[1] Overview of Methods for Analysis and Identification of Flavonoids

By STEPHEN J. BLOOR

The flavonoids are a class of plant secondary metabolites derived from the condensation of a cinnamic acid with three malonyl-CoA groups. All flavonoids arise from this initial reaction, which is catalyzed by the chalcone synthase enzyme. The chalcone is usually converted rapidly into a phenylbenzopyran, and further modification leads to the flavones, isoflavones, flavonols, or anthocyanins (Fig. 1). Additional structural elaboration, mainly through glycosylation but also via acylation or alkylation, gives us the huge variety of flavonoid structures seen throughout the plant kingdom.

Many books and review articles have been written on the subject of flavonoids, their occurrence, and analysis. The series "The Flavonoids: Advances in Research"[1–3] provides the most comprehensive coverage of occurrence and structural variation, whereas more specific texts or articles relate to the analysis of flavonoids.[4–7] Although the general methodology used for analysis and identification of flavonoids has much in common with the techniques used for many other groups of natural products, a number of useful techniques have evolved that can provide shortcuts enabling the rapid identification of flavonoid type and substitution pattern. Despite the plethora of flavonoid structures presented in the scientific literature, the number of common, basic structural units remains limited; the flavone and flavonol compounds are by far the most common structural types and so are the main focus of this article. Another particular advantage the analyst has in flavonoid analysis is the distinctive UV (or UV–VIS) spectra of these

[1] J. B. Harborne and T. J. Mabry (eds.) "The Flavonoids-Advances in Research." Chapman and Hall, London, 1982.

[2] J. B. Harborne (ed.) "The Flavonoids-Advances in Research since 1980." Chapman and Hall, London, 1988.

[3] J. B. Harborne (ed.) "The Flavonoids-Advances in Research since 1986." Chapman and Hall, London, 1994.

[4] K. R. Markham and S. J. Bloor *in* "Flavonoids in Health and Disease" (C. A. Rice-Evans and L. Packer, eds.), pp. 1–33. Dekker, New York, 1998.

[5] T. J. Mabry, K. R. Markham, and M. B. Thomas "The Systematic Identification of Flavonoids." Springer-Verlag New York, 1970.

[6] K. R. Markham "Techniques of Flavonoid Identification." Academic Press, London, 1982.

[7] P. M. Dey and J. B. Harborne (eds.) "Methods in Plant Biochemistry," Vol. 1. Academic Press, London, 1989.

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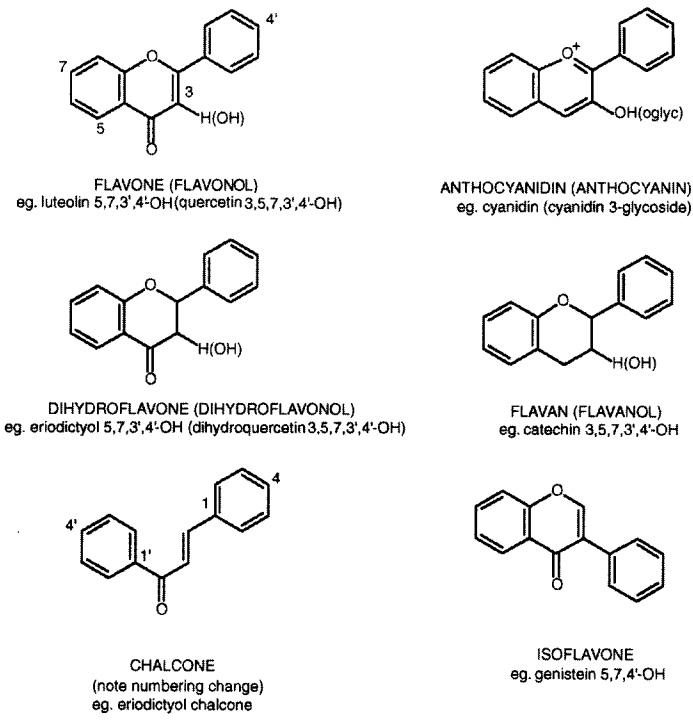

FIG. 1. Basic flavonoid structural types.

compounds where minor differences in structure are often seen as significant differences in their UV spectra, enabling rapid diagnosis of certain structural features. Modern instrumental techniques enable us to gain much information regarding the mass and UV–VIS spectra of individual components in a complex mixture. A combination of some more traditional analytical techniques combined with these modern techniques enables at least a partial identification of most flavonoid components without large-scale purification of the individual compounds. Although more specific examples of applications are described elsewhere in this volume, this article gives a general overview of the typical procedures used to determine type and quantities of flavonoid compounds.

