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The Systematic Identification of Flavonoids

With 325 Figures

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Chapter I describes in detail the two-dimensional paper chromatographic analysis of flavonoids. Although sufficient pure material can usually be eluted from the paper chromatograms to obtain the ultraviolet spectra, in some instances the purification of the flavonoids can only be achieved by other techniques such as thin-layer or column chromatography. In Chapter II the column and thin-layer chromatographic procedures which are commonly employed in our laboratory for the separation of flavonoids are described. Chapter III, the last chapter in Part I, presents gas and paper chromatographic procedures for the identification of the sugar moieties in flavonoid glycosides together with comments on the various methods available for determining the structures of flavonoid aglycones.

Part II presents data and procedures for the ultraviolet spectral analysis of flavonoids. Once a pure flavonoid is obtained, its ultraviolet spectra in methanol alone and methanol with each of five diagnostic reagents are always recorded. Chapter IV outlines the steps for obtaining the UV data while Chapters V, VI and VII present, respectively, the UV spectral curves with interpretations for flavones and flavonols; isoflavones, flavanones, and dihydroflavonols; and chalcones and aurones. For each of the 175 flavonoids examined in the present investigation a set of six UV spectra are presented along with R_f values in the solvents used for two-dimensional paper chromatography and spot colors when viewed on paper under ultraviolet light alone and ultraviolet light in the presence of ammonia vapor.

Finally in Part III we have discussed procedures for obtaining and interpreting NMR spectra of flavonoids (Chapter VIII) as well as presenting 128 NMR spectra (Chapter IX). Most of the NMR spectra were determined for the trimethylsilyl ethers of the flavonoids, all of which are soluble in carbon tetrachloride.

Only with two flavonoids, hymenoxin and scaposin, both of which are highly oxygenated and methoxylated flavones, have we found it necessary to resort to the total synthesis of a flavonoid in order to establish its structure; in almost all other cases the information recorded here was sufficient for the complete structure analysis.

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Preface

About 1958, the late Professor R. E. ALSTON and Professor B. L. TURNER, both of the Department of Botany, The University of Texas at Austin, initiated a general systematic investigation of the legume genus *Baptisia*. They found that flavonoid patterns, as revealed by two-dimensional paper chromatography, were valid criteria for the recognition of the *Baptisia* species and for the documentation of their numerous natural hybrids. Later, they showed that the flavonoid chemistry could be used for the analysis of gene flow among populations. At that time no attempt was made to even partially identify the flavonoids which were detected chromatographically. Nevertheless, it soon became apparent that the full value of the chemical data for systematic purposes required knowledge of the structures of the flavonoids.

In 1962, one of us (T. J. M.) in collaboration with Drs. ALSTON and TURNER began the chemical analysis of the more than 60 flavonoids which had been chromatographically detected in the 16 *Baptisia* species. In the intervening years, a number of chemists and botanists, including Drs. K. BAETCKE, B. BREHM, M. CRANMER, D. HORNE, J. KAGAN, B. KROSCHEWSKY, J. MCCLURE, H. RÖSLER, and J. WALLACE, participated in the development of techniques and procedures for the rapid identification of known flavonoids and in the structure determination of new flavonoids. In addition, the flavonoid chemistry of many plants other than *Baptisia* was investigated.

Two of us (K. R. M. and M. B. T.) joined the group in 1965 and were recipients of Post-doctoral Fellowships from the University of Texas at Austin during the period (1965 – 1967) when most of the information presented in this volume was assembled. This volume presents, for the most part, procedures which were most useful in our flavonoid studies, together with our collection of ultraviolet and nuclear magnetic resonance spectra of flavonoids. Thus, no attempt has been made to describe all the information available in the literature regarding the isolation and identification of flavonoids (J. B. HARBORNE's "Comparative Biochemistry of the Flavonoids" provides an excellent summary of the literature up to 1966). Moreover, a number of classes of flavonoids are either not treated at all (anthocyanins) or are only covered briefly (for example, chalcones and aurones). The quantity of data presented for each of the various classes of flavonoids corresponds roughly to the frequency with which we have encountered them.

The book is divided into three parts (I, II and III); the first deals mostly with the isolation and purification of flavonoids while the second and third comprise a spectra section in which flavonoid UV and NMR spectra are discussed.

