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The Flavonoids

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Preface

This new work in *The Flavonoids* series provides a comprehensive review of the primary scientific literature published between 1986 and 1991. It can also be regarded as a third supplement to the original *Flavonoids* volume published in 1975 under joint editorship with T.J. and H. Mabry. The first supplement *Advances in Research* appeared in 1982 and covered the 1975–80 literature, while the second supplement *Advances in Research Since 1980* was published in 1988 and covered the 1981–85 literature. All the chapters in this volume are self-contained and provide a treatment of the most recent literature against the background of what has gone before. Methodology of flavonoid analysis has been largely omitted, since two key works have recently been published in this area: *Methods in Plant Biochemistry*, vol. 1, *Plant Phenolics* edited by J.B. Harborne (Academic Press, 1989) and *Carbon-13 NMR of Flavonoids* edited by P.K. Agrawal (Elsevier, 1989). There is, however, a chapter on ^{1}H nuclear magnetic resonance spectroscopy, since this has not been covered in recent years.

Chapters 1–9 review the chemistry of the new structures reported during 1986–91, in relationship to earlier known structures, and the authors discuss the natural distribution of each class of flavonoid. Function is considered here in relationship to the role of flavonoids in flower colour (Chapter 13), their effect on man and other mammals (Chapter 15) and their effects on insects (Chapter 14). Biosynthesis is discussed in Chapter 11, and recent experiments in the molecular biology of flavonoids are included. An important review of the genetics of flavonoid production (Chapter 12) provides the first modern up-to-date account of the mode of inheritance of anthocyanin pigments in plants.

As editor, I am once again deeply indebted to the contributors, who have carried out their demanding assignments in an exemplary fashion. The pace of flavonoid research has not slackened at all in recent years, and it is hoped to produce a fourth supplement, probably in modified form, before the turn of the century. I would therefore welcome comments, criticisms and

suggestions from readers about this continuing series. In the meantime, I am most grateful to the staff of Chapman & Hall for their generous support and interest in this endeavour.

JEFFREY B. HARBORNE
December, 1992

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The anthocyanins

DIETER STRACK and VICTOR WRAY

1.1 INTRODUCTION

1.1.1 Occurrence

Anthocyanins are the most important group of water-soluble plant pigments visible to the human eye. With a few exceptions, e.g. the betalains, they are universal plant colorants and largely responsible for the cyanic colours of flower petals and fruits. They may also occur in roots, stems, leaves and bracts, accumulating in the vacuoles (Wagner, 1982) of epidermal or subepidermal cells. They are also found infrequently in the petal mesophyll, e.g. in most members of the Boraginaceae (Harborne, 1988b). In a recent study on the histological distribution of anthocyanins in spathes of various *Anthurium* species, specific distributions of anthocyanin-containing cells of the ad- and abaxial epidermis, hypodermis and mesophyll were observed (Wannakrairoj and Kamemoto, 1990). The authors suggest that these specific distributions might even be of value in establishing intrasectional species relationships. An interesting tissue compartmentation has been reported by Miyazaki *et al.* (1991), who identified different patterns of hydroxybenzoyl and hydroxycinnamoyl acylated anthocyanins in the tuber periderm and flesh, respectively, of two cultivars of *Ipomoea batatas*.

The anthocyanins are usually in solution within the vacuole, although they may sometimes be located in spherical vesicles, called 'anthocyanoplasts' (Pecket and Small, 1980), in which structural modifications of the C_{15} nucleus may occur (Merlin *et al.*, 1985). Development of these vesicles, involving light-dependent anthocyanin accumulation, has been observed in cells of *Ipomoea batatas* suspension cultures (Nozue and Yasuda, 1985). They were also detected in vacuoles from *Vitis vinifera* suspension cultures (Cormier *et al.*, 1990). Anthocyanoplasts are usually detected in vacuoles, although they may also appear as membranous vesicles in the cytoplasm (Nozzolillo and Ishikura, 1988). The presence or absence of anthocyanoplasts in vacuoles can provide additional data useful to taxonomic studies (Nozzolillo and McNeill, 1985), and has been exemplified with seedlings from 31 taxa of *Phaseolus* and *Vigna*. Anthocyanoplasts were found in all tested members of the *Vigna* subgenus *Ceratotropis*.