For the purposes of analysis, the flavonoids can be basically classified into three types: flavonoid glycosides, nonpolar flavonoids (aglycones, methylated or alkylated flavonoids), and anthocyanins. Each type requires a different analytical technique. The proanthocyanidins,[8] especially the oligomeric forms, would constitute a fourth class but are not discussed in detail here.

[8] L. J. Porter *in* "The Flavonoids: Advances in Research since 1986" (J. B. Harborne, ed.), pp. 23–53. Chapman and Hall, London, 1994.

Chromatographic Analysis

Thin-Layer Chromatography (TLC) [or Paper Chromatography (PC)]

Historically, paper chromatography has been the preferred method for flavonoid analysis, and relative mobility data are available for a large variety of compounds.[9,10] However, not all laboratories are equipped to perform PC, but TLC on cellulose sheets (e.g., Schleicher and Schuell, Keene, NH, Avicel) is a useful alternative. Cellulose TLC is especially useful for quick analyses of materials containing flavonoids, especially flavonoid glycosides. The relative mobility and appearance of spots under UV, before and after spraying with various reagents, enable a good approximation of structural type. Two solvent systems are routinely used for such preliminary analyses: 15% acetic acid (acetic acid : H_2O, 15 : 85) and TBA (*t*-BuOH : acetic acid : H_2O, 3 : 1 : 1). Rough relative mobilities for various flavonoid types are shown in Table I. Typically, plastic-backed sheets are cut to a suitable size, e.g., 5 cm wide × 7 cm high, the samples are applied as small 1- to 2-mm spots, allowed to dry, and the sheets developed in a glass tank (a slide-staining jar is useful for small sheets). The relative mobilities in TBA and 15% acetic acid should give a rough guide as to flavonoid type. Sheets are dried and viewed under UV light (366 nm). The significance of the spot color and the behavior on exposure to ammonia vapor are detailed in Table II. The sheet is then sprayed with NA reagent (a 1 % solution of diphenylboric acid–ethanolamine complex in methanol), dried, and again viewed under UV light. Most flavonoids show some color, but most significantly 3′, 4′-dihydroxyflavones or flavonols are orange and the 4′-hydroxy equivalents are yellow-green. If desired, a fingerprint type of two-dimensional chromatogram can be prepared by applying one sample only on a spot in one corner of the sheet, developing the sheet with TBA in one dimension, and then, after drying, developing in the second dimension with 15% acetic acid. Generally, most flavonoids will be separated on two-dimensional TLC and their spot characteristics can be noted. Silica TLC is also a useful screening system for flavonoid compounds.[11] Ethyl acetate–formic acid–acetic acid–H_2O (100 : 11 : 11 : 27) gives a good range of mobilities for flavone and flavonol glycosides (R_f : diglycosides < monoglycosides < aglycones) and, as discussed earlier, most spots can be visualized with the NA reagent. Anthocyanins are best analyzed by TLC using a quite acidic solvent system. A useful general eluting solvent for cellulose TLC of these compounds is HCl : formic acid : H_2O (1 : 1 : 2).[12] The intensely colored spots (violet for trihydroxylated B-ring, red for dihydroxy and orange for mono-) do not require spraying, and generally the R_f increases as the degree of glycosylation increases.

[9] K. R. Markham and R. D Wilson *Phytochem. Bull.* **20,** 8 (1988).
[10] K. R. Markham and R. D Wilson *Phytochem. Bull.* **21,** 2 (1989).
[11] H. Wagner, S. Bladt, and E. M. Zgainski "Plant Drug Analysis." Springer-Verlag, Berlin, 1983.
[12] O. M. Andersen and G. W. Francis *J. Chromatogr.* **318,** 450 (1985).