Before an analysis of the flavonoids in a given plant is initiated, we place in the University of Texas at Austin Herbarium a voucher specimen representing the plant population under investigation. The importance of properly vouchering the plant material before beginning the chemical studies cannot be over emphasized for only in this way can later investigators ascertain with certainty the plant for which the chemical results are reported.

Our first step in a typical investigation of the flavonoids in a plant is to extract the flavonoids from a few dried leaves with methanol or aqueous methanol; the extract is then used to determine the two-dimensional paper chromatographic flavonoid pattern.

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Part I

The Isolation, Purification and Preliminary Identification of Flavonoids

Chapter I

The Two-Dimensional Paper Chromatographic Analysis of Flavonoids

Two-dimensional paper chromatography represents one of the best methods for the rapid separation of mixtures of flavonoids from crude methanol or methanol-water extracts of dried plant material. Furthermore, sufficient quantities of the separated compound for both hydrolytic and ultraviolet spectral analyses often can be isolated from about 30 chromatograms. The present discussion will emphasize the chromatographic techniques routinely employed in this laboratory for the analysis of flavonoids; however, a number of other procedures and variations have been published elsewhere [*1*, *2*].

I-1. Reagents and Materials

Paper

Whatman 3 MM chromatographic paper (46 × 57 cm) has proved to be satisfactory for both qualitative and quantitative analysis of crude plant extracts containing complex mixtures of flavonoids.

The TBA and HOAc Solvent Systems

a) *TBA* 3:1:1 Solution of reagent-grade tertiary butanol:reagent-grade glacial acetic acid:water.

b) *HOAc* 15 Ml of reagent grade glacial acetic acid mixed with 85 ml of H_2O.

The TBA and HOAc solvent systems were satisfactory for the two-dimensional paper chromatographic analysis of most flavonoid extracts encountered in our laboratory. The TBA solvent is unstable when stored for long periods, and it is recommended that it be prepared fresh each month and stored in the dark.

Chromatographic Cabinet (Chromatocab)

Chromatocabs[1] may be constructed according to the plans presented in Fig. 1. A chromatocab is required for each solvent system. The plans call for (i) a glass window in one end of the chromatocab for viewing chromatograms, (ii) a foam neoprene gasket around the top of the cabinet to ensure air-tight sealing of the lid, and (iii) a completely waxed interior to protect the cabinet and to assist in solvent equilibration. Five of each of the following items[2], with the exception of the anti-siphon rods, are required for each cabinet (thus 10 chromatograms may be developed simultaneously in each cab): 24″ (62 cm) glass solvent troughs, $26\frac{3}{4}$″ (68 cm) glass anti-siphon rods (10 rods are required for each chromatocab), $23\frac{1}{2}$″ (59.7 cm) glass anchor rods and $26\frac{1}{2}$″ (67.5 cm) stainless steel trough holders.

Ultraviolet Viewing Lamp

A long wavelength (3,660 angstroms) ultraviolet lamp[3] equipped with two 15-watt Blak-Ray tubes and covered with a glass plate is satisfactory for viewing the developed chromatograms. It is recommended that protective glasses be worn when working with the UV viewing lamp.

Drying Rack

A wooden frame [24″ (61 cm) × 24″ (61 cm) × 18″ (45.6 cm)], open on all sides and fitted with 10 strings placed at $2\frac{1}{2}$″ (6.35 cm) intervals along the top, is suitable for drying chromatograms in a fume hood. The wet chromatograms are suspended with clothespins.

I-2. Experimental Procedures for the Two-Dimensional Paper Chromatographic Analysis of Flavonoid Mixtures

Experimental details are presented below for the two-dimensional paper chromatographic analysis of the flavonoids present in *Baptisia lecontei* (Leguminosae) and *Hymenoxys scaposa* (Compositae) air-dried plant material[4].

