

Critical Review

Analytical Chemistry of Fruit Bioflavonoids

A Review

Kevin Robards* and Michael Antolovich

Charles Sturt University Riverina, P.O. Box 588, Wagga Wagga 2678, NSW Australia

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Kevin Robards is Associate Professor of chemistry at Charles Sturt University Riverina. He obtained his Ph.D. in analytical chemistry in 1979 from the University of New South Wales after working as a chemist in industry. His research interests are focused on the application of analytical chemistry to the food and environmental sciences.



Michael Antolovich is a lecturer at Charles Sturt University Riverina. He completed his Ph.D. in 1989 at the University of New South Wales, followed by a postdoctoral position at Princeton University working on metalloporphyrins. He returned to Australia on a Research Fellowship. His current research interests are in computational chemistry and its application to food and environmental sciences.



Introduction

The bioflavonoids are aromatic secondary plant metabolites belonging to the class of plant phenolics. Many of the latter were characterized during the classical period of organic chemistry but their recognition as a discrete group of genetically related plant metabolites came in 1957 with the formation in England of the Plant Phenolics Group, later the Phytochemical Society of Europe. The terms 'phenolic', 'polyphenol' and 'phenols' can be precisely defined chemically but, in the present context of plant phenols, such a definition is not entirely satisfactory since it would inevitably include compounds such as oestrone, the female sex hormone which is principally terpenoid in origin. For this reason, a definition based on metabolic origin is preferable, the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism (Fig. 1). The major classes of plant phenols are listed in Table 1. Also included is an example of a fruit in which the constituents of the particular class are abundant. The fruit juice industry has become one of the world's major agricultural businesses with world trade in fruit juices annually exceeding \$10 billion.¹ Further, it is estimated that fruit juice consumption accounts for 25–30% of dietary intake of flavonoids.

The single most important group of phenolics is the flavonoids. They are highly diverse, in both their chemical structure and proposed biological functions.^{2–13} Their metabolic pathways are particularly complex with, in many cases, multiple alternative metabolic fates. Profiling of the components of a pathway over time provides a dynamic view of the metabolic events occurring in the plant. Nevertheless, establishing a biological function for such compounds is often very

difficult and complicated by the fact that the alternative products for a given metabolite may vary from tissue to tissue, from one growth condition to another and in response to environmental stimuli.

Interest in the bioflavonoids is related to their diversity, biological significance as secondary plant metabolites and ecological role,⁹ use as chemotaxonomic markers,¹⁴ impact on fruit quality,¹⁵ physiological effects,^{11,16,17} and industrial applications.¹⁸ In consequence, commercial interest in these compounds is considerable.¹⁹ Their pharmacological properties also account for recent interest in measuring dietary intakes of bioflavonoids which ranged between 23 mg d⁻¹ estimated in The Netherlands and 170 mg d⁻¹ estimated in the USA. Major

dietary sources of flavonoids determined from studies and analyses conducted in The Netherlands include tea, onions, apples, and red wine.²⁰ However, current estimates of daily consumption of flavonoids differ considerably.²¹ All of these aspects justify the intense interest in bioflavonoids which has been manifested over several decades and accounts for the many reviews and monographs^{1-9,22} devoted to various aspects of these compounds.

This review critically examines the analytical chemistry of the bioflavonoids in fruits with emphasis on work of the last decade, although earlier studies are included where they are relevant. Methods used for the analysis of samples other than fruits will be discussed where these illustrate current applications which can be extended to include fruits or which may emerge as important advances over existing methods. The terms bioflavonoid and flavonoid are used interchangeably to encompass all compounds derived from the basic structures in Fig. 2. Where a distinction between the various flavonoids is intended, the name of the specific class of compound or derivative is used. Thus, flavonoid glycoside is used generically whereas flavanoid glycoside denotes a particular class of glycoside. There are many flavonoids present in low concentrations which remain unidentified but whose significance may far outweigh their concentration level. Isolation and structure elucidation of these compounds are the initial steps to understanding their significance and action. Information on their biosynthesis is essential to understanding the interaction between plants and the environment. Methods of characterization and identification follow those in general use for natural substances. Hence, preparation of an extract, biological screening, bioguided fractionation, isolation and structure elucidation constitute the usual approach. For structural elucidation, physical methods based on spectral characteristics feature prominently, although older chemical and biochemical approaches should be considered particularly as adjuncts to spectral analysis. These methods, which include NMR spectrometry, have been treated elsewhere^{6,23} and are not considered in the present discussion. On the other hand, MS is included because of its role as an on-line detection device in GC and, more recently, HPLC. Commercial development of coupled LC-NMR as a complementary technique to LC-MS will provide enormous benefits and stimulate rapid developments in flavonoid chemistry.

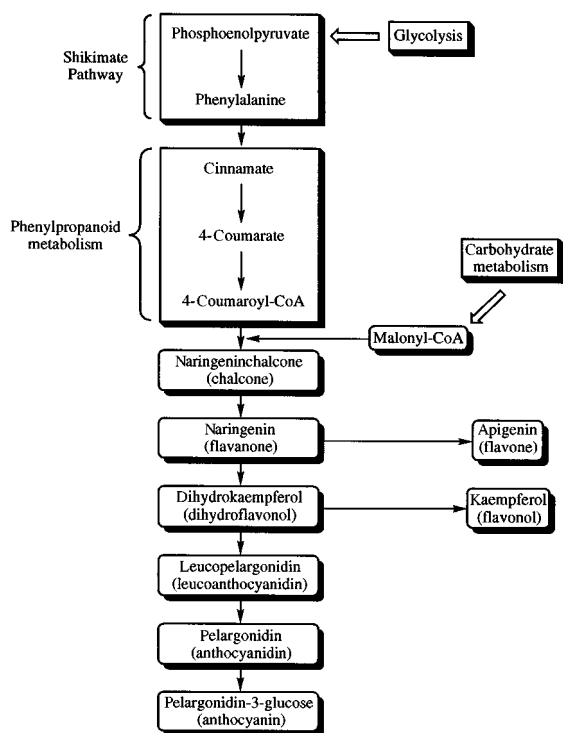


Fig. 1 Biosynthetic pathway leading to the various flavonoid classes.

Table 1 Major classes of phenolics in fruits

Basic skeleton	Class	Examples	Common fruit source
C ₆	Simple phenols Benzoquinones	Catechol, Hydroquinone, resorcinol	
C ₆ -C ₁	Phenolic acids	<i>p</i> -Hydroxybenzoic acid, salicylic acid	Strawberry
C ₆ -C ₂	Acetophenones Phenylacetic acids	<i>p</i> -Hydroxyphenylacetic acid	
C ₆ -C ₃	Hydroxycinnamic acids Phenylpropenes Coumarins Isocoumarins Chromones	Caffeic acid, ferulic acid Eugenol, myristicin Umbelliferone, aesculetin, scopolin	Apple Citrus
C ₆ -C ₄	Naphthoquinones	Eugenin Juglone	Walnut
C ₆ -C ₁ -C ₆	Xanthenes	Mangostin, mangiferin	Mango
C ₆ -C ₂ -C ₆	Stilbenes Anthraquinones	Resveratrol Emodin	Grape Cherry
C ₆ -C ₃ -C ₆	Flavonoids	Quercetin, cyanidin	
(C ₆ -C ₃) ₂	Lignans Neolignans	Pinoresinol	
(C ₆ -C ₃ -C ₆) ₂	Biflavonoids	Agathisflavone	
(C ₆ -C ₃) _n	Lignins		Stone fruits
(C ₆) ₆	Catechol melanins		
(C ₆ -C ₃ -C ₆) _n	Condensed tannins (flavolans)		Persimmon

Chemical Structure

The flavonoids are built upon a C₆-C₃-C₆ flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. Several classes (Fig. 2) are differentiated according to the degree of unsaturation and degree of oxidation of the three-carbon segment. Within the various classes, further differentiation is possible based on the number and nature of substituent groups attached to the rings. The range of known flavonoids is therefore vast, currently exceeding 5000.⁵ These are frequently referred to by trivial names which generally relate in some way to the plant origin. For example, quercetin was originally isolated from *Quercus* and tricetin from *Triticum*. The range of trivial names is immense and can be confusing. Fortunately, excellent summaries have been compiled by Swain²⁴ and Wollenweber and Dietz.²⁵

Additional structural complexity is introduced by the common occurrence of flavonoids as the *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. A common flavonoid such as kaempferol may be found to occur in nature in any one of 214 different glycosidic forms.⁹ In principle, any of the hydroxyl groups can be glycosylated but certain positions favour glycosylation; for example, 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 most commonly encountered sugar with galactose, rhamnose, xylose and arabinose not uncommon and mannose, fructose, glucuronic and galacturonic acids being rare. Disaccharides and even higher are also found in association with flavonoids, the more common being rutinose (6-*O*- α -1-rhamnosyl-d-glucose) and neohesperidose (2-*O*- α -1-rhamnosyl-d-glucose). Acylation of the glycosides in which one or more of the sugar hydroxyls is derivatised with an acid such as acetic or ferulic is occasionally observed. Glycosylation has a profound effect on the flavonoid rendering it more water soluble, permitting storage of the flavonoid in the cell vacuole where they are commonly found. Glycosylation may also occur *via* direct linkage of the sugar to the benzene nucleus (flavonoid *C*-glycoside) by an acid-resistant carbon-carbon bond. This is much less common with a more restricted range of sugars and flavonoid aglycone types. Flavonoid sulfates containing one or more sulfate residues attached to a phenolic or sugar hydroxyl are even less common.

Various groupings are identified within the various classes of flavonoids because of structural similarities. Flavanoids or dihydroflavonoids are so-called because the C-2 and C-3 of their skeleton are hydrogenated. Thus, the flavanoids include flavanones (or dihydroflavones), flavanols (also called 3-hydroxyflavanones or dihydroflavonols) and the dihydrochalcones. Strictly, flavanols and flavans are also included but they are usually treated separately because they do not possess a carbonyl group in their heterocyclic ring. There are only a few naturally occurring flavans (see Fig. 2),^{26,27} although the term is sometimes used collectively to include the flavan-3-ols and flavan-3,4-diols.

Significance

It is estimated²⁸ that about 2% of all carbon photosynthesized by plants, amounting to about 1×10^9 t per annum, is converted into flavonoids or closely related compounds. The flavonoids constitute one of the largest group of naturally occurring phenolics. Quantitative data are provided in Table 2 for various classes of phenolics in fresh fruit. Such data should be handled very cautiously for a number of reasons, the most important being that results display great variability for different cultivars of the same species and also between different authors. Nevertheless, the data do provide a guide to concentration levels and distribution between fruits.

The number of polar, water-soluble flavonoids is considerable, but there are also many lipophilic compounds such as the flavones. The former are sequestered in the vacuole and are always present in conjugated form, frequently in glycosidic linkage, but may be released as the free aglycone during fungal infection or insect grazing. In such cases, they are likely to be considerably more toxic than the bound form, to the invading organism. This is seen in the case of quercetin, which is an effective inhibitor of enzyme activity of many types,⁹ whereas the related quercetin *O*-glycosides (*e.g.*, rutin) cause negligible inhibition.

Function in Plants

In some cases, flavonoid function may well be related to primary metabolism. Some flavonoids may have an indirect effect on plant growth while others may protect the more vulnerable cell constituents from damaging radiation by virtue of their strong UV absorption.³¹ In general, however, the search for a function for these compounds has focused on the interaction that may take place between the plant and other living organisms and, in particular, on the effects of flavonoids on microorganisms which may infect plants and on animals which graze on plants.

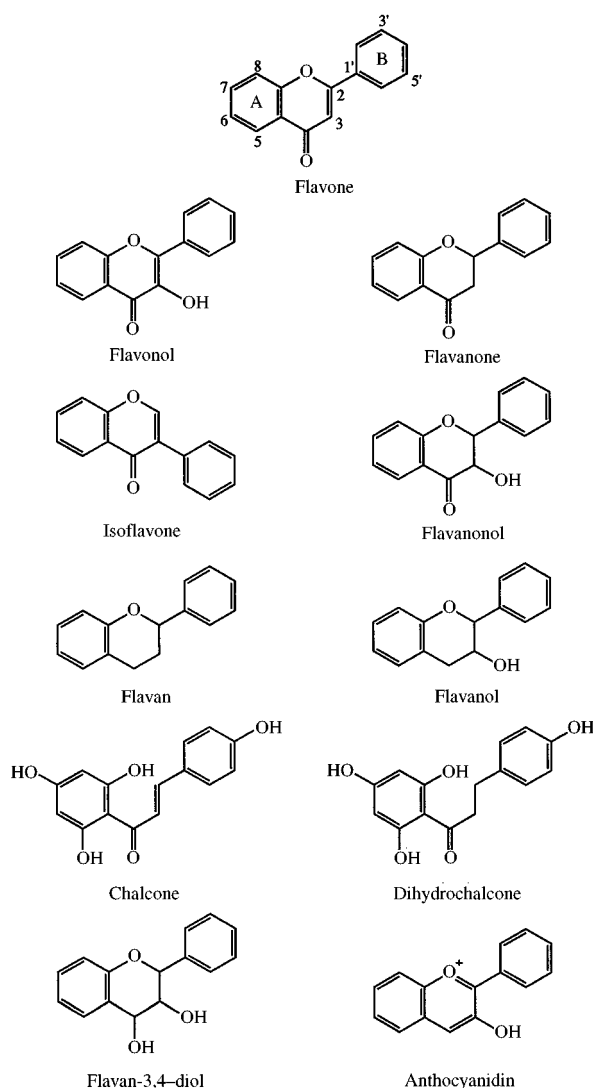


Fig. 2 Structures of the various classes of flavonoids.