TABLE I
TWO-DIMENSIONAL PC MOBILITY[a] OF VARIOUS FLAVONOID TYPES IN TBA AND 15% ACETIC ACID[b]

15% Acetic acid mobility	TBA mobility	Flavonoid types commonly encountered
Low	Med-high	Flavone, flavonol, biflavone, chalcone, aurone aglycones, and anthocyanidins
Low+	Med-low	Flavone mono-*C*-glycosides
Low+	Med	Flavone mono-*O*-glycosides and flavonol 7-*O*-glycosides
Med-low	Low	Anthocyanidin 3,5-diglycosides (*p*-coumaroyl)
Med-low	Med-low	Anthocyanin 3-monoglycosides
Med-low	Med	Anthocyanin 5-monoglycosides
Med	Med-low	Flavone di-*C*-glycosides
Med	Med	Flavone *C*-glycosides (*O*-glycosides of)
Med	Med	Flavonol 3-diglycosides
Med	Med-high	Flavonol 3-monoglycosides
Med+	Low	Anthocyanidin 3,5-glycosides
Med+	High	Isoflavone, dihydroflavone, dihydroflavonal aglycones, and cinnamic acids
Med-high	Low	Flavonoid sulfates
Med-high	Med	Anthocyanidin 3,5-diglycosides (acetyl)
Med-high	Med-high	Flavonol 3,7-diglucosides
High	Med-low	Flavonol 3-triglycosides
High	Low	Flavonol 3-tetraglycosides
High	Med	Isoflavone mono- and diglycosides
High	Med-high	Dihydroflavonol 3-glycosides

[a] Mobility code (typical R_f range): low, 0 to 33 med, 0.33 to 0.65; high, 0.66 to 1.0; "+" indicates high end of range. Hyphenated categories indicate a range covering both.

[b] Reproduced from K. R. Markham and S. J. Bloor, in "Flavonoids in Health and Disease" (C. A. Rice-Evans and L. Packer, eds.), pp. 1–33. Dekker, New York, 1998, with permission.

HPLC

The distinctive UV–VIS spectra of most flavonoids and the widespread availability of high-performance liquid chromatography (HPLC) systems with multiwavelength capability, or the ability to record on-line spectra, has meant HPLC is now the method of choice for flavonoid analysis.[13–15] Most often separations are performed using a reversed-phase column (RP-18) and a gradient elution system starting with a predominantly aqueous phase and introducing an increasing proportion of an organic solvent such as methanol or acetonitrile. As most flavonoids are ionizable, some acid is usually added to the mobile phase to control the pH.

[13] K. Vande Casteele, C. Van Sumere, and H. Geiger *J. Chromatogr.* **240,** 81 (1982).

[14] C. Van Sumere, P. Fache, K. Vande Casteele, L. De Cooman, and E. Everaert *Phytochem. Anal.* **4,** 279 (1993).

[15] M. C. Pietrogrande and Y. D. Kahie *J. Liq. Chrom.* **17,** 3655 (1994).

TABLE II
INTERPRETATION OF TYPICAL FLAVONOID SPOT COLORS ON TWO-DIMENSIONAL PC[a]

Spot color (366 nm)	Spot color (NH_3)	Common structural types indicated[b]
Dark purple	Yellow or yellow-green shades	Flavones (5- and 4′-OH)
		Flavonols (3-OR and 4′-OH)
	Red or orange	Chalcones (2′-OH, with free 2- or 4-OH)
	Little change	Flavone *C*-glycosides (5-OH)
		Flavones (5-OH and 4′-OR)
		Flavones (5-OH and 3,4′-OR)
		Isoflavones (5-OH)
		Dihydroflavones (5-OH)
		Dihydroflavones (5-OH)
		Biflavonyls (5-OH)
Yellow fluorescent	Little change	Flavonols (3-OH)
	Red or orange	Aurones
Magenta, pink, yellow fluorescence	Blue with time	Anthocyanins/anthocyanidins (Pelargonidin-3, 5-OR, yellow fluorescence)
Blue fluorescence	Yellow-green or blue-green	Flavones (5-OR)
		Dihydroflavones
		Flavonols (3,5-OR)
	No change	Isoflavones (5-OR)
	Brighter blue	Cinnamic acids and derivatives

[a] Reproduced from K. R. Markham and S. J. Bloor, *in* "Flavonoids in Health and Disease" (C. A. Rice-Evans and L. Packer, eds.), pp. 1–33. Dekker, New York, 1998, with permission.
[b] -OR, *O*-glycoside or *O*-alkyl.