1.1.2 Biosynthesis

The enzymology of the late steps in anthocyanin formation is still incomplete. The compounds involved are collectively named 'flavonoids'. More than 4000 known flavonoid structures are divided into 12 classes on the basis of the oxidation level of the central pyran ring. In the pivotal step of flavonoid biosynthesis, ordinarily 4-coumaroyl-coenzyme A, derived from L-phenylalanine in the 'general phenylpropanoid metabolism' (Hahlbrock and Grisebach, 1975), enters a stepwise condensation reaction with three molecules of malonyl-coenzyme A to form the C_{15} chalcone intermediate, the tetrahydroxychalcone (naringenin chalcone). In the following well known 'flavonoidal metabolism', the

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actual precursor for anthocyanin formation, the flavan-3,4-*cis*-diol (leucoanthocyanidin), is formed, which then appears to be converted to the anthocyanidin flavylium cation by a hydroxylation at C-2 followed by two dehydrations. The enzymic conversion of leucoanthocyanidins to anthocyanidins, however, has not yet been demonstrated, and nor has it been for the analogous reaction with the flavan-4-ol leading to 3-desoxyanthocyanidins (Forkmann, 1991, this volume).

1.1.3 Function

Anthocyanins play a definite role in the attraction of animals as pollination and seed dispersal factors, and hence they are of considerable value in the coevolution of these plant–animal interactions. Thus, anthocyanin patterns in a given plant family might be more closely correlated with the pollinator class than with taxonomy, as has recently been discussed in the case of floral anthocyanins among 87 *Penstemon* species (Scrophulariaceae) (Scogin and Freeman, 1987). In particular, the aglycones may be related to pollination ecology, since they constitute the chemical basis of flower colour in angiosperms along with other colour-modifying factors (Harborne, 1988a; Brouillard and Dangles, this volume). Scogin (1988) found, in a survey of anthocyanidins of 146 species of bird-visited flowers, that a 'bird-visitation pigment syndrome' was generally uniform across wide geographical distances. The pigment syndromes of perching and hovering birds were distinct. There was no evidence for pelargonidin enrichment in tropical floras, as suggested by Harborne (1976), at least for hummingbird-visited flowers. The author discusses, from advancement indices and pigment frequencies, that floral adaptation for bird visitation may not be accompanied by great evolutionary advancement in pigment composition. In another survey on anthocyanins of the genus *Erythrina* (Fabaceae), no correlation between floral pigments and class of avian pollinators could be detected (Scogin, 1991).

In contrast to flower pigmentation, the transient appearance of anthocyanins in certain seedcoats, seedlings, leaves, stems and roots does not find an easy explanation. Several speculations on the perception or filtration of light and response to stress factors, including microbial attack, await further thorough studies. Nicholson and coworkers (Nicholson *et al.*, 1987, 1988; Hipskind *et al.*, 1990) found the 3-desoxyanthocyanidins apigeninidin and luteolinidin, as well as the caffeic acid ester of apigeninidin 5-arabinoside, as phytoalexins produced in response to microbial infection. Anthocyanins may also be important factors – with other flavonoids (Harborne, 1988a) – in the resistance of plants in insect attack. Cyanidin 3-glucoside was shown to protect cotton leaves against the feeding of tobacco budworm (Hedin *et al.*, 1983). Several physiological functions for anthocyanins in the general metabolism of plants described in the literature (McClure, 1975; Hrazdina, 1982) are still rather obscure.

1.1.4 Application

Apparently harmless to health, anthocyanins have considerable potential in the food industry as safe and effective food additives (Markakis, 1982). Their annual world production has been estimated to reach 10 000 tons from grapes alone (Timberlake, 1980). Compared to the synthetic colorants, however, anthocyanins have not been extensively used because of their instability towards a variety of chemical and physical factors (Markakis, 1982; Timberlake and Henry, 1986). The increasing number of new polyacylated anthocyanins, displaying marked stability, may prove to be of particular importance for food technology. There will be, however, limitations in the supply of the respective plant materials. Future successful biotechnological processes using cell cultures for the production of these stable anthocyanins is promising, since it can be assumed that such cultures retain the capacity for production of their '*in vivo*'-specific anthocyanins (Seitz and Hinderer, 1988). There are several reports over the last five years supporting this assumption.

1.1.5 Research interests

Anthocyanins still evoke a challenge to the imagination of (bio)chemists (Harborne and Grayer, 1988), who continue to investigate the final steps of anthocyanidin biosynthesis and the mechanisms of anthocyanin deposition. The relationship between biosynthesis and the occurrence of 'anthocyanoplasts' should also be considered.

