

Recent Advances in Polyphenol Research

VOLUME 2

Edited by
Celestino Santos-Buelga,
Maria Teresa Escribano-Bailon
and Vincenzo Lattanzio

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Contents

Contributors

Preface

1 The Visible Flavonoids or Anthocyanins: From Research to Applications

1.1 Introduction

1.2 Copigmentation of anthocyanins

1.3 Formation of inclusion complexes

1.4 Ion-pair formation

1.5 Metalloanthocyanins

1.6 Z-Chalcones: unexpected open cavities for the ferric cation

1.7 Anthocyanin biological activity

1.8 Some thoughts on applications

1.9 References

2 Flavonoid Chemistry of the Leguminosae

2.1 Introduction

2.2 Flavonoid structures in the Leguminosae: trends and distribution

2.3 Advances in analytical methodology applied to Leguminosae flavonoids

2.4 Leguminosae flavonoids and chemosystematics

2.5 Concluding remarks

2.6 Acknowledgments

2.7 References

3 Updating Wine Pigments

3.1 General overview

3.2 Factors that affect wine color intensity and stability

3.3 Chemical transformations of flavonoids

3.4 Final remarks

3.5 Acknowledgments

3.6 References

4 Ellagitannins - An Underestimated Class of Plant Polyphenols: Chemical Reactivity of C-Glucosidic Ellagitannins in Relation to Wine Chemistry and Biological Activity

4.1 Ellagitannins: an underestimated class of bioactive plant polyphenols

4.2 C-Glucosidic ellagitannins: a special subclass of ellagitannins

4.3 Implications of C-glucosidic ellagitannins in wine chemistry

4.4 Biological activity of C-glucosidic ellagitannins

4.5 Conclusion

4.6 Acknowledgments

4.7 References

5 Strategies to Optimize the Flavonoid Content of Tomato Fruit

5.1 Introduction

5.2 The metabolic route to flavonoids in tomato fruit

5.3 The natural biodiversity of flavonoids in tomato

5.4 Metabolic engineering of the flavonoid pathway

5.5 Metabolomics-assisted breeding

5.6 Conclusions and future prospects

5.7 Acknowledgments

5.8 References

6 Biological Activity of Phenolics in Plant Cells

6.1 Introduction

6.2 Synthesis and transports

6.3 Phenolics interact with plasmalemma components

6.4 Phenolics in apoplast

6.5 Phenolics in hyaloplasm

6.6 Phenolics in vacuoles

6.7 Phenolics in mitochondria and chloroplasts

6.8 Phenolics have many emergent roles within the nucleus

6.9 Conclusion

6.10 References

7 Muriel Wheldale Onslow and the Rediscovery of Anthocyanin Function in Plants

7.1 Introduction

7.2 Functional hypotheses for anthocyanins in vegetative tissues

7.3 A modern spin on some old ideas

7.4 Concluding remarks

7.5 Acknowledgments

7.6 References

8 Plant Phenolic Compounds Controlling Leaf Movement

8.1 Introduction

8.2 Endogenous bioactive substances controlling nyctinasty

8.3 The chemical mechanism of rhythm in nyctinasty

8.4 Bioorganic studies of nyctinasty using functionalized leaf-movement factors as molecular probes

8.5 References

9 Red Clover Derived Isoflavones: Metabolism and Physiological Effects in Cattle and Sheep and their Concentration in Milk Produced for Human Consumption

9.1 Introduction

9.2 Phytoestrogens in ruminant feeds

9.3 Red clover as a source of isoflavones

9.4 Metabolism of isoflavones in ruminants

9.5 Equol: the most important metabolite

9.6 Physiological effects and regulatory mechanisms of endogenous estrogens

9.7 Effects of phytoestrogens in sheep reproduction

9.8 Effects of phytoestrogens in cattle reproduction

9.9 Antioxidant capacity of isoflavones

9.10 New outlook

9.11 References

10 Polyphenols as Biomarkers in Nutrition Research: Resveratrol Metabolome a Useful Nutritional Marker of Moderate Wine Consumption