Plant resistance

The early literature contained many suggestions that the flavonoids were involved in disease resistance in plants. Further, the association of increased endogenous flavonoid synthesis with the early stages of infection was recognized but the precise nature of their involvement in disease resistance remained unknown. The development of the phytoalexin theory dramatically altered this situation. Muller³² defined phytoalexins as 'compounds produced after infection under the influence of two metabolic systems, that of the host and that of the parasite, and inhibitory to the parasite.' Most literature reports are concerned with the role of phytoalexins in resistance to disease caused by fungi and fewer relate phytoalexin accumulation to resistance to disease caused by bacteria.³³ By the mid-1960s it became apparent that phytoalexins were produced not only in response to infection but also in response to various forms of physiological stress. This has been an area of intense research activity over the last few decades and has been reviewed by Kuc.³³ A common response of plant cells to stress such as wounding, infection or elicitation is the induced incorporation of phenylpropanoids into the cell wall. However, as stated by Matern and Grimmig,¹⁰ 'The precise role of the phenolic cell wall reinforcement for the protection of plants has . . . remained ill-defined due to *limited analytical knowledge* and the complexity of the cell wall architecture.' The discovery of new analytical methods underpins scientific progress and nowhere is this more evident than in the study of the flavonoids.

Photoprotection

The hypothesis of the protective role of flavonoids against harmful UV radiations is supported by the enhanced levels of flavonoids observed in plants exposed to strong UV radiation.^{34,35} Moreover, photocontrol of flavonoid biosynthesis is induced or increased by UV irradiation.^{36,37} Monici *et al.*³⁵ evaluated the role of kaempferol and pelargonidin as photoprotectors, the compounds being chosen as representative of the widely distributed flavonols and anthocyanidins. They concluded that both compounds contribute to plant protection but *via* distinctly different mechanisms. The action of pelargonidin seems more likely to derive from its strong radical scavenger action whereas kaempferol can be considered a good screen against UV radiation. Their conclusions are supported by *in vivo* experiments.³⁴

Properties and Physiological Activity in Animals

The flavonoids are potent antioxidants, free radical scavengers³⁸ and metal chelators; they inhibit lipid peroxidation²⁰ and exhibit various physiological activities,³⁹⁻⁴⁴ including anti-inflammatory,⁴⁵ anti-allergic, anti-carcinogenic, antihypertensive and anti-arthritis activities.⁴⁶ The biological properties^{12,17,18,21} of quercetin, the most frequently studied flavonoid, are certainly consistent with these activities. Thus, quercetin and other flavonoids modify eicosanoid biosynthesis (anti-prostanoid and anti-inflammatory responses), protect low-density lipoprotein from oxidation (prevent atherosclerotic

Table 2 Food sources and properties of phenolic substances in fruits. Where concentration data are available, they are quoted as extreme values in ripe fruits^{3,5,6,29,30}

Class of phenolic	Specific example	Food source	Biological properties	Concentration/mg kg ⁻¹
Hydroxybenzoic acids		Widely distributed		1-200
Hydroxycinnamic acid derivatives		Widely distributed		1-500
Coumarins		Citrus flavedo		2000-7000
Flavones	Apigenin and luteolin	Found mainly in citrus fruits, red grapes and green beans	Co-pigments in flowers; UV protectants in leaves	
Polymethoxylated flavones	Sinensitin	Citrus flavedo Citrus peel oils		1-10 g l ⁻¹
Flavonols	Quercetin, kaempferol and myricetin	Found in many fruits and vegetables such as kale, spinach, onions, parsley, French beans, endive and apples	Co-pigments in flowers; UV protectants in leaves	
Flavanones	Hesperitin, naringenin	Usually found in citrus fruits such as grapefruit, oranges and lemons		
Flavonol glycosides	Rutin	Widely distributed		2-300
Flavanone glycosides	Hesperidin, neohesperidin and naringin	Citrus	Some have bitter tastes	1000-5000
Anthocyanins (<i>i.e.</i> , anthocyanidin glycosides)	Glycosides of pelargonidin and delphinidin	Coloured berries Other fruits	Red to blue pigments	3000-5000 20-500
Flavan-3-ols (catechins)	+Catechin, epigallocatechin, epicatechin gallate, epicatechin and epigallocatechin gallate	These are the main polyphenols in green tea. Fruits such as apples, cherries and pears also contain limited amounts of catechins		10-300
Chalcones, aurones			Yellow pigments	
Ellagic acid*		Abundant in most berries, especially cranberries, red raspberries and some nuts		

* Not a true flavonoid, but closely related.

plaque formation) as a result of their potent antioxidant action,^{13,47} prevent platelet aggregation (anti-thrombic effects) and promote relaxation of cardiovascular smooth muscle (anti-hypertensive and anti-arrhythmic effects). Free radical formation is considered to play a key role in the development of cancer and coronary heart disease by attack on biomolecules (lipids, proteins, DNA) or the biomembrane. The flavonoids may provide protection⁴⁸ of membranes (including mitochondrial membranes) against the oxidative damage implicated either directly or indirectly *via* DNA damage in neurodegeneration, ageing, and malignant progression to cancer.

In vitro testing has shown that many flavonoids, most notably quercetin, one of the most widespread in the human diet, are mutagenic. However, systematic studies also *in vivo* have produced no clear evidence of carcinogenicity. Moreover, the same compounds which proved mutagenic in the Ames test subsequently proved to inhibit tumour development in several experimental animal models. Quercetin in particular was demonstrated to be a potent anticarcinogen in rodents against skin, colonic and mammary cancers. It also inhibited the induction and progression of human cancers.⁴⁹ These conflicting biochemical activities are very puzzling and it was suggested that phenols could test positive as a mutagen because of artefactual errors due to the test conditions.

The role of flavonoids in carcinogenesis and genotoxicity has been extensively studied^{43,50–53} with particular interest in the flavonoids in wines and other beverages.^{54,55} Hence attention has generally focused on the anti-tumour activity of the flavones luteolin and apigenin and the flavonols, quercetin, kaempferol and myricetin.^{56–58} Solimani *et al.*⁴⁹ used flow linear dichroism to study the interaction between DNA and a series of flavonoids whose biological activity spanned a wide range of potency, namely quercetin (the most active), morin, rutin, naringin and 2,3-dihydroquercetin (inactive). The biologically active flavonoids bound DNA by intercalation and their affinity for DNA followed the same sequence of the potency of their activity.

The hypotensive effects of flavonoids have been demonstrated in animal experiments. Thus, of four flavonoid glycosides⁵⁹ extracted from orange peel, two decreased the blood pressure of rats. Potentially pharmacologically active agents were also studied⁶⁰ in the peel of *Citrus unshiu*. Flavonoid glycosides were isolated from an aqueous extract of peel by precipitation with ethanol and sequential extraction with hexane and butanol. Six flavonoids were isolated from the crude extracts by column chromatography on silica gel as (i) limocitrin-3- β -D-glucose, (ii) limocitrin-3- α -L-rhamnose, (iii) 3,6-di-c-glucosylapigenin, (iv) narirutin, (v) rutin and (vi) narcissin. Compounds (iii) and (v) exhibited hypotensive effects in rats. These findings are supported by epidemiological studies.^{20,61} Inhabitants of certain regions of France show⁶² increased longevity and decreased incidence of cardiovascular disease compared with the USA despite consumption of fats at comparable levels, which are correlated with increased risk of heart attacks. This observation has been termed the French paradox and is attributed, in part, to the routine consumption of wine⁶³ and other flavonoid-rich foods in the Mediterranean area.

Flavonoids are found ubiquitously in plants and thus are part of the human diet (Table 2). Dietary flavonoids consist⁶⁴ mainly of anthocyanidins, flavonols, flavones, catechins and flavanones. They are absorbed from the gastrointestinal tracts of humans and animals and are excreted either unchanged or as flavonoid metabolites in the urine and faeces. Accurate data on population-wide intakes of flavonoids are not available but important dietary sources are vegetables, fruits and beverages, the last accounting for at least 25–30% of the total daily flavonoid intake.⁶⁵ Early estimates of flavonoid ingestion were based mainly on food analyses using techniques of doubtful accuracy. For example, Kuhnau⁶⁵ estimated the dietary intake

of flavonoids as 1 g d⁻¹ in the USA. More recent estimates are considerably lower but significant variations are expected reflecting different diets. Using data of the Dutch National Food Consumption Survey 1987–88,⁶⁶ the average total intake of the flavonoids quercetin, kaempferol, myricetin, apigenin and luteolin among 4 112 adults was determined as 23 mg d⁻¹. This intake exceeded that of the antioxidants β -carotene and vitamin E and thus flavonoids represented an important source of antioxidants in the human diet. The most important flavonoid was the flavonol quercetin (mean intake 16 mg d⁻¹) with the most important sources of flavonoids being tea (48% of total intake), onions (29%) and apples (7%). Flavonoid intake did not vary between seasons and did not correlate with total energy intake, but was weakly correlated with the intake of vitamin A, dietary fibre and vitamin C. However, data on flavonoid absorption from the gut are limited, as are data on the effects of degradation by intestinal microorganisms. The amount of flavonoid that remains biologically available⁶⁷ may not be of sufficient concentration to explain the beneficial effects seen with the Mediterranean diet. Moreover, synergistic and antagonistic effects may occur. More research is needed for further elucidation⁶⁸ of the mechanisms of flavonoid absorption, metabolism and biochemical action. Large-scale epidemiological evaluations of the effects of the flavonoids on chronic diseases are needed to assess the findings from experimental studies.

Uses

Certain flavonoids are used for their pharmacological properties whilst others have important industrial applications. For example, naringin and neohesperidin can be converted into their corresponding dihydrochalcones with a strong sweetening capacity.¹⁸ There are, however, unexplored opportunities for exploiting these compounds. The diversity of the flavonoids in agronomic lines and their role in plant resistance⁶⁹ suggest that they could be exploited for increased food production. Also, the widespread distribution of flavonoids in plants could be explored for their increased use¹² in medicine and disease control. The full exploitation of this potential will depend on enhancing the levels of these metabolites in plants¹⁸ by regulating the associated processes of growth and cell differentiation. For instance, dietary modulation of the susceptibility to disease appears to be a possibility.

Chemotaxonomy

Flavonoid data on various plants demonstrate their value in appreciating plant variability and its phylogenetic significance at species or infraspecific level. Flavonoid compounds are particularly convenient for this purpose as they are widely distributed among plants and are chemically stable. They show a structural diversity due to differences in oxygenation, methylation and glycosylation processes. Moreover, flavonoid profiles are now relatively easy to establish using high-performance techniques discussed later. Thus, flavonoids have been widely used^{70–75} in chemotaxonomy particularly on the genera *Ribes* (e.g., blackcurrant, gooseberry), *Rubus* (e.g., blackberry, raspberry), *Vaccinium* (e.g., cowberry) and *Vitis* (grape). Historically, the root of classical genetics is that of the flavonoid compounds, in particular, of the anthocyanins.⁷⁶

The studies of Torre and Barrit,⁷⁷ which examined the quantitative distribution of anthocyanin pigments in numerous species of *Rubus* led to the publication⁷³ of fundamental work on 'anthocyanin variation in the g. *Rubus*.' Quantitative variations depended on three major genes which determined the quantity of pigments and the colour of the fruit. The large number of cultivars and species of *Vitis* complicate the situation but two groups were distinguished⁷⁸ dependent on the presence

or absence of acylated anthocyanins. Factorial analysis of anthocyanin data in different grape varieties has been used⁷⁹ to identify the criteria to be retained to enable characterization of a cultivar. The total anthocyanin content made possible⁸⁰ the characterization of species of the genus *Rubus*.

In the case of citrus, polymethoxylated flavones have been measured⁸¹ by HPLC in peel oils of orange, mandarin, tangerine and clementine. Standardized principal component analysis was conducted on seven parameters, namely individual concentration of six flavones plus the sum of these concentrations. Factorial discriminant analysis showed the taxonomic significance of the data in differentiation of orange and mandarin groups, the latter characterized by higher total flavone concentrations, particularly tangeretin and nobiletin. However, most interest has focused on the use of flavanone glycosides as taxonomic markers for citrus systematics,^{14,82} as discussed below.

Apart from its fundamental significance, chemotaxonomy has been applied to the authentication of products such as juices and jams and detecting product substitutions.⁸³ Flavonoid profiles are ideal for this purpose; they are complex and most flavonoids are not commercially available. As an illustration, pattern recognition of flavanone glycoside profiles has been used⁸⁴ to detect the addition of grapefruit juice to orange juice.

Fruit Quality

Flavonoids may contribute to fruit quality in a number of ways, *e.g.*, by contributing to sensory attributes such as colour and flavour and through the contribution of some specific flavonoids to bitterness of certain fruits.^{85–88} In fruits such as apples, the flavonoids contribute^{89,90} to the texture of the fruit. They are also involved in the formation of undesirable brown pigments in fresh fruit following bruising or cutting and/or during storage⁹¹ as the result of enzymatic oxidation of endogenous phenolics into quinones which then polymerize into brown products. Apart from economic considerations, which are considerable, browning reactions are responsible for some phenomena affecting colour, taste and nutritional value of the plant products. In addition, this reactivity can affect the resistance characteristics of fruits and vegetables against storage fungi. The susceptibility of apples to browning illustrates the complex interactions between polyphenol oxidase activity and phenolic content. In some instances, enzymatic activity has been identified as the main factor in browning whereas in others the phenolic content has been highlighted.⁹² Amiot *et al.*⁹³ distinguished soluble and insoluble brown pigments, the latter correlating with the flavan-3-ol content of the apple. In fruit juices, flavonoids may contribute to sediment formation by combination of polyphenols such as catechins with proteins to form sediments which cause undesirable hazes in products including wine and fruit juices.⁹³

Biosynthesis

An understanding of the essential features of flavonoid biosynthesis is important to understanding their diversity and to the design of sound analytical procedures.