The order of elution from most polar through to least polar means triglycosides (and higher glycosides) are eluted early, along with most anthocyanin glycosides, followed by di- and monoglycosides and then acylated or alkylated glycosides and aglycones. The requirement for a low pH solvent system for anthocyanins, a high organic modifier content for aglycones, and an extended gradient system to separate the many possible mono- and diglycosidic combinations means no one solvent system will give optimal separation of all flavonoid types.

Three useful gradients solvent systems are outlined. All are designed for use with the same RP-18 column [Merck Lichrospher 100 RP-18 endcapped (5 μm, 11.9 × 4 cm) or Supersphere (4 μm)]. The first is a general-purpose solvent system with high acid content and relatively fast total analysis time for routine analysis of plant extract, including those containing anthocyanin pigments. Table III gives a list of retention times for a variety of flavonoid compounds using this system. Note the bunching together of the aglycones at the end of the chromatographic run. The second system is designed for maximum resolution of flavone and flavonol

TABLE III

HPLC RETENTION TIMES FOR Flavonoids[a,b]

Anthocyanidin glycosides	Anthocyanidins	Flavonoid aglycones	Flavone/flavonol glycosides	Retention time (mins.)
Delphinidin 3,5-di-*O*-glucoside				7.6
		Dihydromyricetin		8.9
Cyanidin 3,5-di-*O*-glucoside				9.7
Delphinidin 3-*O*-glucoside				11.2
Delphinidin 3-*O*-rutinoside				12.7
Cyanidin 3-*O*-glucoside				13.5
			Vicenin-2	14.2
Peonidin 3,5-di-*O*-glucoside				14.6
	Delphinidin			15.3
		Dihydroquercetin		15.5
Petunidin 3-*O*-glucoside				15.5
Malvidin 3,5-di-*O*-glucoside				15.6
			Quercetin 3-*O*-sophoroside	16.1
			Saponarin	17.0
Peonidin 3-*O*-glucoside				17.8
			Luteolin 3′,7-*O*-diglucoside	18.2
			Kaempferol 3-*O*-sophoroside	19.3
Malvidin 3-*O*-glucoside				19.3
	Cyanidin			19.5
			Luteolin 3′-*O*-glucoside	19.6
			Luteolin 5-*O*-glucoside	19.6
			Vitexin	19.8
			Luteolin 7-*O*-glucoside	20.4
		Dihydrokaempferol		20.7
			Quercetin 3-*O*-glucoside	21.0
			Kaempferol 3-*O*-glucoside	21.0
	Petunidin		7-*O*-rhamnoside	21.4
			Kaempferol 3-*O*-rutinoside	22.0
			Isovitexin	22.2
	Pelargonidin			23.5
Malvidin 3-*O*-glucoside,				23.8
5-*O*-(6-acetylglucoside)		Myricetin		24.0
			Quercetin 3-*O*-rhamnoside	24.2
			Quercetin 3-*O*-rutinoside	24.9
			Kaempferol 3-*O*-glucoside	25.0
	Peonidin			25.7
	Malvidin			27.0
			Apigenin 7-*O*-neohesperidoside	27.7
			Kaempferol 3-*O*-rhamnoside	29.2
		Quercetin		31.7
		Luteolin		33.1
		Narigenin		33.3
		Kaempferol		38.4
		Apigenin		38.6
		Tricin		39.5
		Chrysoeriol		39.6
		Isorhamnetin		39.9

[a]Reproduced from K. R. Markham and S. J. Bloor, *in* "Flavonoids in Health and Disease" (C. A. Rice-Evans and L. Packer, eds.), pp. 1–33. Dekker, New York, 1998, with permission.

[b]HPLC column and conditions as outlined in text [solvent system (1)].

glycosides. A slow increase in acetonitrile content between 0 and 52 min is used, but most glycosides elute between 25 and 40 min. The third solvent system is useful for nonpolar flavonoids, such as those found on the leaf surface, and uses a high methanol content.