10.1 Introduction

10.2 Characteristics of nutritional biomarkers

10.3 Strengths and limitations of biological biomarkers over dietary estimation

10.4 Resveratrol: a useful biomarker of wine consumption

10.5 References

11 Translation of Chemical Properties of Polyphenols into Biological Activity with Impact on Human Health

11.1 Introduction

11.2 Polyphenols as antioxidants: the earlier notions

11.3 Beyond “global” antioxidation: alternate biological activities for polyphenols with impact on human health

11.4 References

12 Mitigation of Oxidative Stress and Inflammatory Signaling by Fruit and Walnut Polyphenols: Implications for Cognitive Aging

12.1 Introduction

12.2 Oxidative stress/inflammatory interactions

12.3 Nutritional interventions

12.4 References

13 Antiatherosclerotic Effects of Dietary Flavonoids: Insight into their

Molecular Action Mechanism at the Target Site

13.1 Introduction

13.2 Flavonoids in the diet and their antioxidant/prooxidant activity

13.3 Absorption and metabolism of dietary flavonoids in the digestive system

13.4 Oxidative LDL theory and antioxidant activity of flavonoids in plasma

13.5 Antioxidant and “beyond” antioxidant activity of flavonoids in the artery

13.6 Activated macrophages as potential targets of dietary flavonoids as antiatherosclerotic factors

13.7 Conclusion

13.8 References

Index

Recent Advances in Polyphenol Research

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A series for researchers and graduate students whose work is related to plant phenolics and polyphenols, as well as for individuals representing governments and industries with interest in this field. Each volume in this biennial series will focus on several important research topics in plant phenols and polyphenols, including chemistry, biosynthesis, metabolic engineering, ecology, physiology, food, nutrition, and health.

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Celestino Santos-Buelga, Maria Teresa Escribano-Bailon, and Vincenzo Lattanzio

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Recent Advances in Polyphenol Research

Volume 2

Edited by

Celestino Santos-Buelga

Professor, Food Chemistry

Department of Analytical Chemistry, Nutrition and Food Science

Faculty of Pharmacy

University of Salamanca, Spain

Maria Teresa Escribano-Bailon

Lecturer, Food Technology

Area of Food Technology

Technical School of 'Zamora'

University of Salamanca, Spain

Vincenzo Lattanzio

Professor, Plant Biochemistry and Physiology

*Department of Agro-Environmental Sciences, Chemistry
and Plant Protection*

Faculty of Agricultural Sciences

University of Foggia, Italy

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Dedication

*To Edwin Haslam - a very good friend of Groupe Polyphénols
- whose studies of plant polyphenols (vegetable tannins)
were "seminal" in the development of this area of science.*

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Contributors

Cristina Andres-Lacueva, *Nutrition and Food Science Department, XaRTA, INSA, Pharmacy Faculty, University of Barcelona, Av. Joan XXIII, s/n. 08028, Barcelona, Spain.*

Yves Baissac, *University of Montpellier 2, UMR-188 DIA-PC, Rhizogenesis team, Laboratory of Plant Biochemistry and Physiology, CC 024, Bât.15, Place Eugène Bataillon, F-34095 Montpellier cedex 05, France.*

Luc P.R. Bidel, *INRA, UMR-188 DIA-PC, Rhizogenesis group, University Montpellier 2, CC 024, Place E. Bataillon, F-34095 Montpellier cedex 05, France.*

Arnaud G. Bovy, *Plant Research International, P.O. Box 16, 6700AA Wageningen, The Netherlands and Centre for BioSystems Genomics (CBSG), P.O. Box 98, 6700PB, Wageningen, The Netherlands.*

Raymond Brouillard, *Laboratoire de Chimie des Polyphénols, Faculté de Chimie (CNRS-UMR 7177), Université Strasbourg 1, 4, rue Blaise Pascal, 67070 Strasbourg, France.*