Anthocyanins played an important role in the classical work of plant genetic studies (Alston, 1964). And they are still important markers for geneticists, as discussed for example in a review on the inheritance of anthocyanin pigmentation in the cultivated *Solanum tuberosum* (De Jong, 1991). Anthocyanins are also of increasing interest to molecular geneticists and plant breeders in the fascinating field of modern molecular biology. A series of regulatory genes acting upon the structural genes of enzymes involved in anthocyanin biosynthesis have been identified (Dooner *et al.*, 1991). The techniques of gene transfer have allowed manipulation of flower colour, e.g. the noted experiments with *Petunia* (Forkmann, 1991). However, there are numerous limitations, the most crucial being the phenomenon of variegation and unstable expression of the newly introduced genes (Forkmann, 1991), and considerable effort is still required to elucidate the mechanism of gene expression in plants.

There is also a growing interest in anthocyanin-

producing cell cultures (Seitz and Hinderer, 1988), e.g. cultures from *Daucus* (Dougall and Vogelien, 1990; Hopp and Seitz, 1987; Ozeki and Komamine, 1986; Ozeki *et al.*, 1987, 1989; Takeda, 1988, 1990; Vogelien *et al.*, 1990; Zwayyed *et al.*, 1991), *Catharanthus* (Hall and Yeoman, 1986, 1987), *Centaurea* (Kakegawa *et al.*, 1987, 1991; Takahashi *et al.*, 1991), *Euphorbia* (Bahadur and Reddy, 1987), *Perilla* (Zhong *et al.*, 1991; further cell-culture references are also found here), *Petunia* (Hagendoorn *et al.*, 1991) and *Vitis* (Hirasuna *et al.*, 1991; Table 1.3). In general, 'it is without question that plant cell cultures have become a central, indispensable vehicle in secondary metabolic research' (Zenk, 1991).

1.1.6 Recent advances

The classical techniques in anthocyanin analysis are still important (Strack and Wray, 1989). However, methodological advances have been considerable within the review period (1986–91, including some work from 1992). They include further developments of sophisticated nuclear magnetic resonance (NMR) techniques, in particular the use of two-dimensional (2D) homonuclear and heteronuclear correlation techniques, mass spectrometry (MS), where the exploitation of the newer ionization techniques of fast atom bombardment and ion spray have been particularly successful, and its on-line application in high-performance liquid chromatography (HPLC).

The objective of the following sections is to summarize the recent advances in analytical procedures, in structure elucidation and in work on anthocyanin occurrence. In addition, some selected earlier classic work will be cited in connection with recent problems.

1.2 ANALYTICAL PROCEDURES

1.2.1 Extraction and chromatography

(a) *Extraction and stability*

Increasing numbers of reports on labile highly acylated anthocyanins, especially those with aliphatic acids, make it necessary to perform mild pigment extractions with methanol or ethanol containing weak acids such as acetic (Harborne and Boardley, 1985), tartaric (Philip, 1974) or citric (Main *et al.*, 1978; Strack *et al.*, 1986) instead of HCl, the most widely used acidic component in earlier work. Also, small amounts of more volatile stronger acids, e.g. 0.5–3% trifluoroacetic acid (TFA) for extractions of the most complex polyacylated anthocyanins found by Goto and coworkers, are applicable. TFA can easily be removed during pigment concentration. Generally this should be performed with care to avoid acid-dependent pigment degradation, and it is recommended that the genuine structures be confirmed after concentration procedures and isolation of individual compounds by cochromatography with the crude extracts.

In short preparative extractions, the addition of an acidic component is not always necessary. However, for quantitative analysis, the extraction method should be thoroughly checked for the particular plant material and the particular pigments. In each case, the extraction procedure for structure elucidation or for analytical/quantitative purposes has to be optimized and adapted to the problem under consideration, including awareness of possible artifactual results (Strack and Wray, 1989). Factors affecting anthocyanin stability during extraction and purification, such as pH, temperature, oxygen, light, enzymes, nucleophilic agents, sugar derivatives and copigments, have recently been discussed by Jackman and Smith (1993).

(b) *High-performance liquid chromatography*

Classical chromatographic techniques, such as layer chromatography (paper chromatography (PC) and thin-layer chromatography (TLC)) and open column chromatography (CC), which still play a major role in analytical analyses of anthocyanin patterns and isolation procedures, have recently been reviewed by us (Strack and Wray, 1989).

HPLC of anthocyanins in both analytical/quantitative and (semi)preparative applications is now standard. Apart from the improved resolution of anthocyanin patterns compared with other separation methods, the simultaneous rapid monitoring of qualitative and quantitative data is its own recommendation in a wide range of applications, e.g. identification of cultivars (HPLC fingerprints) or in biochemical work on anthocyanin metabolism.