1. Elution (0.8 ml/min) is performed using a solvent system comprising solvents A (1.5% H_3PO_4) and B [acetic acid: $CH_3CN : H_3PO_4 : H_2O$ (20 : 24 : 1.5 : 54.5)] mixed using a gradient starting with 80% A, linearly decreasing to 33% A after 30 min, 10% A after 33 min, and 0% A after 39.3 min (column temperature 30°).
2. Solvents A (water adjusted to pH 2.5 with H_3PO_4) and B (CH_3CN) are mixed in a gradient (0.8 ml/min) starting with 100% A, linearly decreasing to 91% A after 12 min, 87% A after 20 min, 67% A after 40 min and then held at 67% A for 2 min, then a linear decrease to 57% A after 52 min, and then finally to 0% A at 55 min (column temperature 24°).
3. Elution (1.0 ml/min) is performed using a solvent system comprising 5% formic acid in water (A) and methanol mixed according to a gradient, starting with 65% A, linearly decreasing to 55% A after 10 min, held at 55% A until 20 min, then linearly decreased to 20% A at 55 min, and 5% A at 60 min (column temperature 30°).

The utility of HPLC is best illustrated by an example such as that shown in Fig. 2. A mixture of flower extracts has been used to present a broad spectrum of compound type. Detection in the range of 340–360 nm is suitable for flavones and flavonols (e.g., 352 nm for chromatograms in Fig. 2). In the crude mixture the series of peaks can be grouped based on analysis of on-line spectra (Fig. 3), as luteolin or apigenin flavone glycosides, kaempferol glycosides, an obvious chalcone glycoside, and possibly some aromatically acylated flavonoid glycosides. Alkaline hydrolysis (see later) removes any acyl groups, dramatically increasing the relative level of one of the kaempferol glycosides, confirming that the acylated late-eluting compounds are acyl derivatives of compound 3 (Fig. 3). Acid hydrolysis (see later) to cleave *O*-glycosides gives the expected peaks for luteolin and kaempferol, but the peaks assigned to apigenin glycosides are still present, indicating these are flavone *C*-glycosides. This example demonstrates the considerable amount of information that can be gleaned from a few small-scale experiments.

Quantification of flavonoids is another forte of HPLC in combination with UV detection. In Fig. 2a, a rough estimate of the flavonoid level can be arrived at by comparing integration data for that chromatogram with that from the injection of a known amount of a readily accessible standard such as rutin (quercetin 3-rutinoside) run under the same conditions. A more rigorous quantification will involve the use of several standard compounds; in this case, a flavone glycoside