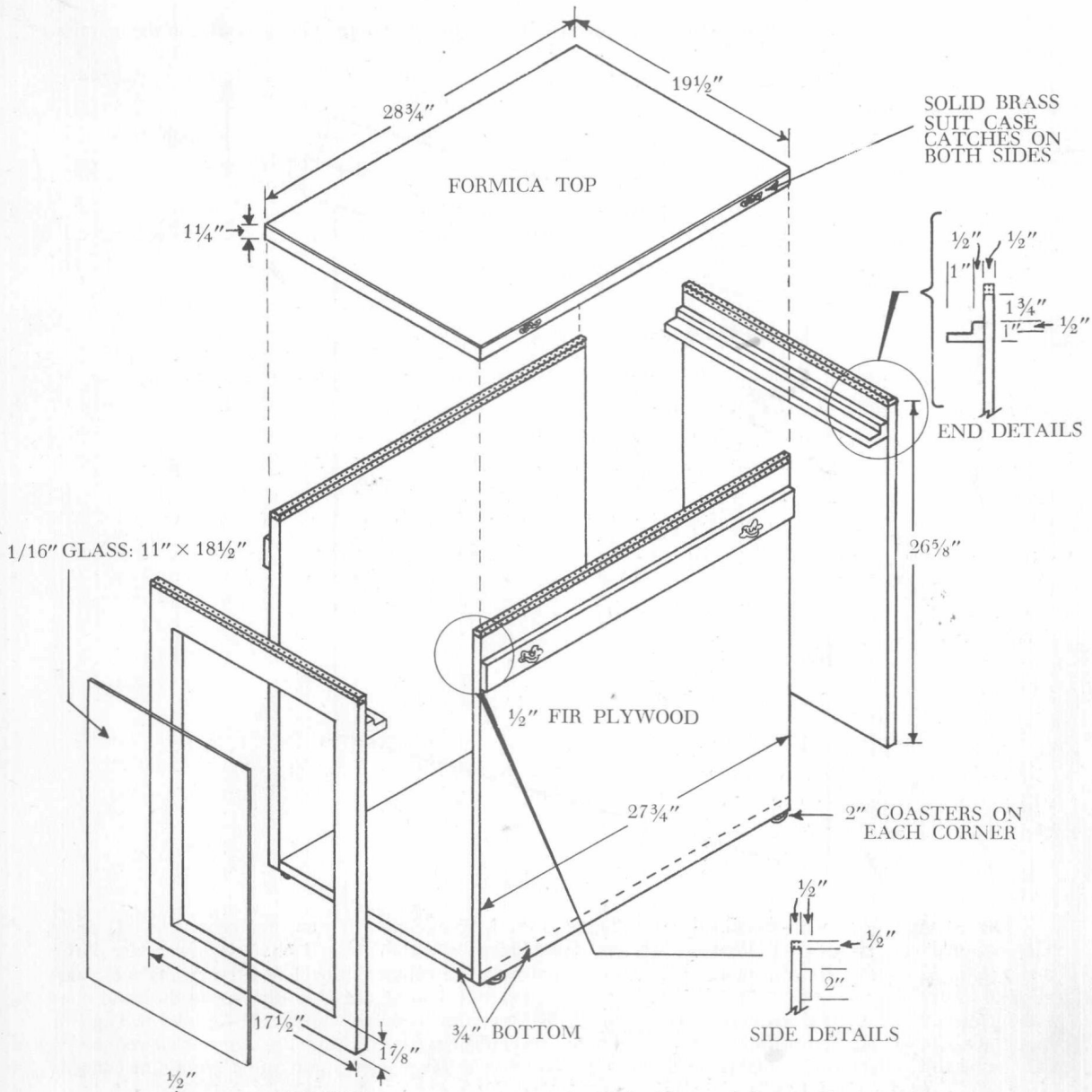
(A) The Two-Dimensional Paper Chromatographic Analysis of *Baptisia lecontei* Flavonoids [3]. Dried stem and leaf material (24 g), which had been finely ground in a Waring Blendor, was extracted at room temperature for 3 days with cold 25% aqueous methanol (180 ml). After the plant material was removed by filtration, the extract, on evaporation under water pump vacuum, yielded a sticky green residue (5.3 g). (Although the methanol present in the extract is readily removed under water-pump vacuum with the aid of a rotary evaporator, the residual water is best removed under oilpump high vacuum.)

[1] Suitable cabs can be purchased from Kensington Scientific Corp., Oakland, Calif.

[2] The items may be obtained from E.H. Sargent and Co., Dallas, Texas.

[3] We used model XX-15 lamps marketed by W.H. Curtin and Co., Houston, Texas.

[4] Results similar to those described here were also obtained with fresh plant material.