There is no HPLC system that can solve all problems. In each case the solvents and gradient profiles, especially when dealing with complex natural mixtures with a wide range of anthocyanin polarity, should be optimized (Strack and Wray, 1989). Goiffon *et al.* (1991) have studied the various parameters affecting the retention of anthocyanins on a reversed-phase column (C_{18}). They were able to establish rules governing the chromatographic behaviour of anthocyanins that allow the prediction of non-overlapping elutions.

From the vast number of published HPLC data, some essential features are noteworthy (compare Harborne and Grayer, 1988; Strack and Wray, 1989). The overall polarity and the stereochemistry of the anthocyanins are the key factors for separation on the most popular reversed-phase materials (C_{18}-derivatized silica column

supports). The following separation factors are well documented.

(i) *Substitution (hydroxyl and methoxyl groups) of the anthocyanidin.* B-ring substitutions of the common structures give the elution order delphinidin < cyanidin < petunidin < pelargonidin< peonidin < malvidin; hydroxyl groups increase, while methoxyl groups decrease mobility. Depending on the solvent system used, cyanidin/petunidin and peonidin/malvidin are critical pairs to resolve.

(ii) *Nature, position and number of sugars attached to the anthocyanidins.* In general, glycosylation increases mobility in the order 3,7-diglycosides < 3,5-diglycosides < 3-glycosides (compare HPLC of cyanidin diglucosides from orchid petals; Strack *et al.*, 1989). This is not a strict rule, since the nature of the sugars can markedly affect pigment mobility, e.g. 3-galactosides elute earlier than 3-glucosides followed by the 3-rutinosides. That HPLC successfully separates 3-glucosides and 3-galactosides, which is difficult to achieve by PC or TLC, has made it possible to correct some earlier misidentifications (Sakata *et al.*, 1986).

(iii) *Sugar acylation with phenolic acids (hydroxycinnamic acids or hydroxybenzoic acids) or aliphatic acids (e.g. malonic acid).* Acylation increases retention time. The polarities of these acids determine the order of elution. Thus a caffeoyl conjugate elutes earlier than a 4-coumaroyl one. A malyl conjugate should elute earlier than the corresponding malonylated pigment. And, again, this is not a strict rule. Pigment conformation, e.g. intramolecular copigmentation, has a strong effect on retention times. This has recently been demonstrated with hydroxycinnamic acid-acylated anthocyanins from *Daucus carota* (Glässgen *et al.*, 1992a). The unusual elution sequence sinapoyl < feruloyl < 4-coumaroyl trisaccharide of cyanidin observed by these workers must be ascribed to intramolecular copigmentation.

HPLC coupled with a new sophisticated detection system is shown in applications of photodiode-array detection of anthocyanins (Andersen, 1985; Hebrero *et al.*, 1988, 1989; Hong and Wrolstad, 1990). This has been one of the most important advances in HPLC in the last ten years. Using this method, the sample is scanned every few milliseconds, generating ultraviolet/visible (UV/Vis.) spectral data and calculating the absorbance maximum. In addition, the purity of each peak can be examined, providing different spectra for the possible impurity and the pure component. HPLC–photodiode-array detection has recently been successfully used in analyses of the anthocyanins from cell suspension cultures of *Daucus carota* (Glässgen *et al.*, 1992a), applying the classical strategy of UV/Vis. spectroscopic interpretations (Strack and Wray, 1989).

Another recent methodological development is the direct coupling of HPLC to a mass spectrometer (HPLC–ion spray mass spectrometry, see below). Loading samples up to 5 μl with flow rates up to 200 μl min^{-1} on narrow-bore reversed-phase columns (e.g. 100 × 2 mm) and using ion spray for interfacing HPLC with MS gave excellent results for the structure determination of anthocyanin mixtures (Glässgen *et al.*, 1992b).

1.2.2 General identification and miscellaneous methods

Non-spectroscopic methods such as hydrolysis and HPLC are valuable tools (Strack and Wray, 1989), which are even applicable when dealing with highly complex pigments (Shi *et al.*, 1992a). These authors identified a cyanidin triglucoside with three molecules of ferulic acid and an extra terminal glucose; the ratios of acyl groups to cyanidin were determined by quantitative HPLC using internal standards. HPLC analysis of polyacylated (hydroxycinnamic acids) anthocyanins with a photodiode-array detector enables the determination of the ratios of the absorbance between the maxima of the UV and visible regions and thus allows the estimation of the number of acyl groups of individual pigments from the chromatogram (Idaka *et al.*, 1987a).

