c. data	19
2.3.3 Solvents and the improvement of spot resolution	21
2.3.4 R_f values	23
2.3.5 Spray reagents and high-sensitivity detection	24
2.3.6 Preparative p.c. for the isolation of flavonoids	25
2.3.7 Hints on solvent evaporation	25
2.4 Column chromatography—for large-scale separations	26
2.4.1 Techniques of column packing and sample application	26
2.4.2 Selection of column packing	28
2.4.3 Solvent selection and column elution	29
2.5 Other useful chromatographic techniques	31
2.5.1 Thin-layer chromatography (t.l.c.) for rapid micro-analysis	31
2.5.2 Paper electrophoresis for analysis of flavonoid bisulphates and glucuronides	32
2.5.3 High performance liquid chromatography (h.p.l.c.) for quantitative microanalysis	33

2.6	Recrystallization techniques	34
-----	------------------------------------	----

3

Ultraviolet-visible Absorption Spectroscopy

3.1	Introduction	36
3.2	The flavonoid spectrum—general	37
3.3	Additional information gained by use of shift reagents	39
3.3.1	Preparation of shift reagents	39
3.3.2	Stepwise procedure for use of shift reagents	40
3.3.3	Interpretation of the spectra	40
3.3.4	Spectra exemplifying shift reagent effects	45
3.4	Sources of reference spectra	49

4

Hydrolysis and the Analysis of Glycosides

4.1	Acid treatment	52
4.1.1	Standard procedure for hydrolysis of <i>O</i> -glycosides	52
4.1.2	Modified procedures for mild hydrolysis and acid labile aglycones	53
4.1.3	Sugar analysis by paper chromatography	53
4.1.4	Sugar analysis by gas-liquid chromatography	55
4.1.5	Flavonoid <i>C</i> -glycoside/ <i>O</i> -glucuronide recognition	56
4.1.6	Bisulphate detection	56
4.2	Enzymic hydrolysis	57
4.2.1	β -Glucosidase	58
4.2.2	Pectinase	58
4.2.3	Anthocyanase	58
4.2.4	β -Glucuronidase	58
4.3	Alkaline hydrolysis	59
4.3.1	Hydrolysis of glycosides	59
4.3.2	Removal of acyl groups	59
4.4	Summary	60

5

Chemical Methods used in Flavonoid Structure Elucidation

5.1	Derivatization techniques	62
5.1.1	Trimethylsilyl ether derivatives	62
5.1.2	Acetylation	63
5.1.3	Permethylation (and perdeuteromethylation)	63
5.1.4	Partial methylation (ethylation) with diazomethane (diazoethane)	64
5.1.5	Methylation (or ethylation) with dimethyl (or diethyl) sulphate	65

5.1.6	Isopropylidene derivatives	66
5.1.7	References to other useful derivatization techniques	67
5.2	Degradative techniques	67
5.2.1	Demethylation (and de- <i>C</i> -glycosylation) methods	67
5.2.2	Cleavage of disaccharides intact from flavonoid 3- <i>O</i> -diglycosides	67
5.2.3	Cleavage of sugar moiety intact from a flavonoid <i>C</i> -glycoside	68
5.2.4	Alkaline degradation of isoflavones to deoxybenzoins ..	68
5.2.5	Alkaline oxidation of isoflavones to benzoic acids (derived from B-ring)	69
5.2.6	Alkaline cleavage of flavonoids (general)	69
5.2.7	Potentially useful interconversions of flavonoid types ...	70
5.2.8	Colour tests for flavanones and dihydroflavonols	70

6

The Usefulness of n.m.r. and m.s. data in Flavonoid Structure Elucidation

6.1	Proton magnetic resonance (^1H -n.m.r.) spectroscopy	72
6.1.1	Typical applications	72
6.1.2	Introduction to ^1H -n.m.r. spectroscopy	72
6.1.3	Sample size and solvents	74
6.1.4	Example spectra and their interpretation	75
6.2	Carbon-13 magnetic resonance (^{13}C -n.m.r.) spectroscopy	78
6.2.1	Typical applications	78
6.2.2	Introduction to ^{13}C -n.m.r. spectroscopy	78
6.2.3	Sample size and solvents	82
6.2.4	Example spectra and their interpretation	82
6.3	Mass spectroscopy	86
6.3.1	Typical applications	86
6.3.2	Introduction to mass spectroscopy (m.s.)	86
6.3.3	Interpretation of flavonoid aglycone mass spectra	87
6.3.4	Interpretation of flavonoid glycoside mass spectra	90
6.3.5	Field desorption mass spectrometry (f.d.m.s.)	93

7

Confirmation of Flavonoid Structure by Direct Comparison 94

References 99

Subject Index 105

1

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 (I) 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