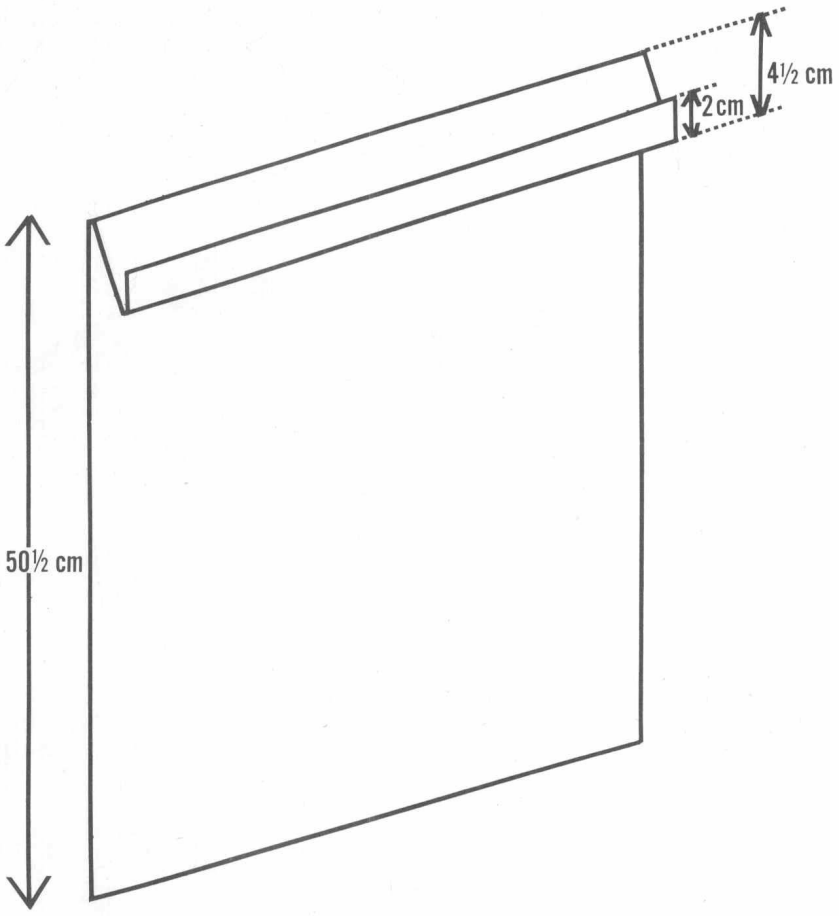


1. Nail sides and bottom in place.
2. Glue neoprene foam gasket and window (one end only) in place.
3. Coat inside of cabinet and top with paraffin wax.
4. Coat outside with clear finish.

Fig. I-1. Plans for construction of a chromatocab

About 0.1 g of the residue was dissolved in 1 ml of methanol (containing a minimum of water to effect solution). This solution was then spotted (using an ungraduated pipette) on the lower right-hand corner of a sheet of Whatman 3MM chromatographic paper. A hair drier was used for solvent evaporation between repeated applications of the solution to the paper. The final spot, which appeared deep purple when viewed under a 3,660 angstrom UV lamp, was about 4 cm in diameter and 10 cm from each edge of the paper. The

chromatogram was folded along the 46 cm edge adjacent to the spot containing the flavonoids in the manner shown in the following sketch:



The chromatogram was developed descendingly in the long direction in a chromatocab (Fig. I-1) using TBA as solvent (see Section I-1). When the solvent front reached to within about 3 cm of the lower edge of the paper (after 22 – 26 hr), the chromatogram was removed from the cabinet, attached to the drying rack, and allowed to dry in a fume hood. The dry chromatogram was folded along the edge adjacent to the band containing the flavonoids and then developed descendingly in the second direction with the HOAc solvent. This run required about 4 hr for completion. The dried two-dimensionally developed chromatogram was viewed in UV light alone and in the presence of ammonia fumes (the mouth of a 100 ml widemouth bottle containing concen-