Flavonoid biosynthesis involves the interaction of at least five different pathways, namely the glycolytic pathway, the pentose phosphate pathway, the shikimate pathway that synthesizes phenylalanine, the general phenylpropanoid metabolism that produces activated cinnamic acid derivatives (4-coumaroyl-CoA) and also the plant structural component lignin and finally the diverse specific flavonoid pathways. The last three should be viewed as segments of a single unit, that of aromatic metabolism. An overview is presented in Fig. 1 with detailed discussions available elsewhere.^{76,94,95} Enzymes responsible

for the formation of different flavonoid classes and for structural modifications, such as hydroxylation, methylation, glycosylation and acylation, have been identified.⁹⁵ Moreover, amino acid and nucleic acid sequences are now available for several of these enzymes.

Sources

Flavonoids are characteristic constituents of green plants with the possible exception of algae and hornworts. They occur in virtually all parts of the plant but the quantitative distribution varies between different organs of the plant^{96,97} and within different populations of the same plant species. This variability is largely controlled by genetics but other factors include maturity, climate, position on the tree, rootstock and agricultural practices. In the case of processed products, technological processes to which fruits are exposed may impact significantly on the flavonoid content. For example, a fivefold increase in phloretin glycosides was measured⁹⁸ in diffusion-extracted apple juices relative to pressed juices; whereas quercetin glycosides were barely detected in pressed juices, a range from 29.9 to 51.8 mg l⁻¹ was found in the diffusion-extracted juices. The effects of processing on the flavonoid content of numerous other juices have been reported. These include the content of flavonols in red raspberry juice⁹⁹ and polymethoxylated flavones in commercial *versus* hand-squeezed orange juice.⁹⁶

Despite the complexity and diversity of the genetically controlled flavonoid distributions of plants, some general observations are possible. An important and interesting feature of these distributions is the strong tendency for taxonomically related plants to produce similar types of flavonoids. Thus, three of the numerous classes of flavonoids are widespread and quantitatively dominant: flavonols, anthocyanins and flavan-3-ols, the last present as both monomers and in condensed forms (tannins). The other classes, notably flavones, flavanones, flavanonols, chalcones and dihydrochalcones, are important only in particular fruits. For example, the most important flavonoids in citrus are the flavanones. Table 3 summarizes the major flavonoids found in various fruits; data are for ripe fleshy fruits. Common and systematic names for the more frequently encountered flavonoid aglycones are given in Table 4. It needs to be borne in mind that compounds found in various parts of the fruit may differ; in particular, compounds associated with the leaves may well be different from those present in flowers, stems, roots or fruits. Indeed, the type of phenolic associated with the surface of plants, *e.g.*, in leaf waxes, is usually different from that occurring within the plant. Surface flavonoids are usually highly methylated and lack sugar substitution.¹⁰²

Modern techniques reveal that most plants are likely to contain 5–15 major flavonoids with anything from 20 upwards of minor flavonoids; newer, higher resolution techniques may reveal this to be an underestimate. Nevertheless, a limited number of flavonoids are usually characteristic of most fruits. Hence Fernandez de Simon *et al.*^{101,103} established the phenolic composition of juices or nectars of orange, apple, pineapple, peach, apricot, pear and grape. They concluded that hydroxycinnamic acid esters with tartaric acid are typical of grape, phloridzin is typical of apple and isorhamnetin glycosides are typical of pear. Myricetin is only found in peach and luteolin and apigenin glucosides are found only in orange. Apricot could be detected by the presence of two coumarins and pineapple by the presence of sinapic acid and the absence of phenolics (benzoic acids and aldehydes, flavan-3-ols, flavonols, chalcones, cinnamic acids and their derivatives).

Anthocyanic Pigments

The anthocyanins are pigments which give most fruits their red, violet and blue colour,^{104,105} although the red colour of some

fruits (*e.g.*, orange, tomato) is caused by carotenoid pigments rather than anthocyanins. The latter are glycosides which release the anthocyanidin aglycone by hydrolysis.²⁹ The aglycones exist in cationic form in acidic medium with numerous mesomeric forms. Interest in the anthocyanic pigments and their stability¹⁰⁶ can be attributed to their contribution to the colour of many processed products, including jams and juices. Nonetheless, anthocyanins have not been used extensively as additives in the food industry¹⁰⁷ because of their instability towards a variety of chemical and physical factors (*e.g.*, pH and light), the difficulty of purification and their limited commercial availability. However, the discovery of more stable acylated anthocyanins will probably see the realization of their considerable potential¹⁰⁸ as safe food additives.

Six anthocyanidins are widespread and commonly contribute to the pigmentation of fruits. Cyanidin is the most common and, in terms of frequency of occurrence,¹⁰⁹ is followed in decreasing order by delphinidin, peonidin, pelargonidin, petunidin and malvidin. There are few fruits which do not contain cyanidin and, in a number of fruits, *e.g.*, peach and pear, it is the

single dominant aglycone. In other fruits, two aglycones are characteristic, *e.g.*, cyanidin and pelargonidin in raspberry cultivars. Lowbush and highbush blueberries are unusual in containing significant amounts of the five aglycones¹¹⁰ delphinidin, cyanidin, petunidin, peonidin and malvidin. These were present as non-acylated glucosides and galactosides with the corresponding acetylated anthocyanin also occurring in several cultivars.

The anthocyanidin glycosides are characteristic of a fruit. Quantitative variations occur in the anthocyanin content of ripe fruits^{111,112} in response to climatic factors, light and temperature in particular. Fruits, in contrast to other plant organs, are characterized² by a relatively large amount of monoglycosides compared to diglycosides. Glycosylation of anthocyanidins almost always occurs at the 3-position with glucose, arabinose and galactose the most common sugar moieties. Hence the most common anthocyanins in fruit consist of the 3-monoglucosides of cyanidin, delphinidin, peonidin, pelargonidin and petunidin, cyanidin 3-galactoside, cyanidin 3-arabinoside plus the single diglycoside cyanidin 3-rutinoside. Among these pigments, cyanidin 3-glucoside is the most

Table 3 Major flavonoids found in selected fruits^{100,101}

Fruit	Major flavonoids				
	Flavonols	Anthocyanins	Flavan-3-ols*/flavanones	Chalcones	Others
Apple	Quercetin, quercetin glycosides including rutin, kaempferol	Cyanidin glycosides including acylated derivatives	(+)-Catechin, (-)-epicatechin	Phloretin derivatives, notably phloridzin	
<i>Citrus</i> — Sweet orange		Glycosides of pelargonidin, peonidin, delphinidin, petunidin	Hesperidin, narirutin, eriocitrin, narirutin-4'-glucoside		Flavones: sinensetin, nobiletin, tangeretin isosinensetin
Grapefruit			Naringin, narirutin, hesperidin, neohesperidin		Flavones: tangeretin, polymethoxylated flavones
Lemon	Rutin, limocitrol, limocitrin, isolimocitrol		Hesperidin, eriocitrin		Flavones: diosmin, luteolin-7-rutinoside
Grape	Quercetin, kaempferol, glycosides of quercetin kaempferol, myricetin, isorhamnetin, including rutin	Glycosides of cyanidin, peonidin, delphinidin, petunidin, malvidin	(+)-Catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin		Flavanonols: glycosides of dihydroquercetin and dihydrokaempferol
Pear	Quercetin, glycosides of quercetin, isoquercetin kaempferol and isorhamnetin	Cyanidin glycosides	(+)-Catechin, (-)-epicatechin	Arbutin, phloretin glucoside	
<i>Stone fruit</i> — Peach	Myricetin, quercetin, kaempferol, quercetin and kaempferol glycosides, including rutin	Cyanidin glycosides	(+)-Catechin, (-)-epicatechin		
Plum	Glycosides of kaempferol and quercetin	Glycosides of cyanidin, peonidin	(+)-Catechin, (-)-epicatechin		
Apricot	Quercetin, quercetin 3-O-glucoside, rutin, kaempferol glycoside				
Sweet cherry	Glycosides of kaempferol and quercetin	Cyanidin glycosides	(+)-Catechin, (-)-epicatechin		
Tomato	Kaempferol and quercetin glycosides		Naringenin, naringenin glycosides	Chalconaringenin	

* Associated mainly with skin and seeds.

abundant in both fruits and other plant organs. Other anthocyanins are limited in their distribution. Anthocyanins identified in red raspberry, for example, were^{113,114} cyanidin-3-sophoroside, cyanidin-3-glucuronoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside and pelargonidin-3-glucuronoside. These pigments were relatively unstable and degraded during storage and fermentation.¹¹⁵ The major anthocyanin in raspberry, cyanidin-3-sophoroside, is also the most stable pigment whilst cyanidin-3-glucoside is considerably less stable. The anthocyanin patterns of raspberry cultivars were distinguished by quantitative rather than qualitative differences. Polymerized pigments in raspberry juices indicated a history of processing or storage abuse. Anthocyanins are the major phenolics in dark-coloured cherry genotypes¹¹⁶ with total anthocyanin content ranging from 82 to 297 mg per 100 g of pitted cherry. Total anthocyanin is considerably less at 2–41 mg per 100 g of pitted fruit in light-coloured cherries. The 3-rutinoside and 3-glucoside of cyanidin are the major anthocyanins with the same glycosides of peonidin as minor anthocyanins. Another minor anthocyanin is pelargonidin-3-rutinoside.

In addition to glycosylation, acylated anthocyanins are found fairly often in fruits, the situation being particularly complex in grapes^{75,117–121} where the 3-monoglucosides corresponding to the five aglycones can all be acylated by acetic or *p*-coumaric acid.

Flavonols

These compounds are very widespread in higher plants where they occur usually as *O*-glycosides in the leaves and outer parts of the plant, while only trace amounts are found in parts of the plant below the soil surface. There are far fewer detailed quantitative studies on fruit flavonols than on anthocyanins particularly in relation to genetic and environmental variability. Nevertheless, over 200 flavonol aglycones have been identified in plants, although only four of these, quercetin, kaempferol,

myricetin and isorhamnetin, are common in fruits. Glycosylation occurs preferentially at the 3-hydroxyl group^{122,123} and the predominant types in fruits are 3-*O*-monoglucosides in the following order: 3-glucosides > 3-galactosides > 3-rhamnosides > 3-glucuronides. The only diglycosides observed with any frequency in fruit are the 3-rutinosides of quercetin and kaempferol. Complete characterization of a flavonol monoglycoside requires a knowledge of whether the sugar–aglycone bond is an α or β linkage and whether the sugar is in furanose or pyranose form. In general, it has been found that sugars with a *d*-configuration, namely glucose, galactose, xylose and glucuronic acid, are usually linked to the aglycone by β bonds whilst α linkages occur to *l*-arabinose and *l*-rhamnose. This is illustrated by the characteristic flavonoid glycosides of apple,^{98,124,125} which include quercetin α -*l*-arabinofuranoside, β -*d*-galactopyranoside, β -*d*-glucopyranoside, α -*l*-rhamnopyranoside and β -*d*-xylopyranoside.

The occurrence of flavones and flavonols has been thoroughly reviewed^{64,126} The flavonol content of Rosaceae fruits, *e.g.*, strawberry, raspberry and blackberry,^{127,128} is dominated by quercetin and kaempferol and their glycosides. The flavonol glycoside profile does not differ greatly between the various Rosaceae fruits, but does differ from flavonol glycoside profiles of stone fruits. Apricots, plums and peaches contain^{129,130} kaempferol and quercetin glycosides. The main glycoside in apricots is rutin, followed by kaempferol-3-rutinoside present at a considerably lower concentration (by a factor of 10). Small amounts of 3-glucosides and 3-galactosides of kaempferol and quercetin and quercetin-3-rhamnoside are also present with traces of more highly glycosylated flavonols, although with considerable varietal variation in amount present. The main glycosides in peaches are 3-glucosides and 3-galactosides of kaempferol and quercetin. In addition, kaempferol-3-rutinoside, quercetin-3-rutinoside and quercetin-3-rhamnoside are present. Traces of triglycosides, present in very small amounts, have been incompletely identified as kaempferol- and quercetin-3-glucosyl-7-diglucoside and 3-galactosyl-7-diglucoside.

Flavan-3-ols

The monomeric flavan-3,4-diols, referred to as leucoanthocyanidins, are frequently found in the woody tissues including the bark of trees but are not major compounds in fruits. On the other hand, the flavan-3-ols are important constituents of fruits in oligomeric or polymeric forms as proanthocyanidins or condensed tannins. However, the monomers are also important natural products in their own right.⁹³ Among these, (+)-catechin, (–)-epicatechin, (+)-gallocatechin and (–)-epigallocatechin are found in fruits. These are generally found in fruits in free rather than glycosylated forms, which distinguishes them from other flavonoids. The catechins are important in so far as they are the natural substrates of polyphenol oxidases and are therefore involved in browning phenomena. They are also the monomer units for the procyanidins.

Flavanones

In most of the plant kingdom, flavanones occur in small amounts compared with other flavonoids, yet they are the predominant flavonoid in citrus. In terms of its flavonoid composition, citrus is exceptional, some citrus flavanones being found nowhere else. Four aglycones are common, namely naringenin, eriodictyol, isosakuranetin and hesperetin. Further, citrus flavanones usually occur as glycosides whereas in other plants flavanones are seldom found in glycosidic form.¹⁵ Glycosylation occurs at position 7 either by rutinose or neohesperidose, disaccharides formed by a glucose and rhamnose molecule differing only in the type of linkage 1 → 6 or 1 → 2. This has formed the basis for classification of citrus.