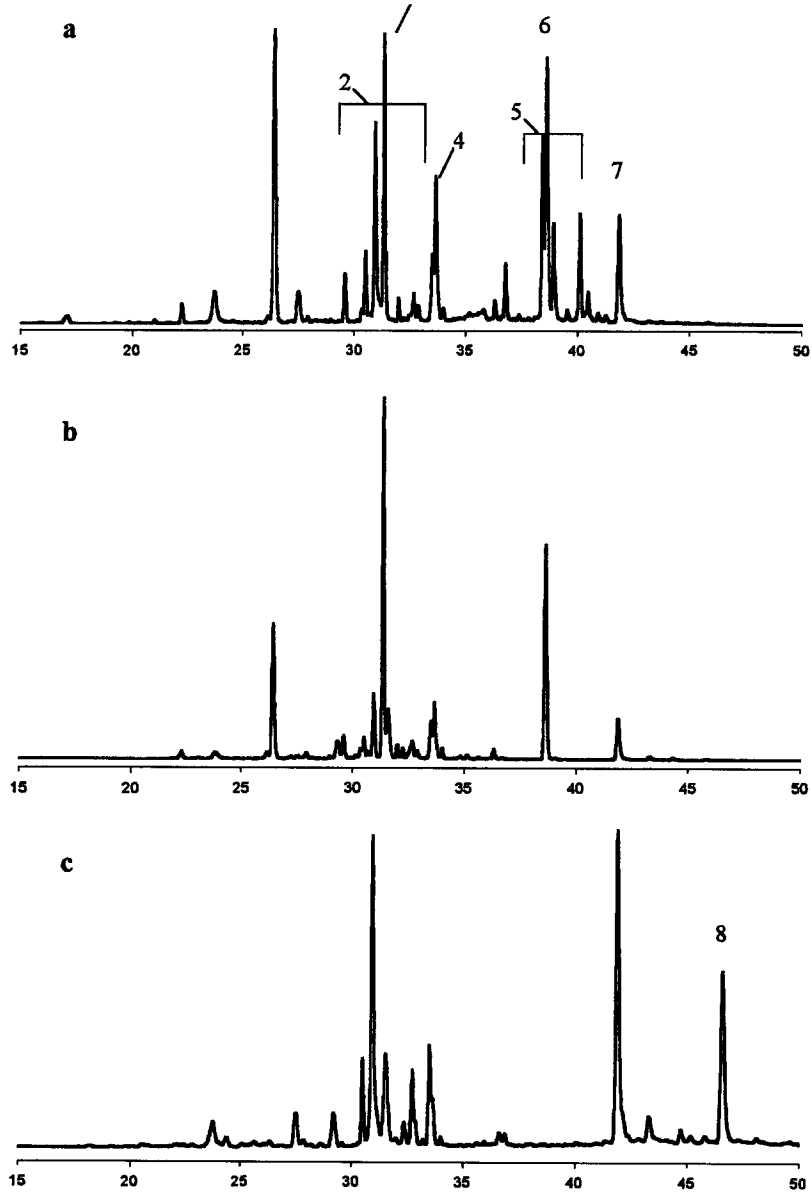

FIG. 2. HPLC chromatograms [absorbance at 352 nm vs. time (min)] of (a) a flavonoid mixture showing seven different groups of compounds: a kaempferol triglycoside [1], a set of apigenin glycosides [2], a kaempferol diglycoside [3], a luteolin glycoside [4], a set of acylated kaempferol glycosides [5], a chalcone [6], and luteolin [7]; (b) the alkaline hydrolysis product of the same mixture shows a large relative increase in peak 3 and loss of the acylated kaempferol glycoside peaks; and (c) the acid-hydrolyzed mixture showing luteolin and kaempferol [8]. Peaks due to apigenin glycosides are still present, showing these are apigenin *C*-glycosides.

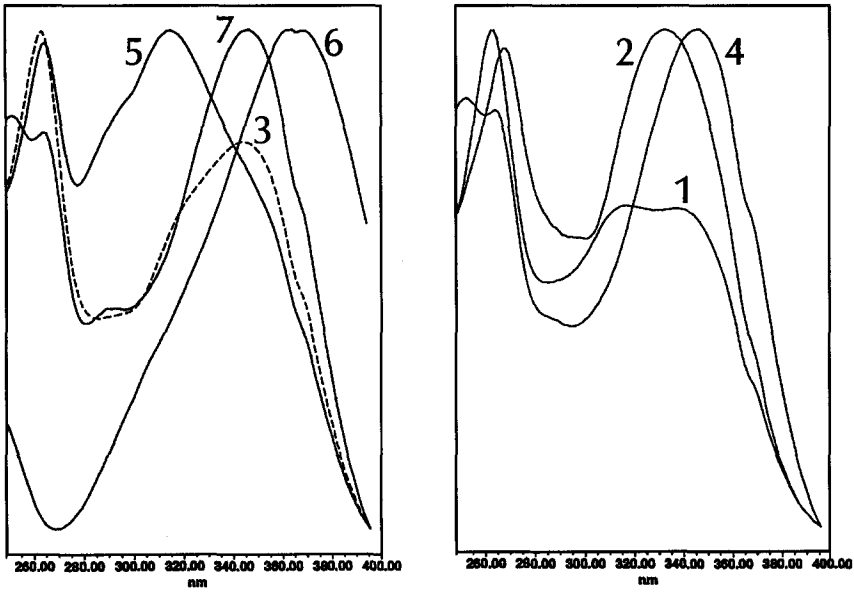

FIG. 3. On-line UV spectra of selected peaks from Fig. 2a. Peak numbers as in Fig. 2.

and chalcone glycoside should also be used. Suitable compounds are available commercially (e.g., Roth, Apin Chemicals).

Sample Preparation

Most flavonoids are extracted readily from source material by alcohol or alcohol/water mixtures. Prior knowledge of the nature of the flavonoids is of course helpful in determining the most efficient extraction technique. Aglycones and nonpolar flavonoids in a product such as bee propolis are best extracted by neat alcohol; plant surface flavonoids can be washed from leaves or stems with ethyl acetate. Glycosides, which are the major vacuolar flavonoids, require more water, and anthocyanins are usually extractable with water containing an organic acid (e.g., 5% formic acid). A useful general solvent for extraction of most flavonoid types from plant tissue is methanol : H_2O : acetic acid, 70 : 23 : 7. Solid source material should be well ground to facilitate maximum extraction. For most plant material, an extract of 50 mg of plant material with 2–5 ml of extraction solvent will yield an extract suitable for TLC or HPLC analysis. The addition of sufficient alcohol to denature the proteins (ca. 50%) to the extraction solvent will allay concerns regarding degradation due to enzyme action when extracting fresh tissue.