Although few new data on methods other than NMR spectroscopy and MS have become available in the review period, the use of infrared (IR) spectroscopy, resonance Raman (RR) spectrometry and circular dichroism (CD) absorption spectroscopy has been reviewed (IR, RR, CD – Strack and Wray, 1989; RR – Merlin *et al.*, 1987; CD – Goto *et al.*, 1986; Goto, 1987). CD spectra have been obtained from living flower petals and offer an insight into details of the association and conformational properties of the anthocyanin pigments *in situ* (Hoshino, 1986). Isotachophoresis has been applied for the analysis of anthocyanins with very similar structures (Hiraoka and Yoshitama, 1986; Tsuda and Fukuba, 1989). Merlin (1990) reports on developments of IR and RR from improvements in Fourier-transform spectrometers.

1.2.3 Spectroscopic methods

(a) *Nuclear magnetic resonance spectroscopy*

The continuing rapid developments in instrumentation, in particular those involving nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), over the last five years, have given considerable impetus to structural elucidation in all fields of natural product chemistry. For the anthocyanins this has meant that structures as large and complex as the heavenly blue anthocyanin from *Ipomoea tricolor* or ternatin A1 from *Clitoria ternatea* (see (*1.6*)) have been determined. In general, such determinations do not require either

lengthy derivatizations or degradations, although some of the classic techniques allow ready structure confirmation.

We have reviewed elsewhere the physical techniques currently available for structural elucidations, and emphasis was placed on the type of information available from the various types of spectroscopy (Strack and Wray, 1989). Here we consider the more recent advances. Modern one- and two-dimensional (1D and 2D) NMR spectroscopy continues to be the most powerful method for structural elucidation in solution. The increasing availability of high-field superconducting magnets (up to 14 T; T = tesla) allows detection of ever-decreasing amounts of compounds, and, providing pure samples can be obtained, the unambiguous structures of compounds in the low milligram range can be successfully investigated. Spectra from 1D and 2D ^{1}H homonuclear correlation spectroscopy (COSY) are now routinely used for characterization of spin systems and, in conjunction with the newer 2D total correlation spectroscopy (TOCSY), also known as homonuclear Hartmann–Hahn spectroscopy (HOHAHA) (Summers *et al.*, 1986), allow ready identification of the number and nature (substitution pattern, configuration and/or conformation) of sugar moieties and the aromatic system of substituents and aglycones (Van Calsteren *et al.*, 1991). Often the ^{1}H shift assignments can be confirmed by the observation of characteristic long-range couplings in the 2D COSY spectrum; in particular, H-6 can be distinguished from H-8 as the latter shows a small coupling to H-4 (Glässgen *et al.*, 1992a). Substituent positions on the aglycones and sequential fragment information have been obtained in numerous cases by the use of 1D ^{1}H nuclear Overhauser difference spectroscopy (Kondo *et al.*, 1987; Strack and Wray, 1989) or from 2D nuclear Overhauser enhancement spectroscopy (NOESY) (Terahara *et al.*, 1990c).

More recently, in those cases where larger amounts of compound were available, 1D ^{13}C and 2D heteronuclear correlation spectroscopy have been used to gain such information. The ^{13}C shifts belonging to protonated carbons have been assigned by 2D correlation with the ^{1}H shifts via $^{1}J(CH)$ using ^{13}C detection (Andersen *et al.*, 1991a, b) or the more sensitive inverse ^{1}H detection (Van Calsteren *et al.*, 1991). Subsequent long-range correlations via $^{2}J(CH)$ and $^{3}J(CH)$, again via ^{13}C detection (COLOC; e.g. Andersen *et al.*, 1991a) or the more satisfactory ^{1}H detection (Van Calsteren *et al.*, 1991), usually provide an overdetermined system of correlations from which the unambiguous structure can be deduced. As a consequence of these and other investigations, a considerable number of valuable ^{13}C shift data for various substituted anthocyanidins are now available, and these are summarized in Table 1.1.