Table 4 Common and systematic names of selected flavonols, flavones and flavanones

Common name	Systematic name
<i>Flavonols</i> —	
Quercetin	3,3',4',5,7-Pentahydroxyflavonol
Kaempferol	3,4',5,7-Tetrahydroxyflavonol
Myricetin	3,3',4',5,5',7-Hexahydroxyflavonol
Isorhamnetin	3'-Methylquercetin
Quercetagetin	3,3',4',5,6,7-Hexahydroxyflavonol
<i>Flavones</i> —	
Tangeretin	4',5,6,7,8-Pentamethoxyflavone
Heptamethoxyflavone	3,3',4',5,6,7,8-Heptamethoxyflavone
Nobiletin	3',4',5,6,7,8-Hexamethoxyflavone
Sinensetin	3',4',5,6,7-Pentamethoxyflavone
Scutellarein	4',5,6,7-Tetramethoxyflavone
Isosinensetin	3',4',5,7,8-Pentamethoxyflavone
Quercetogetin	3,3',4',5,6,7-Hexamethoxyflavone
Chrysin	5,7-Dihydroxyflavone
Apigenin	4',5,7-Trihydroxyflavone
Luteolin	3',4',5,7-Tetrahydroxyflavone
Diosmetin	4'-Methyllyuteolin
Tricetin	3',4',5',5,7-Pentahydroxyflavone
<i>Flavanones</i> —	
Naringenin	4',5,7-Trihydroxyflavanone
Eriodictyol	3',4',5,7-Tetrahydroxyflavanone
Hesperetin	3',5,7-Trihydroxy-4'-methoxyflavanone
Dihydroquercetin	3,3',4',5,7-Pentahydroxyflavanone
Dihydrofisetin	3,3',4',7-Tetrahydroxyflavanone
Dihydrorobinetin	3,3',4',5',7-Pentahydroxyflavanone

Thus, most commercial citrus cultivars^{19,131,132} contain only the non-bitter rutosides whereas sour orange and pummelo have only bitter flavanone neohesperidosides.¹³³ Grapefruit are considered as hybrids because they contain both flavanone rutosides and neohesperidosides.¹³⁴ Further distinction is possible on the basis of the predominant flavanone; in the case of sweet oranges, mandarins, lemons and citrons this is hesperidin, whereas naringin is the major flavanone in grapefruit and pummelo.

It is now accepted that naringin is absent from sweet orange varieties. Nevertheless, the evidence is contradictory¹³⁵ and a recent publication¹³⁶ reported concentration data for naringin in a number of sweet orange varieties. The latter data were obtained by HPLC using coulometric array detection. In our experience, naringin is generally absent but in some sweet oranges a peak co-elutes with naringin using typical reversed-phase conditions. Our preliminary results suggest that this peak is not naringin, a fact noted also by Ooghe *et al.*,¹³⁷ but the true identity of the peak remains unknown. Nevertheless, it does account for the confusion about the presence of naringin in sweet orange. Resolution of this issue is important as naringin is used as a chemotaxonomic marker in distinguishing sweet orange from other citrus cultivars.

Data on flavanones in other fruits are fragmentary. Of the phenolics identified in the fruit cuticles of tomato cultivars,¹³⁸ free naringenin, naringenin 7-glucoside (prunin) and the corresponding chalcone, chalconaringenin, were abundant. These were synthesized mainly during the climacteric and were largely bound to the cutin matrix. The composition of the flavonoid fraction was controlled by the spectral quality of incident radiation, red light favouring the formation of chalconaringenin.

Flavones

These compounds are not common in fruits and are never predominant. Citrus is again a special case containing a number of polymethoxylated flavones as minor flavonoids. Some of these, *e.g.*, nobiletin and sinensetin (sweet orange peel) and tangeretin (tangerine oil), have been known for some time. Concentrations are very high in the flavedo and they are readily isolated from the essential oil of citrus fruits but are also identifiable in the juice.¹³⁹

Other Flavonoids

Other flavonoids are either not widely distributed in fruits or are present as minor components of the total flavonoid content. This section is intended to provide an indication of flavonoid diversity rather than a comprehensive treatise on flavonoid distribution. Nevertheless, the flavanonols, particularly dihydrokaempferol and chalcones, should be noted. The latter are important in relation to stability of anthocyanins since the chalcone form results from an endergonic reaction.¹⁴⁰

Analysis

Many analytical procedures have been developed for flavonoid compounds reflecting the varied reasons for undertaking the analysis. In some instances, profiling of the flavonoid content is necessary to examine process-related variability in flavonoid composition, whereas quantification is the ultimate goal in many cases. In other instances, isolation and identification of unknown flavonoid compounds is demanded. The design of the analytical procedure will depend very much on the intent of the analysis. For example, in both profiling and quantification studies, the most successful approaches have been based on chromatography. Indeed, a sophisticated high-resolution technique is mandatory because of the number and diversity of flavonoids. For this reason and because it avoids the need for

derivatization, HPLC has found widespread acceptance in this role. Where identification is required, spectrometric methods are likely to assume more importance, particularly in coupled modes with HPLC or GC. The methods which follow are for soluble flavonoids and specifically exclude lignins and condensed tannins, which raise special problems.

Total Phenols

As the amount of individual flavonoids in fruits is usually low, they have often been recorded unspecifically as 'total phenolics.' Various enzymatic methods have been reported¹⁴¹ for this purpose. Historically, however, total phenolics were most conveniently assessed by spectrophotometric measurement¹⁴² on a simple extract of the plant or fruit material. There are a number of difficulties associated with such measurements and their continued value is debatable. First, exhaustive extraction with alcoholic and aqueous alcoholic solvents is likely to leave behind much tannin and other phenolics bound at the cell wall in which case measurement of 'total phenolics' is in reality confined to the soluble fraction. Second, the diversity of phenolics means that the selection of a reagent and/or absorbing wavelength will be a compromise, although this will be less of a problem where a single class of phenolics predominates. Results are expressed in terms of molar equivalents of a commonly occurring flavonoid, *e.g.*, hesperidin¹⁴³ or an appropriately chosen mixture of flavonoids.¹⁴⁴

Colorimetric procedures rely on the reaction of the flavonoid with one of a number of reagents¹⁴³ of varying selectivity. Folin-Ciocalteu reagent,^{63,121,145} which has been used before and after precipitation of flavonoids in acidic methanol,¹⁴⁶ and vanillin are the classic reagents. Swain and Goldstein¹⁴⁷ have reviewed methods relating to direct measurement and recommended the Folin reagent for total phenols and vanillin where catechins and proanthocyanidins are the major substances. The Folin reagent is widely used but will react with compounds other than the target phenols and interfering reductants must be removed prior to assay. Newer reagents include Prussian Blue,¹⁴⁸ 4-(dimethylamino)cinnamaldehyde and a rapid browning test in oxidative medium.¹⁴⁶ With few exceptions, the inherent problems of direct spectrophotometric measurement relegate such methods to one of historical interest only. Nevertheless, a highly selective spectrophotometric method has been developed¹⁴⁹ for quercetin based on its oxidation reaction in neutral solution with *N*-bromosuccinimide in the presence of phenol to give a violet chromogen measurable at 510 nm.

All phenols absorb radiation in the UV region (Table 5). For flavonoids, the spectrum typically consists of two absorption

Table 5 UV absorption maxima³⁰

Compound class*	Absorption maxima/nm	
	Band II	Band I
Simple phenols	266–295	
Phenolic acids	235–305	
Hydroxycinnamic acids	227–245, 310–332	
Hydroxycoumarins	<i>ca.</i> 210, 250–260, 280–303	312–351
<i>Flavonoids—</i>		
Flavones, biflavones	250–280	310–350
Isoflavones	245–275	310–330
Flavonols	250–280	350–385
Flavanones	275–295	310–330
Chalcones	240–260	365–390
Aurones	240–270	390–430
Anthocyanins	265–275	465–560

* Usual solvent is methanol with the exception of methanolic HCl for anthocyanins.

maxima in the ranges 240–285 nm (band II) and 300–550 nm (band I). The precise positions and relative intensities of these bands provide valuable information on the nature of the flavonoid and its oxygenation pattern.¹⁵⁰ Flavonoids such as 6- and 8-hydroxyflavonols, chalcones and aurones are characterized by a band extending into the visible region with longwave maxima from 380 to 430 nm whereas the anthocyanins absorb in the visible region, usually at wavelengths above 500 nm. Ionization with alkali normally causes a bathochromic shift of 15–50 nm with an increase in absorbance. The limited use of direct spectrophotometric measurements, whether in the visible or UV region, can be attributed in part to the lack of specificity of such methods. In general, they lead to an overestimation of 'flavonoid' content.¹⁴⁶ Specificity can be enhanced in direct spectrophotometric methods by derivative spectrometry. For instance, chrysin and quercetin were determined¹⁵¹ spectrophotometrically using first- and second-derivative spectra in a method that requires no preliminary separation of the flavanoids.

Continued interest in UV measurements can be attributed largely to the widespread popularity of this technique as a detection method in HPLC. The practising flavonoid chromatographer may gain much useful information from an examination of the earlier literature and application of its lessons. For example, Hostettmann *et al.*¹⁵² demonstrated the use of HPLC with UV and post-column derivatization for the characterization of phenolics. An excellent treatise on such approaches including the use of shift reagents¹⁵³ was given by Markham.²³

Sample Preparation

Sample preparation encompasses a number of steps in the overall analytical scheme, from selection of a sample through extraction of the flavonoid to clean-up or purification. The ultimate goal of these procedures is the preparation of a sample extract uniformly enriched in all components of interest and free from interfering matrix components. Various procedures have been used at each stage reflecting the range of sample types and physico-chemical properties of the various flavonoids. The need for analyte recovery is ultimately related to the limited specificity and sensitivity of analytical procedures. The procedure must allow quantitative recovery of the flavonoids whilst avoiding any chemical modifications in the analytes which result in artefacts and unnecessarily complicate subsequent steps. For example, heat-sensitive components such as coumarins may be degraded by use of elevated temperatures and labile glycosides may be hydrolysed in some situations. Apart from its analytical implications, the extraction and recovery of phenolics^{120,121} can be of critical technological interest in processes such as wine production.

Flavonoid aglycones are polyphenols and as such share the properties of phenols such as solubility in alkali resulting from their slightly acidic nature. However, if left in alkali in the presence of oxygen many will degrade. Historically, recovery of flavonoids by liquid extraction of the fruit¹⁵⁴ has been common. Flavonoids are generally stable compounds and may be extracted from the dried, ground plant material with cold or hot solvents. Suitable solvents for this purpose are aqueous mixtures with ethanol, methanol, acetone and dimethylformamide. Extractions have been performed on freeze-dried extracts of the fruit or, alternatively, by maceration of the fresh, undried fruit with the extracting solvent.¹²⁴ In the latter case, the required proportion of water is lower.

The above procedure is unsuitable for anthocyanins and the less polar aglycones such as flavanones, polymethoxylated flavones, isoflavones and flavonols. The latter are more soluble in solvents such as chloroform, ethoxyethane and ethyl acetate–methanol, although flavonols have been successfully extracted

with aqueous alcohol.¹⁵⁵ In contrast, the anthocyanins are traditionally recovered as the flavylium cation by extraction with cold methanol containing hydrochloric acid.¹⁵⁶ Caution is necessary with acylated anthocyanins, which are frequently labile in solutions containing mineral acid, and this is one of the reasons why the relatively common acylated pigments have been overlooked in earlier studies.¹⁵⁷ Replacement of hydrochloric acid with weaker acids, either formic or acetic acid, allows the recovery of these compounds.^{75,158,159} Care must be exercised to ensure that the acetylated derivatives are in fact natural and not an artefact of the extraction process.¹⁶⁰ With the most labile anthocyanins, the use of non-acidified solvents is probably a sensible precaution. Alternatively, solid-phase extraction on C₁₈ cartridges has been used.¹⁶¹ When the adsorbed anthocyanins are subsequently eluted with an alkaline borate solution, a class separation is achieved. It appears that those anthocyanins possessing *o*-dihydroxy groups (cyanidin, delphinidin, petunidin) form a charged borate complex, resulting in a more hydrophilic species. This complex is preferentially eluted from the reversed-phase cartridge while those anthocyanins not containing *o*-dihydroxy groups (pelargonidin, peonidin, malvidin) are enriched on the cartridge. On the other hand, elution with 0.01% HCl in methanol produces no fractionation. A more exhaustive clean-up on polyvinylpyrrolidone was also examined. The relative proportions of the anthocyanins was different for the two procedures. Thus, for quantitative analysis the extraction and/or clean-up procedure should be thoroughly checked.¹⁶² Jackman and Smith¹⁶³ have discussed factors such as pH, temperature, oxygen, light, enzymes, nucleophilic agents, sugar derivatives and co-pigments which affect anthocyanin stability.

Solvent extraction has also been used for flavonoid recovery from fruit juices. Thus, polarity differences in citrus juice components have been exploited⁸⁴ in a comprehensive recovery scheme for (carotenoids), polymethoxylated flavones and flavanone glycosides based on extraction with solvents of graded polarity. After dilution with methanol, the juice was extracted with hexane to remove the carotenoids. Further extraction of the juice with dichloromethane isolated the polymethoxylated flavones, which were chromatographed by reversed-phase LC with an acetonitrile–methanol–water mobile phase and detection at 280 nm. The flavanone glycosides remaining in solution were chromatographed on a C₁₈ column with an acetonitrile–water mobile phase and detection at 280 nm. Isolation of polymethoxylated flavones in citrus juices has been performed by extraction with organic solvent following addition of sodium hydroxide^{164,165} to eliminate the possible interfering lactones. A comparative study of several solvents with regard to their effectiveness in extracting polymethoxylated flavones from intact and NaOH-treated juices has been published.¹⁶⁵ In terms of total flavones, methyl isobutyl ketone was only slightly less efficient than benzene but was more effective for specific flavones. This data demonstrate the need to consider carefully any recovery problem^{101,103} on an individual basis. It was also concluded that the addition of NaOH to the juice leads to degradation of the polymethoxylated flavones and artefact formation.