In many cases, some pretreatment of extracts is advisable prior to analysis. Chlorophyll can be removed by washing the aqueous alcoholic extract with hexane or diethyl ether, or the source material may be preextracted with such a solvent. Excessive amounts of sugars (e.g., in fruit extracts) may be removed by solid-phase extraction, e.g., on a RP SPE column. In this case, most of the alcohol in the extract should be evaporated, the sample made up to >90% water, and applied to the preconditioned cartridge. The cartridge is washed with water and the flavonoids eluted with alcohol. Some extracts have excessive quantities of nonflavonoid phenolic acids, which overwhelm the flavonoids, making analysis difficult. These can often be removed by SPE with cellulose. The sample is dried, reconstituted in 1% acetic acid in water, and applied to a small cellulose column; washing with 2% acetic acid removes most of the phenolic acids and the flavonoids are recovered by washing the column with alcohol.

Structural Analysis by Hydrolysis

The almost limitless range of flavonoid glycosides means that for most analyses the starting point for identification is the recognition of the flavonoid aglycone. Acid hydrolysis will cleave most sugars from flavonoid glycosides.[6,16] For this type of hydrolysis the flavonoid (0.25–0.5 mg) mixture is dissolved in 300 μl 2 *N* HCl : methanol (1 : 1 v/v) or in trifluoroacetic acid (TFA) : methanol (1 : 1 v/v), sealed in a screw-top polypropylene tube, and heated on a steam bath for 30 min. The sample can then be analyzed directly by TLC or HPLC, and the aglycone identified by comparison with common standard compounds. For TLC, better results are obtained if the posthydrolysis mixture is extracted with an equal volume of ethyl acetate or isoamyl alcohol. The upper layer is evaporated to dryness, redissolved in methanol, and analyzed. The lower aqueous layer can be used for analysis of sugars. Most common flavonoid *O*-glycosides undergo hydrolysis under these conditions; however, glycuronides generally require more extreme conditions, e.g., 2 hr at 100°. Flavonoid *C*-glycosides are not cleaved under these conditions, but the sugar can isomerize between the C-6 and the C-8 positions.[6]

Alkaline hydrolysis is generally only employed if the presence of acyl groups is suspected. To avoid oxidation of the flavonoid, these hydrolyses should be performed in the absence of air. A syringe or small sealed polypropylene vial may be used. An equal volume of 2 *M* NaOH is added to the flavonoid sample solution (generally the sample becomes intensely colored) and left at room temperature for 2 hr. The reaction is halted by neutralization with acid (returned to original color). The sample can then be analyzed directly by HPLC or TLC. Once again, better analytical results are achieved if the hydrolysis mixture is partially purified, in this case RP SPE is best. Comparison of chromatograms of crude and hydrolyzed

[16] J. B. Harborne, *Phytochemistry* **4**, 107 (1965).

material will show which flavonoids are acylated (see section on HPLC). Aromatic acyl groups, from acid or alkaline hydrolysis, such as *p*-coumaric or caffeic acid are detected easily by HPLC, whereas the less easily detected aliphatic acids are more suited to TLC or (after derivatization) gas chromatography.

Enzymatic hydrolysis is used occasionally with flavonoids and can provide selective cleavage of 3-*O*- or 7-*O*-glycosides.[6,13] These hydrolyses can be performed by dissolving the sample (0.3–0.5 mg) in water (500 μl), adding about 10 mg enzyme (e.g., β-glucosidase), and standing at room temperature. The sample is then analyzed as described earlier.

Analysis of Flavonoids by UV–VIS Spectroscopy

A special feature of most flavonoid compounds is the fact that their UV–VIS spectra can be very diagnostic. Often the basic flavonoid structural type is indicated by the spectra of individual compounds in a mixture obtained from on-line detection in HPLC or appearance under UV light on TLC or paper chromatograms. A considerable amount of additional information can be gained from the use of shift reagents; however, the use of these reagents is only really applicable for purified flavonoids.[5,6]

Individual flavonoids are dissolved in methanol (concentration such that the maximum absorbance is between 0.05 and 1.00 AU), and the basic spectrum is measured. Most flavonoids show a band in the 210- to 290-nm region (band II) and a second band at 320–380 nm (band I). Compilations of spectral data are available for comparison.[5,17] For anthocyanins, the latter band is in the visible region (490–540 nm).[18]