Caption to Fig. I-2a on p. 7

Spot No.	Compound	Spot No.	Compound
1.	Apigenin (II)	5d.	Probably liquiritigenin (4′,7-Dihydroxy-dihydroflavone)
2.	4′,7-Dihydroxyflavone (IV)		
3.	Luteolin (I)	5e.	Orobol (X)
3a.	4′,7-Dihydroxyflavonol (VI)	6.	3′,4′,7-Trihydroxyflavone 7-O-glucoside (IIIa)
4.	3′,4′,7-Trihydroxyflavone (III)		
4a.	Fisetin (V)	7.	4′,7-Dihydroxyflavone 7-O-glucoside (IVa)
5.	Pseudobaptigenin (XI)		
5a.	Calycosin (XIII)	8.	Luteolin 7-O-rutinoside (Ib)
5b.	Daidzein (XII)	8a.	Luteolin 7-O-glucoside (Ia)
5c.	Genistein (IX)	8b.	Fisetin 7-O-rhamnoglucoside (Vb)

trated ammonium hydroxide was held in contact with each spot for about 5 sec). All spots which were detected by this procedure were circled with a lead pencil (Fig. I-2a). The isolation of these compounds by column chromatography and the methods used for identifying them are described in Chapter II [*3*].

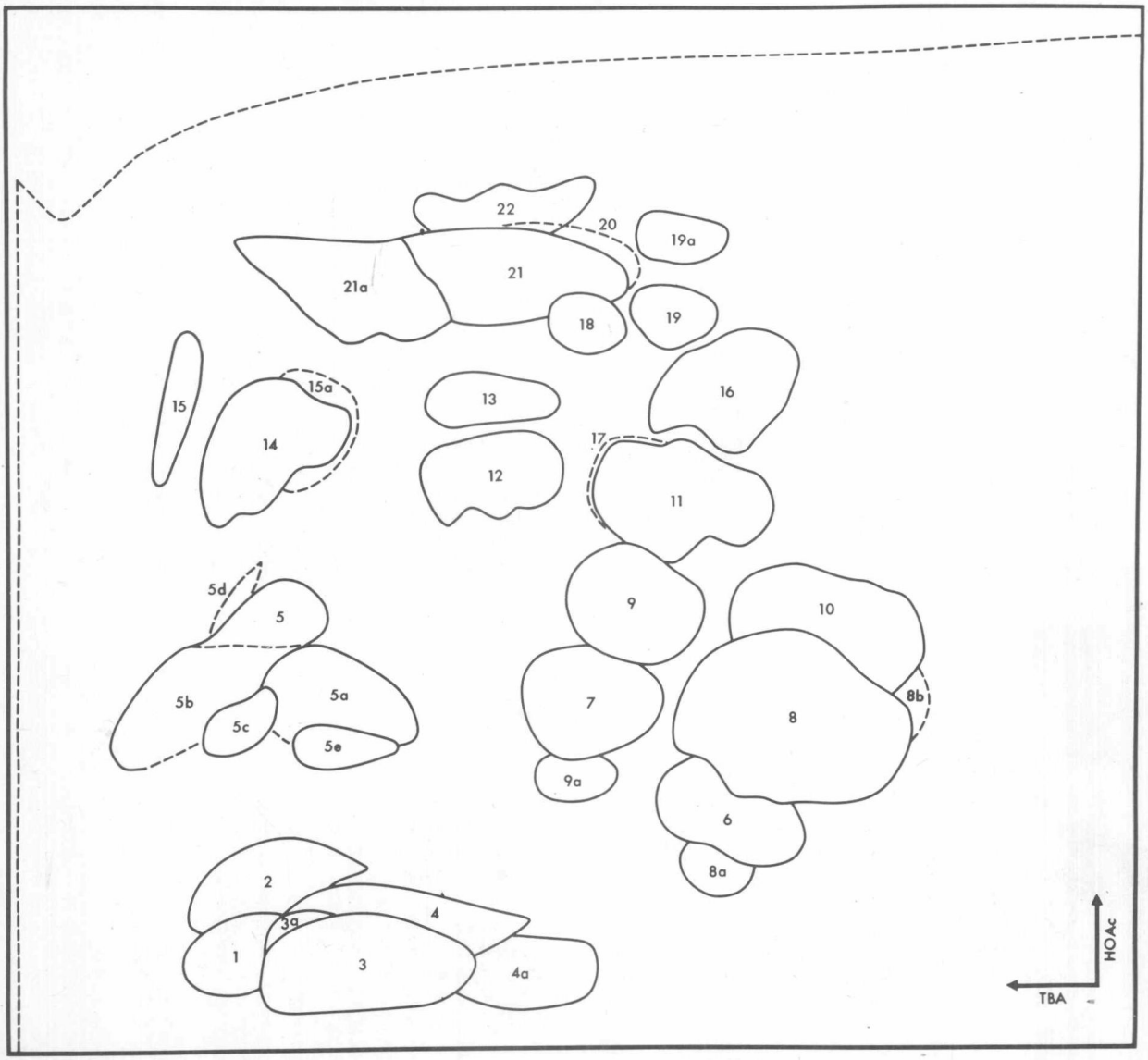
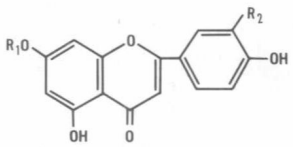
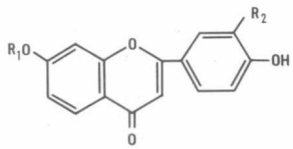