Ideally, fruit juices require minimal sample preparation beyond filtration.¹⁶⁶ For example, flavonol glycosides and phenolic acids were determined¹²¹ in grape juice directly after filtering. Ultracentrifugation followed by filtration has been employed for flavonoid recovery from citrus.^{167,168} Poor recoveries can be attributed to low solubility of certain flavonoids²¹ and also to sorptive losses on the filtration medium. These effects have been thoroughly investigated by Widmer and Martin.¹⁶⁹ In other instances, some form of preliminary sample processing has been deemed desirable.¹³² Solid-phase extraction on mini-cartridges has been employed^{97,170,171} in an attempt to minimize the effects of sample

preparation on extract integrity. Flavonoids were recovered by elution with methanol from a Sep-Pak C₁₈ cartridge¹³² following elution of sugars with aqueous methanol. Recoveries compared favourably with those achieved by simple filtration. However, with cloudy juices in particular, both filtration and solid-phase extraction may be ineffective in recovering flavonoids located in suspended juice solids, even though these may represent a large fraction of the total flavonoids present. Under these circumstances, solvent extraction may be a preferable alternative. Hesperidin, the major flavonoid of sweet orange, presents a specific problem because of its low solubility in aqueous media. Addition of dimethylformamide to the juice has been used^{137,172} in an effort to improve solubility, but in this case some early eluting peaks are lost in the chromatogram. This also results in sample dilution with decrease in sensitivity. Heating of the juice has been used¹³⁷ to increase hesperidin solubility, although it remains unclear whether total or soluble hesperidin is important. Buffering of the sample in the pH range 4.5–5.0 prior to extraction has been recommended¹⁷³ to overcome problems of the pH dependence of flavanone glycoside recovery.

With relatively few exceptions (*e.g.*, refs. 21 and 169), methods of sample preparation have not been systematically investigated. In these circumstances, it becomes very important when comparing results from different methods to consider the extraction procedure.¹⁷⁴

Sample clean-up

The need for this step varies depending on the sample type, method of extraction and subsequent procedure. In some instances, recovery and clean-up will have been combined in a single step. Nevertheless, the original extract, particularly if aqueous alcohol has been used as extracting solvent, will generally contain numerous non-flavonoid substances which can interfere in later stages of the analysis. Carotenoid and chlorophyll pigments can be removed by liquid–liquid extraction with hexane of the aqueous extract after removal of organic solvents.^{17,176} Potential loss of lipophilic flavonoids must be monitored. Column chromatography has been widely investigated for preliminary fractionation of sample extracts. Suitable stationary phases include silica gel, alumina, microcrystalline cellulose and DEAE-cellulose, magnesium silicate, polyamide and Sephadex.

Silica is especially suited to the separation of less polar isoflavones, flavanones and (usually highly alkylated) flavone and flavonol aglycones. For instance, 5,7-dihydroxyflavanone was isolated¹⁷⁷ by elution from silica with chloroform–ethyl acetate (1 + 1). However, traces of iron(III) in the adsorbent may cause irreversible sorption of flavonoids. In contrast to silica, polyamide is a good general-purpose phase suited to separation of flavonoids of varying polarity although ideal for glycosides^{124,178,179} Polyamide 6 minicolumns were used¹²⁸ for the fractionation of flavonols in red raspberry juices. Quercetin glycosides, quercetin and kaempferol were eluted with methanol whilst a second fraction eluted with 0.5% ammonia in methanol contained three flavonol glucuronides, two flavonol forms, aglycones, ellagic acid and its derivatives. Polyamide was also chosen¹²⁴ in conjunction with Sephadex resins for the isolation of flavonoid glycosides of Spartan apple peel. Microcrystalline cellulose is ideal for separation of glycosides from one another or from aglycones and for the separation of the less polar aglycones. Separation of flavanoid glycosides from flavonol and/or flavone glycosides is usually difficult but can be achieved on cellulose columns using water as eluent. The more water-soluble flavanoids are less strongly retained and elute from the column first. Sephadex LH-20 is useful for final clean-up of flavonoid extracts^{180,181} but can also be applied to initial fractionations.¹⁸⁰

Hydrolysis and glycoside analysis

Hydrolysis of glycosides¹⁸² is used as an aid to structural elucidation and characterisation as discussed by Markham.²³ Three types of hydrolytic treatment are used for this purpose, acidic, enzymatic and alkaline. Hydrolysis has also been used to minimize interferences in subsequent chromatography¹⁷⁹ and as an aid to simplifying chromatographic data,^{183–186} particularly in instances where the appropriate standards are unavailable. In this role, chemical treatment has been more common because it is less selective and more exhaustive. There is considerable variation in the lability of the glycosidic bond under hydrolytic conditions. Hydrolysis methods when used for purposes other than characterization/ structural elucidation of unknown flavonoids result in a reduction in information content. Hence, a sample extract containing, say, five *O*-glucosides of a single aglycone plus the free aglycone will produce after acid hydrolysis a single HPLC peak. The advantages in terms of simplicity of interpretation and quantification are apparent. Hence, acid and base hydrolysis simplified¹⁸⁷ the complex HPLC profile of red raspberry juice phenolics dramatically. Minor differences were observed in the profiles resulting from the two treatments following sample preparation on Sep-Pak C₁₈ cartridges.

The rate of acid–base hydrolysis of glycosides depends on acid–base strength, the nature of the sugar and the position of attachment to the flavonoid nucleus. For example, glucuronides resist acid hydrolysis whereas by comparison glucosides are cleaved rapidly. *C*-Glycosides generally remain intact although structural rearrangements can occur in presence of hot acids¹⁸⁸ owing, for example, to a Wessely–Moser rearrangement which has the effect of interconverting 6- and 8-*C*-glycosides.¹⁸⁹ The five major flavonoid aglycones quercetin, kaempferol, myricetin, luteolin and apigenin were determined¹⁹⁰ in freeze-dried vegetables and fruits after acid hydrolysis of the parent glycosides. The aglycones were separated by reversed-phase HPLC, the identity of the eluted compounds being confirmed by UV photodiode-array detection. Completeness of hydrolysis and extraction were optimized by systematically testing different conditions such as acid concentration, reaction period and methanol concentration in the extraction solution using samples containing various types of flavonoid glycosides. Optimum hydrolysis conditions were presented for flavonol glucuronides, flavonol glycosides and flavone glycosides. Recoveries of the flavonols quercetin, kaempferol and myricetin ranged from 77 to 110% and of the flavones apigenin and luteolin from 99 to 106%.

Thin-layer Chromatography

Prior to the advent of chromatography, analysis of flavonoids was a difficult proposition. The advent of paper chromatography revolutionized the analysis of natural products and many paper chromatographic (PC) methods were developed for flavonoids in the 1950s and 1960s. Excellent compilations are available but are largely of historical interest, having been supplanted by TLC. TLC in its simplest form is inexpensive and is especially useful as a method for the simultaneous analysis of several samples. The usual advantages of TLC, namely speed and an open-bed technique,¹⁹¹ are realized in flavonoid analysis.^{192,193} The selection of a suitable stationary phase and solvent depends on the class(es) of flavonoid to be examined. Hydrophilic flavonoids, for example, can be readily separated by TLC on polyamide¹⁹⁴ or microcrystalline cellulose layers.¹²² Silica gel layers have traditionally been used for the less hydrophilic flavonoids, including methylated flavones and isoflavones. Thus, polymethoxylated flavones were isolated¹⁹⁵ from orange juice concentrates by extraction with benzene and separated on silica gel layers. On the other hand, flavanone glycosides of citrus juices were separated¹⁹⁶ on polyamide

layers. The glycosides were extracted from the juices with ethyl acetate following an initial extraction with ethoxyethane to remove less polar substances. The ethyl acetate extract was subjected to clean-up on a Sephadex G-25 column prior to TLC. Applications of layer chromatography to anthocyanins have been reviewed by Strack and Wray.¹⁹⁷

Detection can be achieved by direct examination in the case of anthocyanins. More generally, direct viewing of the plates under ultraviolet radiation^{101,198} provides a sensitive means of spot location or, alternatively, various spray reagents are available. Useful reagents are aqueous aluminium chloride,¹⁹⁶ sodium borohydride,⁸² diazotised sulfanilic acid and vanillin.²³ Colour reactions which permit the determination of the type of flavonoid have been tabulated.¹⁹⁹ Thus, for example, flavanones and flavanonols are reduced by magnesium and HCl in methanol solution to flavylum ions, which yield intensively orange, red and violet colours. Flavones and flavonols show almost no reaction. Flavones and flavanonols, in turn, may be distinguished by substitution of magnesium by zinc in the test since only flavanonols are reduced to blue-violet compounds.²⁰⁰ More recently, the advantages of diphenyltin dichloride have been demonstrated²⁰¹ for both the qualitative and quantitative analysis of flavones and flavonols on thin-layer plates by forming fluorescent complexes of different colour.

Two-dimensional TLC^{101,127} can be used for difficult separations such as the resolution of critical pairs. Alternatively, the excellent resolutions achieved with TLC can be improved further with high-performance layers²⁰² which are available for a range of adsorbents and reversed-phase materials. HPTLC has been applied to flavanones and dihydroflavonols on silanized silica gel.²⁰³

Gas Chromatography

As originally practised, quantification was difficult with both PC and TLC. The introduction of GC overcame this difficulty and it was inevitable that the new technique be applied to flavonoid analysis. Nevertheless, flavonoids were not an ideal application area for GC, which never assumed the importance achieved in other areas. The main difficulty is the limited volatility of many flavonoids, notably the glycosides, which must therefore be derivatized prior to GC analysis. In a typical analysis, recovery of flavonoids²⁰⁴ from orange juice by elution from polyamide columns was followed by the formation of trimethylsilyl ethers. The results obtained by this method were in good agreement with those for the spectrophotometric determination of rutin at 358 nm and hesperidin and naringin at 284 nm after separation of components by TLC and elution of flavonoids from the layer material.

The polymethoxylated flavones are exceptional in that GC provides a viable alternative for their analysis. For this purpose, packed columns²⁰⁵ employing relatively non-polar phases such as OV-17 on Chromosorb W are unsuitable. On the other hand, high-efficiency open-tubular columns are ideal, producing^{139,206,207} excellent separations of the flavones in orange peel oil. A stationary phase is now commercially available²⁰⁸ which offers improved retention and selectivity for these compounds. The main advantage of such columns, however, is the low stationary phase bleed, which permits operation at elevated temperatures with minimal interference in the detection process. This greatly facilitates the use of coupled GC-MS.

High-performance Liquid Chromatography

HPLC combines the advantages of simultaneous separation and quantification without the need for preliminary derivatization, in most cases. Progress in this technique over the last decade is evident from an examination of the excellent text by Markham²³ published in 1982, which devoted 10 pages to describing uses of

PC for flavonoid analysis but dismissed HPLC in less than 1 page. This situation is now unthinkable. The merits of HPLC are seen in the resolution of the 3-glucoside and 3-galactoside of cyanidin in a 20 min separation. Before the advent of HPLC, this separation required 2 d to achieve by PC.⁶ The data in Table 6 summarize chromatographic methods for flavonoid analysis.

Normal-phase chromatography has been used for the separation of flavonoids in orange juice and skins of ripe tomato.^{213,214} Non-polar components were removed from the plant material by extraction, following which the aqueous phase was subjected to clean-up on a polyamide column. Flavonoids including flavone, flavonol and flavanone aglycones and their glycosides were eluted with methanol prior to acetylation. The recovered flavonoid acetates were separated isocratically on LiChrosorb Si60 using benzene-acetonitrile, benzene-ethanol or iso-octane-ethanol-acetonitrile solvent systems and detected at either 312 or 270 nm. Similarly, polymethoxylated flavones were separated²¹¹ on LiChrosorb Si60 following extraction from orange and tangerine peels. Capacity factors of the flavones were correlated²¹² with the position of the methoxy groups on the flavone skeleton. As an alternative to HPLC on a bare adsorbent, supercritical fluid chromatography allowed²²³ excellent separations of polymethoxylated flavones of citrus oils. Carbon dioxide modified with methanol gave rapid elution of the compounds as sharp, well resolved peaks.

For these normal-phase systems, there is the concern²¹⁷ that highly polar materials may be retained irreversibly on the column, with the result that the separation characteristics could be gradually altered. Thus, reversed-phase chromatography (RPC) has invariably been the method of choice^{217,239-241} for the separation of the flavonoids, usually on C₈^{165,213} or C₁₈ columns^{101,166,186,215,234,242,243} used in conjunction with aqueous mobile phases and methanol, acetonitrile or, less commonly, tetrahydrofuran as organic modifier. The benefits of RPC have been realized for all classes of flavonoids and, indeed, phenolics in general, but RPC has particularly enhanced the separation performance of anthocyanins. In an early application,²⁴⁴ 18 anthocyanins in a mixture of 20 were separated by RPC in 2 h. For the separation of anthocyanin pigments of red raspberry^{108,242} on a C₁₈ column, 15% acetic acid was used as organic modifier. Both the retention properties and the spectral properties obtained with photodiode array detection were used for characterization. More generally, small amounts of acetic acid, formic acid or phosphate buffers (*e.g.*, 50 mm)²³⁴ incorporated in the mobile phase tend to improve separations markedly. Recently, a branched-chain, fluorocarbonaceous, silane-bonded silica gel material has been developed²⁴⁵ for HPLC. This phase is characterized by its ability to resolve polyphenols such as flavonoids as sharp peaks and excellent durability under extreme eluting conditions, in contrast to ordinary hydrocarbon-bonded silica gel columns. Rommel and Wrolstad¹²⁸ found that polymer columns or end-capped C₁₈ columns with high carbon loads were ineffective for the separation of flavonoids of red raspberry juices. However, a C₁₈ column, not end-capped and with a low carbon load, gave a good separation.