The procedure for shift reagent tests and the resultant shifts for flavones or flavonols[5,6] are as follows.

i. Two to three drops of 2.5% NaOMe in methanol are added directly to the cuvette containing the methanolic solution of the flavonoid. A 45- to 65-nm shift in band 1 of flavones and flavonols with no loss of intensity indicates a free 4′-OH. A decrease in intensity of this band indicates a substituted 4′-OH. If band I reduces or degrades after several minutes, then either a free 3,4′-OH or three adjacent OHs are likely, and if a new low intensity band appears at 320–335 nm, a free 7-OH is indicated.

ii. Several milligrams of solid sodium acetate are added to a fresh methanolic solution of the flavonoid. A shift of band II to longer wavelength indicates a free 7-OH.

[17] L. Jurd *in* "The Chemistry of Flavonoid Compounds" (T. A. Geissman, ed.), pp. 107–155. Pergamon Press, Oxford, 1962.

[18] F. J. Francis *in* "Anthocyanins as Food Colors" (P. Markakis, ed.), pp. 181–207. Academic Press, New York, 1982.

iii. Several milligrams of solid boric acid are added to the solution from step ii to diagnose for the presence of ortho di-OH groups. Without these groups the spectrum will revert to the original methanol spectrum. Movement of band I (12–36 nm) indicates an ortho di-OH group(s).

iv. Another methanolic flavonol solution is prepared and tested for response to $AlCl_3$. A few drops of 5% $AlCl_3$ in methanol solution are added, and a shift of band II by 20 to 40 nm again indicates ortho di-OH group. Two to three drops of 20% aqueous HCl are then added to the methanol/$AlCl_3$ flavonoid solution, and a shift of 35 to 70 nm indicates a free 5- and/or 3-OH.

These shift reagents have proven to be extremely useful guides to substitution patterns for a variety of flavonoids. Little material is required and the tests are performed directly in the sample cuvette, taking only a few minutes to complete.

Quantification of flavonoids is often performed by UV–VIS spectroscopy. However, the contribution of interfering compounds in a mixture should be accounted for. Anthocyanins are generally free of interference if the measurements are made at the visible maximum (500–540 nm, measure at pH 1.0). A useful ε value is 30,175 for the common glycoside, cyanidin 3,5-diglucoside.[13] Flavones and flavonol glycosides can be measured at their band I maxima of 340–360 nm and a general ε value of 14,500 used (i.e., in a 1-cm path length cell, a 1 *M* solution will give a absorption value of 14,500 AU; specific values for many flavonoids are available).

For many plant extracts the presence of cinnamic acid derivatives are a problem when quantifying flavonoids using this wavelength range of the UV–VIS spectrum. Most cinnamic acids can be removed by SPE with cellulose (see earlier).

The overview presented here has only skimmed the surface of this subject. There are many steps separating a quick analysis of a mixture and the accurate determination of the structural features of each constituent flavonoid. A number of techniques used in flavonoid analysis such as nuclear magnetic resonance, (NMR), electrophoresis, and liquid chromatography-mass spectrometry (LC-MS) have not been discussed and the reader is referred to more detailed reviews (^{13}C NMR,[19] ^{1}H NMR[20], electrophoresis[21–24]). Some of these methods are also discussed in other articles in this volume that deal with more specific examples of flavonoid analysis.

[19] K. R. Markham and V. M Chari *in* "The Flavonoids: Advances in Research" (J. B. Harborne and T. J. Mabry, eds.), pp. 19–134. Chapman and Hall, London, 1982.

[20] K. R. Markham and H. Geiger *in* "The Flavonoids: Advances in Research since 1986" (J. B. Harborne, ed.), pp. 441–497. Chapman and Hall, London, 1993.

[21] F. A. Tomas-Barberan *Phytochem. Anal.* **6,** 177 (1995).

[22] P. Pietta, P. Mauri, A. Bruno, and C. Gardana *Electrophoresis* **15,** 1326 (1994).

[23] T. K. McGhie and K. R. Markham *Phytochem. Anal.* **5,** 121 (1994).

[24] K. R. Markham and T. K. McGhie *Phytochem. Anal.* **7,** 300 (1996).