Fast interconversion of the quinonoidal bases and carbinol pseudobases, via the flavylium cation, with subsequent ring opening to give the *Z*- and *E*-chalcone pseudobases, was demonstrated by ^{1}H NMR spectroscopy for malvidin 3-glucoside (Cheminat and Brouillard, 1986; Mistry *et al.*, 1991) and 3,5-diglucoside (Santos *et al.*, 1992). Variation of the ^{1}H chemical shifts of malvidin 3,5-diglucoside with pH and concentration has been used to study the self-association of the quinonoidal bases (Hoshino, 1991). The observation of negative nuclear Overhauser effects (NOEs) for H-4 and H-6 at room temperature upon irradiation of the anomeric protons, and normal positive NOEs at 60 °C was ascribed to destacking of the anthocyanin with subsequent increased tumbling at the higher temperature. A similar phenomenon has been observed in 2D NOESY spectra (Nerdal and Andersen, 1991). Although various models appear to be compatible with the NMR chemical shift and CD spectroscopic data, recent quantitative 2D NOESY data, used as distance constraints in a distance geometry algorithm, indicate a head-to-tail model (Nerdal and Andersen, 1991) in contrast to previously proposed models (Goto *et al.*, 1986; Goto, 1987). Direct evidence of intramolecular stacking has been afforded by the observation of long-range NOEs between the anthocyanidin and aromatic acid moieties in diacylated (Goto *et al.*, 1986) and monoacylated compounds (Glässgen *et al.*, 1992a; Yoshida *et al.*, 1991a). ^{1}H chemical shift changes and UV/Vis. spectroscopy have been used to investigate a comprehensive number of intermolecular copigmentation interactions between anthocyanins and a variety of natural phenolics, caffeine, theophylline, adenosine 5′-triphosphate (ATP), deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Mistry *et al.*, 1991), and the self-association of anthocyanidin 3,5-diglucosides (Hoshino, 1992). Further details of these processes will be found in Chapter 13.

(b) *Mass spectrometry*

Fast atom bombardment mass spectrometry (FAB-MS) is currently the method of choice for mass determination of the anthocyanins (for review see Strack *et al.*, 1989). It is particularly useful in those situations where the NMR method encounters characterization difficulties with substituents such as malonyl and oxalyl groups (Strack *et al.*, 1986). The recent introduction of the alternative ion spray (aerospray) (Bruins *et al.*, 1987) soft-ionization technique (IS-MS) also produces $[M]^{+}$ ions in high abundance directly from solutions of anthocyanins, which allows rapid determination of the molecular mass of the pigment. In combination with tandem MS (MS/MS), it allows a fast and convenient method for the determination of the anthocyanin aglycone; and additionally, combined with liquid chromatography (HPLC), provides a rapid and sensitive method for the identification of a series of anthocyanins

Table 1.1 Representative ^{13}C chemical shifts*† of the anthocyanidin moieties of anthocyanins 1–8 in two mixed solvents: deuterated dimethylsulfoxide (DMSO-d_6)/HCl (i) and CD_3OD/CF_3CO_2H (ii)

Cpd/solv.	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1/(i)	161.3	143.7	134.3	157.6	102.7	169.0	94.0	156.0
2/(i)	161.4	144.1	134.7	157.7	102.5	168.6	94.1	155.7
2/(ii)	164.36	145.64	137.03	159.55*	103.50	170.56	95.19	157.75*
3/(ii)	164.81	145.82	135.8	156.83*	106.03	169.62	97.50	156.90*
4/(i)	161.1	144.1	134.4	157.6	102.5	168.4	94.0	155.7
5/(ii)	164.19	145.49	137.35	159.29	103.42	170.7	95.24	157.86
6/(ii)	163.79	146.09†	134.08	156.89*	105.54	169.73	97.55	156.61*
7/(ii)	164.55	146.79	136.16	156.91	105.86	169.73	97.61	157.37
8/(ii)	163.29	145.74	138.22	158.12	103.66	171.3	95.76	159.46

Compounds 1–8 are shown below

Cpd	*C-3*	*C-5*	*C-7*	*C-3'*	*C-4'*	*C-5'*
1	O–Sugar	OH	OH	H	OH	H
2	O–Sugar	OH	OH	OH	OH	H
3	O–Sugar	O–Sugar	OH	OH	OH	H
4	O–Sugar	OH	OH	OH	OH	OH
5	O–Sugar	OH	OH	OMe	OH	H
6	O–Sugar	O–Sugar	OH	OMe	OH	OH
7	O–Sugar	O–Sugar	OH	OMe	OH	OMe
8	O–Sugar	OH	OH	O–Sugar	OH	O–Sugar

*†Assignment of signals in row interchangeable.
‡1, Andersen (1988b); 2, Andersen, O.M., Aksnes, D.W., Nerdal, W. and Johansen, O.-P. (private communication); 3, Van Calsteren, M.-R., Cormier, F., Do, C.B. and Laing, R.R. (private communication); 4, Strack *et al.* (1986); 5, Johansen *et al.* (1991); 6, Andersen, O.M., Opheim, S., Aksnes, D.W. and Froystein, N.A. (private communication); 7, Terahara *et al.* (1990c).

in complex mixtures (Glässgen *et al.*, 1992a, b). Ion spray techniques appear to be ideal for thermolabile compounds such as anthocyanins – and also for the analogous pigments, the betalains in members of the Caryophyllales (Heuer *et al.*, 1992). The alternatives of electrospray (Whitehouse *et al.*, 1985) and thermospray (Blakeley and Vestal, 1983) are also of interest in analysis of natural products.