Stationary and mobile phase effects on retention and selectivity have been examined in several studies.^{209,215,217} Thus, Pietrogrande and Kahie²⁴⁶ compared the retention behaviours of several flavonoid compounds on reversed-phase HPLC columns including phenyl, cyano and octadecyl phases. The selectivity properties of methanol, acetonitrile and tetrahydrofuran as organic modifiers were also reported on each stationary phase. Selectivity depended on both phases but specific stationary phase selectivity effects were more pronounced with methanol; in particular, the phenyl phase showed a greater selective retention for unsaturated flavonoids while octadecyl proved more selective for glycosides. Retention data

Table 6 Conditions used for the chromatographic analysis of flavonoids

Analyte	Sample	Recovery	Column	Mobile phase	Detection	Comments	Ref.
Flavanones, flavanols	Plant material	Extraction, hydrolysis and polyamide column	TLC, silica gel	Dichloromethane-acetic acid-water	Spray reagents		200
Cinnamic and benzoic acids, flavones and glycosides (Umbelliferone, scopoletin), naringin, hesperidin, bergapton	N.A.	N.A.	Bondapak C ₁₈ , 4 × 300 mm	Isocratic: water-acetic acid (methanol) Gradient: acetonitrile-acetate buffer	UV, 280 nm	Substituent effects on elution order	209
Polymethoxylated flavones (×5)	Grapefruit juice	Heat, centrifuge, acidify and ethyl acetate-methanol extraction	Zorbax ODS, 4.6 × 250 mm	Isocratic: THF-water Isocratic: acetonitrile-water	UV, 280 nm and fluorescence, ex 350 nm, em 450 nm UV, 313 nm		210
Polymethoxylated flavones (×5)	Orange and tangerine juices Orange concentrates	Filter, add NaOH then chloroform extraction Filter, add NaOH then benzene extraction	Zorbax C8 4.6 × 250 mm Microbondapak C ₁₈ Zorbax C ₈ , 4.6 × 250 mm	Isocratic: THF-water Isocratic: THF-acetonitrile-water	Stopped flow UV, 313 nm and fluorescence, ex 360 nm, em 415 nm UV, 280 nm	Five major polymethoxylated flavones in juice	164
Polymethoxylated flavones (×16)	Peel extracts	Soxhlet extraction with benzene	LiChrosorb Si60, 4 × 250 mm	Isocratic: heptane-ethanol or propan-2-ol	UV, 280 nm		211, 212
Coumaric acids, flavanones	Tomato skins	Reflux, extraction and hydrolysis	TLC on cellulose or silica gel. GC of derivatives	Various	Spray reagent for TLC; GC-MS		138
Aglycones and flavonoid glycosides (×28)	Tomato skins, orange juice	Centrifuge, elution from polyamide followed by precolumn acetylation	LiChrosorb Si60, 3 × 200 mm	Isocratic: benzene-acetonitrile	UV, various (270 and 300 nm)	Acetylated derivatives	213, 214
Aglycones and flavonoid glycosides (×34)	N.A.	N.A.	Bondapak C ₁₈ , 3.9 × 300 mm	Isocratic: methanol-acetic acid-water	UV, 254 nm	Substituent effects on elution order	215
Catechins, procyanidins	Cider apple	Homogenized, filtered	Spherisorb 5 Hexyl, 5 × 100 mm	Ternary gradient	UV, 280 nm		216
Aglycones and flavonoid glycosides (×141)	N.A.	N.A.	LiChrosorb RP-18, 4.6 × 250 mm	Gradient: formic acid-water-methanol	UV, 280 nm	Substituent effects on elution order	217
Anthocyanins	Elderberry	Heat and pH adjustment	Nucleosil C ₁₈ , 4.6 × 150 mm	Gradient: phosphoric acid-water-tetrahydrofuran	UV/VIS, 254, 340 and 510 nm		218
Anthocyanins	Fruits	Methanol extraction (and silylation for GC)	Aquapore RP-300	Gradient: water-methanol-acetonitrile	VIS, 530 nm		219
Polymethoxylated flavones and glycosides	Citrus peel	Extraction with water at alkaline pH	Ultrasphere C ₈ , 4.6 × 250 mm	Gradient: methanol-acetonitrile-water-acetic acid	UV, 240-400 nm	Structure effects on retention; data on hesperidin complex	19
Flavonol glycosides (40) and sulfates	N.A.	N.A.	Partsil 5 CCS/C ₈ , 5 × 250 mm	Gradient: methanol-acetic acid-water	UV, 365 nm	Structure effects on retention; interference effects	220
Naringin	Orange and tangerine juices	Dimethylformamide (improves solubility but loss in sensitivity and some early eluting peaks < 10 min are absent)	Hypersil ODS, 4.6 × 250 mm	Isocratic: ammonium acetate-acetonitrile	UV		172
Anthocyanidins	Cranberry juice	Diluted juice eluted from SPE C ₁₈ cartridge and filtered	Micropak MCH-10 C ₁₈	Isocratic: water-acetic acid-methanol-acetonitrile	VIS, 530 nm		105
Catechins, procyanidins	Grape seeds	Maceration and extraction	Brownlee Labs C ₁₈ , 4.6 × 250 mm	Gradient: acetonitrile-water	UV, 280 nm		120
Narirutin, naringin, hesperidin, neohesperidin	Citrus juices	Centrifuge and filter or SPE	Zorbax ODS, 4.6 × 250 mm	Isocratic: water-acetonitrile-acetic acid	UV, 280 nm	Data for 52 cultivars	132

Table 6—continued

Analyte	Sample	Recovery	Column	Mobile phase	Detection	Comments	Ref.
Flavonoid glycosides, particularly of quercetin	Apple peel	(Extraction with ethyl acetate, fractionation on polyamide and Sephadex)	Radial Pak RP (C ₁₈)	Gradient: tetrahydrofuran in trifluoroacetic acid	UV, 270 nm		124
Polymethoxylated flavones	Orange and mandarin peel oils	Oil expressed in a hydraulic press	LiChrosorb Si60	Heptane-propan-2-ol	UV, 280 nm		81
Anthocyanins	Bog whortleberry	Filtration and droplet counter-current chromatography	Supelcosil LC18, 4.6 × 250 mm	Gradient: water-methanol-acetic acid	VIS, 515 ± 25 nm		221
(Carotenoids), polymethoxylated flavones, flavanone glycosides	Orange juice	Comprehensive recovery scheme	Zorbax ODS, 4.6 × 250 mm	Gradient: acetic acid-water-methanol	UV, 280 nm		84
Quercetin and phloretin glycosides	Apple skins	Homogenize with methanol, extract non-polar components with hexane and clean-up on C ₁₈	Radial Pak C ₁₈ , 8 × 100 mm		UV, photodiode array	Preparative-scale HPLC; MS data	175
Naringin, neohesperidin	Citrus juices	Centrifuge and filter	Supelco C ₁₈ , 3.6 × 125 mm	Isocratic: water-acetonitrile-acetic acid	UV, 280 nm (photodiode array)		166
Phenolic acids, aglycones, flavonoid glycosides	Bark and leaf of prunus	N.A.	LiChrosorb C ₁₈ , 4.0 × 250 mm	Various	UV, 280 nm	Detailed retention data	222
Polymethoxylated flavones (×9)	Juices	Filter then SPE	Hypersil ODS, 2.1 × 200 mm	Gradient: water-acetonitrile-THF	UV, photodiode array	Concentration in juice (fresh <i>versus</i> concentrate)	170
Polymethoxylated flavones (×5)	Orange juice	Addition of dimethylformamide, heat and centrifuge then SPE on a C ₁₈ cartridge	Hypersil ODS, 4.6 × 250 mm	Isocratic: acetonitrile-water	UV (photodiode array), fluorescence, ex 330 nm, em 430 nm	Juice, peel and pulp wash data	96
Flavonoid glycosides	Citrus	Centrifuge and filter	Superspher, 4.6 × 250 mm		UV, various wavelengths	Flavonoid content of various citrus	167
Cinnamic acids, flavonol glycosides, procyanidins	Pear juice	Filtration or Sephadex LH-20	Supelcosil LC-18, 4.6 × 250 mm	Gradient: phosphate buffer in acetonitrile	UV, 280, 320 nm		123
Flavonol glycosides, phenolic acids, procyanidins	Grape juice	Filtration or Sephadex LH-20	Supelcosil LC-18, 4.6 × 250 mm	Gradient: phosphate buffer in methanol	UV, 280, 320 nm		121
Flavonol glycosides, procyanidins	Apple juice	Filtration or Sephadex LH-20	Supelcosil LC-18, 4.6 × 250 mm	Gradient: phosphate buffer in methanol	UV, 280, 320 nm	Treatment effects	125
Anthocyanins	Cranberry and strawberry	Elution from SPE C ₁₈ cartridge	Supelcosil ODS, 5 × 250 mm, PLRP-S, 4.6 × 250 mm	Isocratic: acetic acid-acetonitrile	UV/VIS, 260, 520 nm	Polymer-based column for low pH	108, 161
Anthocyanin	Red raspberry juice and wine	Filtration	Supelcosil LC-18, 4.6 × 250 mm	Gradient: phosphoric acid-acetonitrile	VIS, 520 nm		115
Polymethoxylated flavones (×8)	Citrus oils	N.A.	Zorbax Si, 4.6 × 250 mm	Gradient: acetic acid-acetonitrile	UV, 313 nm		223, 224
Polymethoxylated flavones (×27)	Orange peel oil	Oil diluted with ethyl acetate	OV-1, 0.32 mm × 50 m	Supercritical fluid chromatography: methanol-carbon dioxide	FID		206, 207
Naringin, narirutin, hesperidin, neohesperidin, prunin	Citrus juice	Methanol elution from polyamide cartridge	Cyclobond 1, 4.6 × 250 mm	Gradient: water-methanol-acetic acid	UV, 280 nm (diode array)	Diastereomer separation	225
Cinnamic acids	Orange and grapefruit	(Hydrolysis), ethyl acetate extraction and silica gel clean-up	LiChrospher RP18, 4 × 250 mm	Isocratic: acetic acid-water-methanol	UV, 300 nm	Distribution in orange and grapefruit sections	226

Table 6—continued

Analyte	Sample	Recovery	Column	Mobile phase	Detection	Comments	Ref.
Narirutin, hesperidin, didymin, narirutin-4- β -glucoside	Citrus	Centrifuge and filter	Supelcosil LC-18, 4.6 \times 150 mm	Gradient: phosphate buffer in acetonitrile	UV, 280 nm		168
Naringin, neohesperidin, neohesperidin	Citrus	Centrifuge and filter	Supelco LC18, 150 mm	Isocratic: water-acetonitrile-acetic acid or phosphate buffer in acetonitrile	UV, 280 nm	Interference effects due to sorptive losses and co-elution; extensive report on filter types	169
Cinnamic derivatives, flavonols, flavan-3-ols, dihydrochalcones	Apple	Freeze-dried material extracted with methanol, dried and extracted with ethyl acetate	Rosil C ₁₈ , 4.6 \times 150 mm	Gradient: water-acetonitrile-methanol containing phosphoric acid	UV, 280, 320 nm		93
Cinnamic and benzoic acids, flavonols, flavan-3-ols, chalcones and glycosides	Juices of orange, apple, pineapple, peach, apricot, pear, grape	Diethyl ether-ethyl acetate extraction after concentration	Novapak C ₁₈ , 3.9 \times 300 mm or 3.9 \times 150 mm	Gradient: acetic acid-methanol-water	UV, 280, 340, 254, 365 nm	Concentration data for a range of fruits	101
Cinnamic acids	Orange juice	Ethyl acetate extraction of alkaline centrifugate	Adsorbosphere C ₁₈ , 4.6 \times 250 mm	Isocratic: various	UV, 300 nm	Solvent optimization	227
Flavanones	Citrus juice	Ethyl acetate extraction, hydrolysis and TMS derivatization	RSL 200BP, 0.25 mm \times 50 m	GC	MS		228
Flavonols (quercetin, kaempferol, myricetin), flavones (luteolin, apigenin)	Strawberry, apple, red currant, apricot, pear, cherry, plum, peach, grape	Extraction of freeze-dried material followed by hydrolysis	Nova Pak C ₁₈ , 3.9 \times 150 mm	Isocratic: acetonitrile-phosphate buffer or methanol-phosphate buffer	UV, 370 nm		186, 190
Anthocyanins	Apples	Homogenization and extraction	Spheri 10-RP18	Gradient: formic acid-methanol-water	UV, 260, 280, 325 nm		229
Anthocyanins	Blackberry juice and wine	Filtration	Supelcosil LC-18, 4.6 \times 250 mm	Gradient: acetonitrile-acetic acid	VIS, 520 nm		104
(Amino acids), cinnamic acids, narirutin, naringin, hesperidin	Apple, orange and grapefruit juices	Centrifuge and filter	M.S.Gel C ₁₈ , 4.6 \times 150 mm	Gradient: phosphate buffer in acetonitrile-methanol	Coulometric array		136
Phenolic acids, flavonols and glycosides	Red raspberry	SPE and hydrolysis	Spherisorb ODS1, 4.6 \times 250 mm (not end-capped, low carbon load phase)	Gradient: acetonitrile-acetic acid	UV, 260, 360 nm		128, 187, 230
Flavonoid aglycones (\times 49)	N.A.	N.A.	PermaBond OV1, 0.25 mm \times 25 m	GC	MS		231
Anthocyanins, anthocyanidins	Raspberry and blackberry juices	Filtration and SPE	Polymer Labs. PLRP-5, 5 \times 250 mm	Gradient: acetic acid-acetonitrile	VIS, 520 nm		114
Flavonols (quercetin, kaempferol, myricetin); flavones (apigenin, luteolin)	Juices of apple, grape, tomato, grapefruit, lemon and orange	Acid hydrolysis and extraction	Nova-Pak C ₁₈	Isocratic: acetonitrile-phosphate buffer	UV, 370 nm	Quantitative data for various juices	54
Flavan-3-ols, procyanidins	N.A.	N.A.	Hypersil ODS, 4 \times 250 mm	Gradient: formic acid-methanol	UV, 280 nm vs. PCR, 640 nm		232
Eriocitrin, hesperidin, naringin, narirutin, neohesperidin	Orange and grapefruit juice	Sample heated, centrifuged and filtered	Novapak RP18, 3.9 \times 150 mm	Gradient: phosphate buffer in acetonitrile	UV, 280 nm	Extraction efficiency	137
Anthocyanins	Red raspberry juice	SPE	Spherisorb ODSII, 4.6 \times 250 mm	Gradient: acetic acid-methanol	VIS, 515 nm	Effect of storage conditions	113