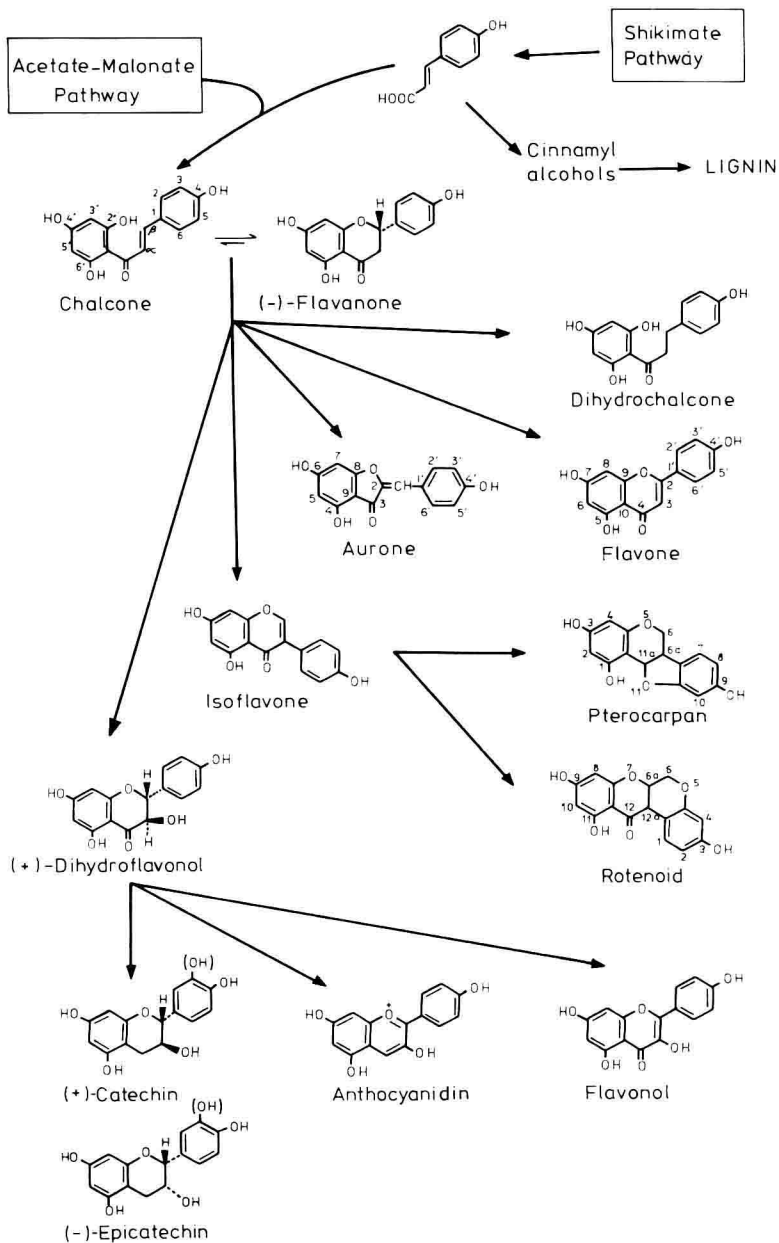
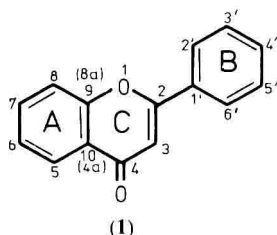


Fig. 1.1. Currently proposed interrelationships between flavonoid monomer types (supported by varying levels of experimental evidence, see Wong, 1976).

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;



isoprenylation of hydroxyl groups or of the flavonoid nucleus; methylenation of *ortho*-dihydroxyl groups; dimerization (to produce biflavonoids); bisulfate 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
<i>Flavones</i>		
Chrysin	5,7-OH	<i>Populus</i>
Baicalein	5,6,7-OH	<i>Scutellaria</i>
Apigenin	5,7,4'-OH	<i>Petroselinum</i>
Acacetin	4'-Me apigenin	<i>Robinia</i>
Scutellarein	5,6,7,4'-OH	<i>Scutellaria</i>
Hispidulin	6-Me scutellarein	<i>Ambrosia</i>
Luteolin	5,7,3',4'-OH	<i>Reseda</i>
Chrysoeriol	3'-Me luteolin	<i>Eriodictyon</i>
Diosmetin	4'-Me luteolin	<i>Diosma</i>
Tricetin	5,7,3',4',5'-OH	<i>Lathyrus</i>
Tricin	3',5',-Me tricetin	<i>Triticum</i>

Table 1.1 (contd)

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

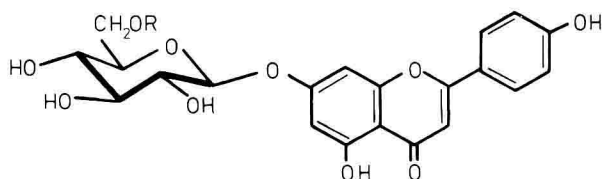
Flavonoid aglycones	Structure	Source
<i>Dihydroflavonols</i>		
Pinobanksin	3,5,7-OH	<i>Pinus</i>
Aromadendron	3,5,7,4'-OH	<i>Eucalyptus</i>
Fustin	3,7,3',4'-OH	<i>Rhus</i>
Taxifolin	3,5,7,3',4'-OH	<i>Pseudotsuga</i>
<i>Biflavonoids</i>		
Agathisflavone	6,8"-biapigenin	<i>Agathis</i>
Cupressuflavone	8,8"-biapigenin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

^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 = OCOCH₃) 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