Two MS/MS experiments are useful in IS-MS situations. A daughter-ion spectrum of each individual peak in the spectrum is recorded to identify the anthocyanin peaks in the spectrum by the detection of $[anthocyanidin]^+$ fragments; and parent-ion spectra of the $[anthocyanidin]^+$ are recorded to detect selectively all anthocyanins containing this aglycone. These methods have been successfully used to identify a series of cyanidin-containing anthocyanins from *Daucus carota* (Glässgen *et al.*, 1992b) and two gallic acid-containing anthocyanins from *Victoria amazonica* (Strack *et al.*, 1992). Tandem FAB-MS of anthocyanins with crude extracts from cell cultures of *Vitis vinifera* (Laing and Cormier, 1990) has been employed for the identification of anthocyanins (Table 1.3) without chromatographic purification of the individual components.

1.3 CHEMISTRY

1.3.1 General aspects

Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins, which are polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium (flavylium cation). They belong to the phenolic class of flavonoids with the typical A-ring benzoyl and B-ring hydroxycinnamoyl systems, with the carbon numbering system shown in the structures of Table 1.2. There are almost 300 naturally occurring structures.

Besides the basic flavylium cation, the 'primary structure', anthocyanins occur in aqueous acidic solution as 'secondary structures', a mixture of the quinonoidal base(s), the carbinol pseudobase and the chalcone pseudobase (Brouillard, 1982). In addition, there are four possible stabilization mechanisms leading

10	*1′*	*2′*	*3′*	*4′*	*5′*	*6′*	*Ref.‡*
111.7	119.2	134.4	117.0	164.3	117.0	134.4	1
111.8	119.5	117.4*	146.2	154.4	116.8*	126.7	1
113.45	121.31	118.56	147.41	155.78	117.48	128.22	2, 3, 4
113.13	121.13	118.71	147.78	156.73*	117.66	129.71	5
111.7	118.3	111.2	146.5	143.1	146.5	111.2	1
113.63	121.14	115.19	149.51	156.37	117.55	128.84	3
113.03	119.58	109.52	149.76†	147.55†	146.16†	114.03	6
113.60	119.66	111.07	149.86	147.19	149.86	111.07	3
114.33	120.27	117.30	147.69	147.19	147.69	117.30	7

Compound

Pelargonidin 3-*O*-β-D-glucopyranoside
Cyanidin 3-*O*-β-D-glucopyranoside
Cyanidin 3-*O*-β-[6-*O*-(*E*-4-coumaroyl)-2-(β-D-xylopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside
Delphinidin 3-*O*-β-D-glucopyranoside
Peonidin 3-*O*-β-D-glucopyranoside
Petunidin 3-*O*-β-[6-*O*-(4-*O*-*E*-4-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside
Malvidin 3,5-*O*-di-β-D-glucopyranoside
Delphinidin 3,3′,5′-tri-*O*-β-D-glucopyranoside

to 'tertiary structures', such as self-association, inter- and intramolecular copigmentation, and metal complex formation. Copigmentation is probably the most efficient protection mechanism, avoiding nucleophilic attack of the quinonoidal structures by water in the slightly acidic medium of most vacuoles.

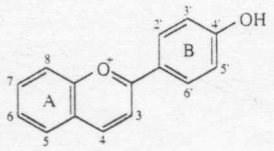
In addition to the 17 anthocyanidins listed previously (Harborne and Grayer, 1988), one new 6-hydroxyl derivative has been found. Besides the known 6-hydroxypelargonidin (aurantinidin) and 6-hydroxycyanidin, the delphinidin analogue has been shown to occur (Saito *et al.*, 1988a). In an investigation of various cultivars of *Alstroemeria* species, it was found that 6-hydroxydelphinidin 3-rutinoside co-occurs only in pink-purple coloured flower petals with the 3-rutinosides of delphinidin, cyanidin and 6-hydroxycyanidin. According to the anthocyanin types present, two other groups of cultivars could be defined. These are one group composed of cyanidin and 6-hydroxycyanidin glycosides (ivory to orange-red in colour) and a second composed of cyanidin and delphinidin glycosides (crimson-pink).