Table 6—continued

Analyte	Sample	Recovery	Column	Mobile phase	Detection	Comments	Ref.
Eriocitrin, hesperidin, naringin, narirutin, neohesperidin, neohesperidin	Lemon, lime, grapefruit, orange	Dilution in dimethylformamide-ammonium oxalate plus heat/centrifuge and filter	Alltech RP18, 4.6 × 300 mm	Isocratic: water-acetonitrile-THF-acetic acid	UV, 280 nm	Data for various citrus; buffer necessary to ensure adequate extraction	173, 233
Cinnamic acid derivatives, flavonoids, anthocyanins	Wine and musts	Filtration	Novapak C ₁₈ , 3.9 × 150 mm	Phosphate buffer in acetonitrile	UV/VIS, photodiode array	Wine characterization	234
Aglycones, flavonoid glycosides (×25)	Citrus fruit	Centrifuge and SPE	LiChrospher RP18, 4.0 × 250 mm (×25)	Gradient: phosphoric acid in methanol	UV, photodiode array	Data for distribution in fruit tissues	97
Polymethoxylated flavones (×6)	Orange juice	Extraction with benzene	Novapak RP18, 3.9 × 150 mm	Gradient: THF-acetonitrile-water	UV, photodiode array, 340 nm		139
Anthocyanins	Blueberries	Homogenize and filter	SuperPac Pep-S, 4 × 250 mm	Gradient: formic acid-water-methanol	VIS, 525 nm		110
Anthocyanins	Cherry	Homogenize and filter	SuperPac Pep-S, 4 × 250 mm	Gradient: formic acid-water-methanol	UV, 280 nm; VIS, 525 nm		235
Cinnamic derivatives, flavonol glycosides	Pear	Freeze-dried material homogenized with methanol, washed with hexane and SPE	Adsorbosphere C ₁₈ , 4.6 × 150 mm	Gradient: acetonitrile-methanol-water-phosphoric acid	UV	UV and MS	122
Narirutin, naringin, hesperidin, neohesperidin, (nootkatone)	Grapefruit, pummelo, tangelo	DMSO extraction of dried fruit	Bondapak C ₁₈ , 4 × 250 mm	Isocratic: water-methanol-acetonitrile-acetic acid	UV, 280 nm		18, 236
Hesperidin, neohesperidin, narirutin	Orange juice	Dilution, centrifuge and filter	Uncoated fused-silica capillary tubings	Borate buffer in acetonitrile (capillary electrophoresis)	UV/VIS		237
Catechins, flavonol glycosides	Apple juice	Centrifuge and ethyl acetate extraction	Spherisorb ODS-2, 4.6 × 250 mm Novapak C ₁₈ 3.9 × 300 mm	Gradient: aqueous phosphate buffer	UV		238
Hesperidin, naringin, narirutin, rhoifolin	Orange, grapefruit	Extraction with methanol heat, centrifuge and filter	Alltima C ₁₈ , 4.6 × 250 mm	Isocratic: water-acetonitrile-propan-2-ol-formic acid or water-tetrahydrofuran	UV, photodiode array	Data for orange and grapefruit; extraction efficiency	21

for flavonoids on a LiChrospher ODS-5 phase with 10 non-congeneric eluents were analysed²⁴⁷ and capacity factors quantitatively related to structural information found from 20 molecular descriptors which included physico-chemical parameters. Structural features reflecting positive charge distribution within a molecule were identified as being of most importance for retention. On a more practical note, a quaternary mobile phase afforded²³³ greater control of selectivity than a ternary mixture.

Under the usual reversed-phase conditions, the more polar compounds are generally eluted first.²⁰⁹ Thus, diglycosides precede monoglycosides, which precede aglycones. The elution pattern for flavonoids containing equivalent substitution patterns¹⁹ is flavanone followed by flavonol and flavone. This elution pattern holds for both aglycones and glycosides. For isomeric compounds which differ in the structure of the saccharides attached at the 7-position, the rutinoside eluted ahead of the neohesperidoside. This relationship was established for the isomeric pairs hesperidin–neohesperidin, naringin–narirutin and eriocitrin–neoeriocitrin. For anthocyanins, molecular structure–retention characteristics have been demonstrated, *e.g.*, in the work of Vande Castele *et al.*²⁴⁸ and Takeda *et al.*²⁴⁹ Goiffon *et al.*²⁵⁰ established rules governing the chromatographic behaviour of anthocyanins on a reversed-phase column. The overall polarity and stereochemistry of the compound are key factors. In particular, the following factors are well documented: substitution of the anthocyanidin B-ring, the position, nature and number of sugar moieties attached to the anthocyanidin and the sugar acylation. Thus, substitution of the B-ring gives the elution order delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin with hydroxyl groups decreasing and methoxyl groups increasing retention. Peonidin–malvidin and cyanidin–petunidin are critical pairs depending on the choice of mobile phase. Glycosylation generally decreases retention in the order 3,7-diglycosides < 3,5-diglycosides < 3-glycosides and 3-galactosides < 3-glucosides < 3-rutinosides, subject to modification by the nature of the sugar moiety. Acylation decreases mobility.

The determination of phenolics in apple juices and cider by the usual technique of RPC involving an aqueous methanol gradient and detection at 280 nm resulted in interference due to co-elution of phenolic acids and procyanidins.²¹⁶ A preliminary fractionation of the phenolic compounds of wine into neutral and acidic groups²¹⁵ before injection into the HPLC column eliminated the problem. Alternatively, co-elution was overcome²¹⁶ by operating the reversed-phase column initially at pH 7, when the phenolic acids were eluted rapidly in an ionized form, followed by a decrease in pH to 2.5 for the remainder of the run. Co-elution and potential interference problems are also possible²²⁰ with certain flavonol glycosides and sulfates. This difficulty was readily resolved by the addition of an ion-pair reagent to the reversed-phase system. Tetrabutylammonium phosphate proved to be suitable and this shifted the elution of the flavonol sulfates to longer retention times whereas the glycosides were unaffected.

Flavanone glycosides can exist as a pair of diastereoisomers by virtue of the carbohydrate attached at the chiral C-2 atom. The benzoylated derivatives²⁵² have been resolved into their diastereoisomers on a silica gel stationary phase by HPLC. Separation of the underivatized diastereomeric forms is also theoretically achievable but has not been generally realized in practice by RPC on alkylsilane phases. With a cyclodextrin-bonded stationary phase the separation has been achieved in the reversed-phase mode²²⁵ with a water–methanol–acetic acid gradient increasing in methanol content.

Despite the obvious successes of RPC, the separations between glycosides and aglycones are not adequate for the resolution of a complex mixture containing many compounds of each group. For this reason, it has become common practice to

separate the various classes in a preliminary extraction step. For example, polymethoxylated flavones were determined in orange juice⁹⁶ following extraction with dimethylformamide and clean-up on a C₁₈ cartridge. Flavones were chromatographed on a Gynkochrom ODS-Hypersil column with UV detection. The amount and distribution of polymethoxylated flavones in juice, peel and pulp wash varied considerably with the content of orange peel being, about 100× higher than that of juice. As an alternative to preliminary fractionation, column flushing has been used¹³⁵ particularly in the analysis of glycosides to remove unwanted groups of compounds at the conclusion of a chromatogram. For example, in a typical flavanone glycoside analysis of whole filtered citrus juice, substances removed during the column wash cycle include free aglycones and carotenoids plus some phenolic acids.

Isocratic elution has been used particularly where members of an individual class of flavonoid are to be determined. For example, polymethoxylated flavones were separated isocratically in the reversed-phase mode¹⁶⁵ using a C₈ column and tetrahydrofuran–acetonitrile–water as mobile phase. Fisher²⁵³ applied an isocratic system of water–acetonitrile (4 + 1 v/v) for the resolution and quantification of flavanone glycosides in citrus juice. However, gradient elution²²⁹ is more common in recognition of the complex flavonoid profiles of many fruits. With isocratic elution, the separation between glycoside and parent aglycone is small,²⁰⁹ but is increased significantly by employing a solvent gradient.²¹⁰ In a typical application, flavonol glycosides and phenolic acids were determined in grape juice¹²¹ by HPLC on a Supelcosil LC-18 column with a methanol gradient in a phosphate buffer. The eluate was monitored at 280 and 320 nm with a photodiode-array detector. The only sample preparation involved was preliminary filtration, although determination of procyanidins required preliminary isolation on a Sephadex LH-20 column. It is not surprising that total phenols as determined colorimetrically with Folin–Ciocalteu reagent at 765 nm showed no correlation ($r^2 = 0.141$) with HPLC results.

Detection

Detection of the eluted species has been based, most commonly, on measurement of absorption of radiation^{186,190,235} at characteristic wavelengths (Tables 5 and 6). Hence 515–520 nm has been widely used for anthocyanidins and anthocyanins whereas various wavelengths in the UV region have been used for other flavonoids, *e.g.*, 280 nm for flavanone glycosides and 313 nm for the polymethoxylated flavones. For comparison, detection wavelengths used for non-flavonoid components include 450 and 465 nm for carotenoids, 200 nm for amino acids, 210 nm for limonoids, 245 nm for ascorbic acid and 214 nm²⁵⁴ for organic acids.

Post-column derivatization has received little attention²³² but offers a number of advantages, including enhanced selectivity. Hostettmann *et al.*¹⁵² devised a post-column reactor in which UV spectra and shifts could be measured by a photodiode-array detector, although an improved system has now been described.²⁵⁵ Anthocyanins are usually separated in the typical reversed-phase system involving acidic mobile phases as their flavylium cations. In this situation, they can be selectively detected at their longer wavelength absorption maxima of 500–550 nm where other interfering phenolics show no absorption. Anthocyanins can also be detected in the UV region as the colourless chalcone, an equilibrium form²⁵⁶ favoured at pH 3.5. This was illustrated with malvidin-3-glucoside,²⁵⁷ which showed a peak at 280 nm with a retention time slightly less than that of the corresponding peak of the cationic form. Developments in photodiode-array detection^{97,98,243} facilitate such studies.

The extensive use of photodiode-array detection can be attributed to the ability to collect on-line spectra²⁵⁸ without using stopped-flow techniques. This is illustrated by the measurement and characterization of flavonols¹²⁸ in red raspberry juices by photodiode-array spectral techniques. Polymethoxylated flavones in citrus juices were separated¹⁷⁰ by RPC following isolation by SPE on a C₁₈ cartridge. The eluting substances were detected with a photodiode array in the region 230–400 nm. The UV spectra of eluting peaks obtained at the apex and both inflection points of the peak were compared and exact coincidence of the three spectra after due allowance for the background absorption was taken as an indication of purity. In most instances, a photodiode array UV detector has been chosen but operation in the visible region²³⁴ provides enhanced scope for the characterization of pigments. For example, anthocyanin pigment profiles of commercial food colouring products were characterized¹⁰⁸ by HPLC with photodiode-array detection. Both the retention properties on reversed-phase HPLC and the spectral properties by photodiode-array detection were used for characterization.

Fluorescence detection is an obvious means of improving sensitivity and selectivity in flavonoid analysis. It is interesting that one of the earliest papers on HPLC²¹⁰ employed this means of detection as an adjunct to conventional UV detection. Stopped-flow scans were used to obtain excitation and emission spectra of the eluted species. This early paper demonstrated the complementary nature of the two methods of detection. The hydroxycoumarins scopoletin and umbelliferone exhibit strong emission at 450–460 nm. The limited stability and light sensitivity of several phenolics were noted and should serve as a warning. A subsequent paper¹⁶⁵ demonstrated widely varying fluorescence intensities for five common polymethoxylated flavones found in orange juice. Discrepancies between quantitative data obtained by HPLC and TLC were noted and an explanation was presented for this variation.

Electrochemical detection of phenolics has been described.^{154,259} Eluted species were characterized by retention data and current–potential responses. In an approach analogous to the more familiar photodiode-array detectors, 16 serial coulometric detectors maintained at different potentials were used¹³⁶ for on-line resolution of co-eluting phenolic and flavonoid compounds and the generation of voltammetric data. Resolution with the detector array was based on ease of oxidation and may be related to differences in structure where availability of electrons and the capacity for charge stabilization differed. The magnitude of the reduction potential for each class of compound corresponded to specific substitution patterns in the sequence catechol > methoxycatechol > monohydroxyl > methoxyl. Twenty-seven standard compounds were resolved in a 45 min run and limits of detection were in the low-ng ml⁻¹ range. The utility of the technique in generating multivariate data for differentiation of juices and juice mixtures was shown.

Coupled Methods

The on-line coupling of methods is of enormous potential because the selectivity can then be tuned in an optimum way, which in turn can be translated to either a faster analysis²⁶⁰ or an improved determination limit. It is difficult to provide an adequate definition of a coupled technique which is valid in all situations. Hence the distinction between coupled and other techniques is based more on historical development than on any fundamental considerations. The combinations of chromatography with either mass or Fourier transform IR spectrometry currently provide the best methods of qualitative and quantitative analysis.

Of these techniques, GC–MS has become routine and is generally carried out with either electron impact (EI) ionization

or chemical ionization (CI) sources, since these are appropriate for the introduction of volatile compounds. Comprehensive information about the mass spectra of the flavonoids has been published.^{261,262} In general, the EI mass spectra of flavonoid aglycones are characterized^{263,264} by intense molecular ion peaks plus significant fragments from both A and B rings that have been classified according to systems developed by Mabry and Markham²⁶⁵ and Grayer.²⁶² The fragmentations often provide sufficient information to determine molecular mass, elemental formula, substitution patterns in the A and B rings and the class of flavonoid. The EI and CI mass spectra of glycosides²⁶⁶ are dominated by the same ions as for the corresponding aglycones;²⁶⁶ the protonated aglycone invariably being the base peak in the CI mass spectra. A distinction with the mass spectra of the aglycones²⁶³ is the relatively weak fragments from fission of the A and B rings.