Fig. I-2a. The two-dimensional paper chromatographic pattern of flavonoids obtained from *Baptisia lecontei* plant material [*3*]

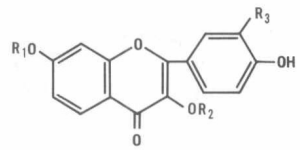
Spot No.	Compound	Spot No.	Compound
9.	Apigenin 7-O-rhamnoglucoside (II b)	16.	Orobol 7-O-rutinoside (X a)
10.	3′,4′,7-Trihydroxyflavone 7-O-rhamnoglucoside (III b)	17.	3′,4′,7-Trihydroxyflavonol 3-O-glucoside (V a)
11.	4′,7-Dihydroxyflavone 7-O-rhamnoglucoside (IV b)	18.	Sphaerobioside (IX a)
12.	Calycosin 7-O-glucoside (XIII a)	19.	Calycosin 7-O-rhamnoglucoside (XIII b)
13.	Daidzein 7-O-glucoside (XII a)	19a.	Daidzein 7-O-rhamnoglucoside (XII b)
14.	Scopoletin (XIV)	20.	(+)-Fustin 3-O-glucoside (VIII a)
15.	(+)-4′,7-Dihydroxy-dihydroflavonol (VII)	21.	Pseudobaptisin (XI a)
15a.	(+)-Fustin (VIII)	21a.	Lecontin (VII a)
		22.	Scopoletin 7-O-glucoside (XIV a)



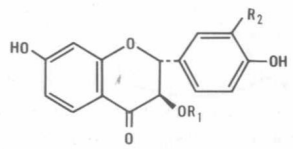
	R_1	R_2	
I	H	OH	luteolin
Ia	glu	OH	luteolin 7-O-glucoside
Ib	rh-glu	OH	luteolin 7-O-rutinoside
II	H	H	apigenin
IIa	glu	H	apigenin 7-O-glucoside
IIb	rh-glu	H	apigenin 7-O-rhamnoglucoside



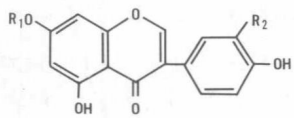
	R_1	R_2	
III	H	OH	3′,4′,7-trihydroxyflavone
IIIa	glu	OH	3′,4′,7-trihydroxyflavone 7-O-glucoside
IIIb	rh-glu	OH	3′,4′,7-trihydroxyflavone 7-O-rhamnoglucoside
IV	H	H	4′,7-dihydroxyflavone
IVa	glu	H	4′,7-dihydroxyflavone 7-O-glucoside
IVb	rh-glu	H	4′,7-dihydroxyflavone 7-O-rhamnoglucoside



	R_1	R_2	R_3	
V	H	H	OH	fisetin
Va	H	glu	OH	3′,4′,7-trihydroxyflavonol 3-O-glucoside
Vb	rh-glu	H	OH	fisetin 7-O-rhamnoglucoside
VI	H	H	H	4′,7-dihydroxyflavonol



	R_1	R_2	
VII	H	H	(+)-4′,7-dihydroxy-dihydroflavonol
VIIa	glu	H	(lecontin)
VIII	H	OH	(+)-fustin
VIIIa	glu	OH	(+)-fustin 3-O-glucoside



	R_1	R_2	
IX	H	H	genistein
IXa	rh-glu	H	sphaerobioside
X	H	OH	orobol
Xa	rh-glu	OH	orobol 7-O-rutinoside