The different colour variations of anthocyanins are only partly due to their substitution patterns. The basic chromophores are the scarlet pelargonidin, the crimson cyanidin and the purple delphinidin (Harborne, 1988b). In an HPLC screening of anthocyanins in varieties of *Iris ensata*, Yabuya (1991) found that the formation of purple, red-purple, blue-purple, light purple and pink flowers depends upon the contributions of malvidin, petunidin and delphinidin to the major anthocyanins. Recent results on the possible *in situ* structures of anthocyanins derived from stabilization mechanisms such as self-association, inter- or intramolecular copigmentation, and complex metallo structures give a more realistic view of the contribution of anthocyanin structure to colour (Goto and Kondo, 1991). Lu *et al.* (1992a) showed that the flower colour of *Pharbitis nil* gradually shifts to the blue region with increasing numbers of caffeic acid residues in polyacylated pelargonidin glycosides.

1.3.2 Glycosides

Anthocyanins occur as 3-monosides, 3-biosides and 3-triosides as well as 3,5-diglycosides and more rarely 3,7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose and xylose. Glycosides containing the latter two pentoses have now been described in about a dozen new reports (Table 1.3). The complex substitutions of the polyacylated lobelinins (see (*1.5*)) and ternatins (see (*1.6*)) are noteworthy. A few more reports of the more rarely found substitution at the 7-hydroxyl have appeared from members of the Commelinaceae and Orchidaceae (Table 1.3).

Table 1.2 Structures of naturally occurring anthocyanidins



(*1.1*)

Anthocyanidin	*Substitution pattern**					
	3	*5*	*6*	*7*	*3'*	*5'*
Common basic structures						
Pelargonidin (Pg)†	OH	OH	H	OH	H	H
Cyanidin (Cy)	OH	OH	H	OH	OH	H
Delphinidin (Dp)	OH	OH	H	OH	OH	OH
Common methylated structures						
Peonidin (Pn)	OH	OH	H	OH	OMe	H
Petunidin (Pt)	OH	OH	H	OH	OMe	OH
Malvidin (Mv)	OH	OH	H	OH	OMe	OMe
Rare 3-desoxy structures						
Apigeninidin (Ap)	H	OH	H	OH	H	H
Luteolinidin (Lt)	H	OH	H	OH	OH	H
Tricetinidin (Tr)	H	OH	H	OH	OH	OH
Rare hydroxylated structures						
Aurantinidin (Au)	OH	OH	OH	OH	H	H
6-Hydroxy-Cy (6OHCy)	OH	OH	OH	OH	OH	H
6-Hydroxy-Dp (6OHDp)	OH	OH	OH	OH	OH	OH
Rare methylated structures						
5-Methyl-Cy (5MCy)	OH	OMe	H	OH	OH	H
Rosinidin (Rs)	OH	OH	H	OMe	OMe	H
Pulchellidin (Pl)	OH	OMe	H	OH	OH	OH
Europinidin (Eu)	OH	OMe	H	OH	OMe	OH
Hirsutidin (Hs)	OH	OH	H	OMe	OMe	OMe
Capensinidin (Cp)	OH	OMe	H	OH	OMe	OMe

*Numbering according to the anthocyanidin carbon numbering system in (*1.1*).
†Abbreviation (used in Table 1.3).

A new monoglycoside, cyanidin 3-xyloside, has been found in fruits of *Aronia melanocarpa* (Rosaceae) and co-occurs with the major components, cyanidin 3-galactoside and 3-arabinoside (Oszmianski and Sapis, 1988). Cyanidin 3-xyloside has also been found recently in the fruits of apple (Mazza and Velioglu, 1992). In the anthocyanins, rhamnoglucosides usually occur as the rutinoside (rhamnosyl-1,6-glucoside); however, Andersen (1988b, 1989b) reported the neohesperidoside (rhamnosyl-1,2-glucoside) as a new disaccharide found in pigments of the receptacles of members of the Podocarpaceae.

There is one new report on the occurrence of rare glucuronosyl derivatives, namely cyanidin 3-malonylglucuronosylglucoside in *Bellis perennis* (Saito *et al.*, 1988b). This pigment, along with cyanidin 3-malonylglucoside, is more stable in neutral solution than cyanidin 3-glucoside, but less stable than cyanidin 3-glucuronylglucoside.

1.3.3 Acylglycosides

Recent advances in analytical procedures (Strack and Wray, 1989) have resulted in numerous reports on anthocyanins variously acylated with hydroxycinnamic acids, hydroxybenzoic acids, acetic acid and some aliphatic dicarboxylic acids, such as malonic, malic, oxalic and succinic acids (Harborne and Grayer, 1988).