Because of limited volatility, analysis of flavonoids and their glycosides by GC and thus GC–MS has not generally found favour. However, GC–MS has been applied²⁶⁷ to the TMS-ether derivatives of flavone and flavonol glycosides. Flavonones have also been examined in citrus juices by GC–MS using EI²²⁸ following derivatization and sample hydrolysis. With anthocyanins, derivatization is an essential step for GC–MS.^{219,268} In contrast, the polymethoxylated flavones possess suitable volatility for GC. Thus, Berahia *et al.*²⁶⁹ analysed 39 polymethoxylated flavones by GC–MS. In addition to the common behaviour of flavones under EI conditions, such as a retro-Diels–Alder reaction which gives a characteristic fragment from the phenyl group of the flavone skeleton, new fragmentation pathways were identified and proposed. Ions characteristic of various substitution patterns were also identified. Improvements in GC column technology have increased the range of flavonoids amenable to GC–MS as the underivatized compounds. For instance, Schmidt *et al.*²³¹ analysed 49 flavones, flavonols, flavanones and chalcones without derivatization by GC–MS. Compared with direct inlet mass spectra, the GC–MS data exhibited the same typical fragmentation patterns but with slight differences in intensities.

Classical mass spectrometric gas-phase ionization techniques such as EI and CI are generally less suitable²⁶⁰ for polar, non-volatile compounds such as the flavonoids. Soft ionization techniques such as laser desorption,²⁷⁰ field desorption (FD),²⁷¹ plasma desorption and particularly fast atom bombardment (FAB or secondary ion emission)²⁷² revolutionized the MS analysis of these compounds. For instance, FD–MS has been applied to the study of flavanone glycosides²⁷³ and FAB–MS to flavanone and dihydroflavonol glycosides.²⁷⁴ These methods also facilitate the coupling of HPLC and MS. The major obstacles to coupled LC–MS²⁷⁵ were the problems of dealing with liquid solvent and of producing gas-phase ions, particularly intact molecular ion species, without the application of heat. The newer soft ionization methods overcome lack of volatility by direct formation or emission of ions from the surface of a condensed phase. Hence they eliminate the need for neutral molecule volatilization prior to ionization and generally minimize thermal degradation of the molecular species.

First-generation LC–MS instruments such as the moving wire or belt interface²⁷⁶ overcame incompatibility between high vacuum and the introduction of solvent by removing the liquid. Early approaches to LC–MS never achieved widespread acceptance. In the second generation, *e.g.*, continuous-flow FAB (or dynamic FAB), soft ionization techniques were coupled with liquid introduction. When introduced, continuous-flow FAB–MS rapidly superseded all other ionization methods for flavonoids and, in particular, anthocyanin studies, as it provided an ideal technique for the analysis of highly polar compounds, without the need for derivatization. It has the advantage of producing a molecular ion plus various fragmentation ions which provide structural information. Nowadays,

interfacing and ionization have merged in third-generation instruments such as the thermospray²⁷⁷ and electrospray²⁷⁸ types.

With spray ionization as distinct from field desorption, ions are generated in an excess of ambient bath gas, not in a vacuum, and are therefore ideal for coping with an LC effluent. The chromatographic eluate passes through a resistively heated stainless-steel capillary tube located in the thermospray probe. A supersonic jet of vapour is created by adjusting the temperature of the capillary to a level where the solvent is partially vaporized. The vapour jet contains an entrained 'mist' of small, statistically generated electrically charged droplets. The droplets continue to vaporize and shrink as they traverse the source until a point is reached where free ions are repelled from the droplet surface and leave the thermospray source through an orifice in a sampling cone. The process is greatly enhanced if the analyte is itself ionic or by the presence of a volatile electrolyte, such as 0.1 M ammonium acetate. Thermospray emerged as a practical LC-MS technique²⁷⁹ applicable to non-volatile samples in aqueous effluents at conventional flow rates, whereas continuous-flow FAB has developed as a technique compatible with microbore LC. However, thermospray is not without its difficulties; in particular, the efficiency of ion production varies widely with compound type and the flow rate and temperature of the inlet tube must be optimized for each different compound class. Moreover, each class of compound requires different conditions for optimum ionisation, and this is further complicated by gradient elution. Thermospray continues to find applications despite its limitations^{280,281} and has recently been reviewed.²⁸² Plasmaspray²⁸³ overcomes a number of these limitations, producing more fragmentation than thermospray and ionizing a wider range of compounds.

Electrospray and its several variations²⁸⁴⁻²⁸⁶ are a newer development using atmospheric pressure ionization mass spectrometers. The type of analytes and, more specifically, their polarity, are the prime factors in the choice of the LC-MS interface. Nonetheless, electrospray and ionspray are undoubtedly the fastest developing approaches²⁸⁷ and have largely replaced thermospray. The power of electrospray as an alternative, highly sensitive soft ionization technique for the investigation of polar, non-volatile and thermolabile molecules such as anthocyanins has been demonstrated.^{288,289} Electrospray operates with flow rates at less than 10 $\mu\text{l min}^{-1}$ using microbore columns or a conventional column equipped with an effluent splitter. Ionspray ionization allows higher flow rates of 2-200 $\mu\text{l min}^{-1}$,^{268,290} when used with narrow-bore (e.g., 100 \times 2 mm id) reversed-phase columns, as illustrated by the structure determination of anthocyanins.²⁸⁹ The chromatographic eluate enters the ion source through a capillary maintained at a high potential. The strong electric field generated by this potential causes the eluate to be expelled from the capillary as a plume of charged droplets. As solvent evaporates from the small droplets, a critical size is reached where repulsion between the charged entities in the droplet (mobile phase, electrolyte and sample ions) becomes greater than the surface tension forces holding them into the droplet and they are ejected into the surrounding gas. The ionized species enter the mass analyser through a skimmer cone. Mass spectra generated by all three techniques have a similar form, *i.e.*, dominated by pseudo-molecular ions with little or no fragmentation. Hence, the ES mass spectrum typically shows the molecular cation M^+ , aglycone ion and ions associated with the solvent, although fragmentation can often be induced by raising the cone voltage. Acid (acetic or formic) is often added to mobile phases in positive ion electrospray as a source of protons to assist ionization. Sensitivity is improved when the organic content in the mobile phase exceeds 20%.

Atmospheric pressure chemical ionization (APCI) is a further development of electrospray in which the nebulized sample is

ionized through a corona discharge and analytes become electrically charged by chemical ionization. APCI, which is compatible with flow rates of up to 2 ml min^{-1} , was used²⁹¹ to determine various isoflavones. The negative ion mode provided quality mass spectra which gave not only the molecular mass of the isoflavones, but also their molecular structures. Deuterium oxide was used to induce peak shifts in the mass spectra to determine the number of exchangeable hydrogen atoms in each molecule.

On rare occasions, MS can provide data sufficient for full flavonoid structure analysis, but more generally it is used to determine molecular mass and to establish the distribution of substituents between the A- and B-rings. This situation will improve with new developments. Tandem mass spectrometry (MS-MS)^{292,293} has been applied successfully to problems involving trace analysis of citrus flavanones and metabolite identification.²⁹⁴ Positive CI-MS-MS was superior to EI-MS-MS for the detection of a common fragment ion for flavanones at m/z 153. Using this approach, the flavanones, naringenin and hesperitin were detected in human urine after citrus ingestion. Glycosides were labile under the experimental conditions, probably during ionization. MS-MS, particularly in combination with LC and soft ionization techniques,²⁸¹ can be expected to improve significantly the separations of complex samples. The information available from such methods can be expected to increase with the development of newer technologies, including collisionally induced dissociation spectra.²⁸¹ For the latter, alternating low and high orifice voltages are used in which no fragmentation occurs at low voltage and fragmentation is induced at the high orifice voltage. This permits the simultaneous measurement of molecular mass and structural characterization.

A multi-dimensional chromatographic system combining on-line HPLC with high-resolution GC-MS has been described²⁹⁵ for the analysis of complex mixtures. The system was not applied to flavonoid analysis but gave excellent results for the characterization of neroli oil. Other developments still in their infancy, at least in applications to flavonoid analysis, are the coupling of GC and FTIR spectrometry and capillary electrophoresis (CE) with MS. Trimethylsilyl derivatives of several flavonoids were studied²⁹⁶ by GC-FTIR. The correlation between retention and gas-phase IR data was successfully used in structural identification of compounds having very similar chromatographic behaviour. The shift of the carbonyl frequency gave information on the presence of substituting agents.

In combination with the inherent sensitivity and selectivity of mass spectrometry, coupled CE-MS becomes a very powerful technique. The correspondence between CE and electrospray ionization flow rates provides the basis for an extremely attractive technique. Hence, various isoflavones were separated²⁹⁷ on an uncoated fused-silica column using 25 mM ammonium acetate buffer and negative electrospray ionization MS detection. This approach permitted the determination of molecular mass of the isoflavones and also the presence of various functional groups according to observed losses from the $[M - H]^-$ ion during collision-induced dissociation effected by adjusting the MS parameters.

Other Techniques

Several other techniques have been examined for application to flavonoid analysis. For example, a quantitative method using radioimmunoassay^{131,298,299} gave the naringin concentrations in orange flavedo and albedo. Chemiluminescence has also been studied. Hence, a number of flavonoids, when excited by hydroxyl radical, emit light with an intensity consistent with that of the radical-scavenging activities of these compounds. Thus, chemiluminescence was reported³⁰⁰ to decrease in the

order rutin > myricetin = isoquercitrin > quercetin > kaempferol > isorhamnetin for the major flavonols and nasunin > rubrobrassicin > delphinidin > cyanidin = malvin > malvidin for anthocyanins. Based on these data, chemiluminescence warrants closer examination, particularly as an on-line detection technique for HPLC where the inherent sensitivity and selectivity will be optimally exploited.

Capillary (zone) electrophoresis (CE or CZE) is a high-performance technique³⁰¹ in which separation is achieved on short, uncoated, fused-silica capillary tubes (100 cm 50–100 µm id). In a typical application, orange juice was analysed^{237,302,302} by CE using a 35 mm sodium borate buffer containing 5% acetonitrile at 21 kV with high-speed scanning detection for UV and visible spectra.

The injection volume is critical as a 1 m capillary of 75 µm id contains about 5 µl of buffer, hence, the sample volume must be less than 50 nl to avoid overload. In this instance, a 10 s hydrodynamic injection produced optimum results. The only sample preparation required was dilution and filtration. The method permitted the simultaneous analysis of a broad range of charged water-soluble molecules whilst non-polar compounds such as carotenoids moved with the electroosmotic flow without being separated. As the flavonoids are charged molecules in alkaline media, they were separated in this study.

Capillary electrophoresis is not applicable to the separation of uncharged solutes. Despite a superficial resemblance to HPLC, separation in CE depends on differences in electrical properties of the analytes rather than differences in solute distribution between two phases. Thus, most forms of CE are not considered as chromatography. The exception is a technique developed by Terabe and co-workers^{304,305} called micellar electrokinetic capillary chromatography (MECC), which is a hybrid of electrophoresis and chromatography and is a true chromatographic process. This technique involved introduction to the buffer of a surfactant [*e.g.*, sodium dodecyl sulfate (SDS)]³⁰⁶ at a concentration exceeding that of the critical micelle concentration. At this point the surfactant ions begin to aggregate and form spherical particles whose hydrocarbon tails are in the interior of the sphere with the charged ends exposed to water. The micelles constitute a stable second phase that is capable of solubilizing non-polar solutes into the hydrocarbon interior of the micelles. With SDS as surfactant, the surface of the micelles has a large negative charge, giving them a large electrophoretic mobility towards the positive electrode. However, most buffers are characterized by a high electroosmotic flow rate toward the negative electrode such that the micelles are carried towards that electrode also, but at a much reduced rate. Hence, the system consists of a faster moving aqueous phase with a slower moving micellar phase and solutes will distribute themselves between the aqueous phase and the interior hydrocarbon phase of the micelles. Solute polarity determines the position of the resulting equilibria and hence the migration rate.

The migration behaviour of flavonoids in MECC has received little attention.³⁰⁷ Factors affecting resolution and selectivity have been identified^{103,307} as applied voltage, capillary temperature, electrolyte concentration and nature (complexing or non-complexing buffers), buffer pH, micelle concentration and nature (SDS or cetyltrimethylammonium bromide)³⁰⁸ and the addition of organic modifiers to the running buffer (organic solvents, cyclodextrins, urea, cholate). Organic solvents modified the interaction between micelles and solutes thus altering retention and resolution.³⁰⁷ When methanol was used,³⁰⁹ either as a sample solvent or as a constituent of the buffer, the most hydrophobic flavones appeared as double or triple peaks in the electropherograms. These double peaks disappeared when acetonitrile was used instead of methanol. Micelles in MECC provide both ionic and hydrophobic interactions, the extent of each depending on the buffer pH. The separation of selected flavonoids was improved by SDS at pH

8.3 but there was little or no effect at higher pH. At pH 10.5, the separation was mainly regulated by ionization of the hydroxyl groups and borate complexation of the carbohydrate residues. Similar effects have been reported³¹⁰ for the separation of flavonoid glycosides. A correlation was generally observed^{309,311} between the migration order in MECC of flavone aglycones and the elution order previously reported for reversed-phase HPLC.

Applications of both CE and MECC to the analysis of secondary plant metabolites have been reviewed.^{312,313} The advantages and criticisms of CE were examined in relation to HPLC, it being concluded that CE will become an indispensable tool, together with HPLC and GC, in phytochemical laboratories since these techniques are in many ways complementary,³¹¹ and problems that are difficult to solve by HPLC, can often be solved using CE. Indeed, MECC enjoys a number of advantages over HPLC, including very high efficiencies; plate numbers exceeding 10⁵ are not uncommon. The ease with which the stationary phase can be altered by changing the micellar composition of the buffer compares with the need to change columns in HPLC.

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