Stefan Chassaing, *LSPCMIB-Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cedex 09, France.*

Marc Coumans, *University of Montpellier 2, UMR-188 DIA-PC, Rhizogenesis team, Laboratory of Plant Biochemistry and Physiology, CC 024, Bât.15, Place Eugène Bataillon, F-34095 Montpellier cedex 05, France.*

Patrick Dumas, *INRA, UMR-188 DIA-PC, Rhizogenesis group, Institut de Recherche pour le Développement (IRD), 911, Avenue Agropolis, F-34394 Montpellier cedex 05, France.*

Paulo Figueiredo, *Universidade Atlântica CEIDSS, Antiga Fábrica da Pólvora de Barcarena, Oeiras, 2730-036 Barcarena, Portugal.*

Victor A.P. de Freitas, *Chemistry Investigation Centre, Department of Chemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal.*

Yves Glories, *Institut des Sciences de la Vigne et du Vin, Bordeaux-Aquitaine (UMR-INRA 1219), Université de Bordeaux, 210 Chemin de Leysotte CS 50008, 33882 Villenave d'Ornon, France.*

Victoria Gómez-Roldán, *Plant Research International, P.O. Box 16, 6700AA Wageningen, The Netherlands and Netherlands Consortium for Systems Biology (NCSB), Kruislaan 318, 1098SM Amsterdam, The Netherlands.*

Kevin S. Gould, *School of Biological Sciences, Victoria University of Wellington, P.O. Box 600, Wellington, New Zealand.*

Robert D. Hall, *Plant Research International, P.O. Box 16, 6700AA Wageningen, The Netherlands, Centre for BioSystems Genomics (CBSG), P.O. Box 98, 6700PB, Wageningen, The Netherlands, and Netherlands Consortium for Systems Biology (NCSB), Kruislaan 318, 1098SM Amsterdam, The Netherlands.*

Géraldine Isorez, *Laboratoire de Chimie des Polyphénols, Faculté de Chimie (CNRS-UMR 7177), Université Strasbourg 1, 4, rue Blaise Pascal, 67070 Strasbourg, France.*

Christian Jay-Allemand, *University of Montpellier 2, UMR-188 DIA-PC, Rhizogenesis team, Laboratory of Plant Biochemistry and Physiology, CC 024, Bât.15, Place Eugène Bataillon. F-34095 Montpellier cedex 05, France.*

James A. Joseph, *USDA-ARS, Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111, USA.*

Michael Jourdes, *Institut des Sciences de la Vigne et du Vin, Bordeaux-Aquitaine (UMR-INRA 1219), Université de Bordeaux, 210 Chemin de Leysotte CS 50008, 33882 Villenave d'Ornon, France.*

Yoshichika Kawai, *Department of Food Science, Graduate School of Nutrition and Bioscience, the University of Tokushima, Kuramoto-cho 3 Tokushima, Japan.*

Lea Kontio, *Department of Chemistry, Organic Chemistry Laboratory, University of Helsinki, 00014 Helsinki, Finland.*

Marie Kueny-Stotz, *Laboratoire de Chimie des Polyphénols, Faculté de Chimie (CNRS-UMR 7177), Université Strasbourg 1, 4, rue Blaise Pascal, 67070 Strasbourg, France.*

João Laranjinha, *Center for Neurosciences and Cell Biology and Faculty of Pharmacy, University of Coimbra, Health Sciences Campus, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal.*

Dorothee Lefeuvre, *Université de Bordeaux, Institut des Sciences Moléculaires (CNRS-UMR 5255), Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, 33607 Pessac cedex, France.*

Nuno Mateus, *Chemistry Investigation Centre, Department of Chemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal.*

Kaeko Murota, *Department of Food Science, Graduate School of Nutrition and Bioscience, The University of Tokushima, Kuramoto-cho 3 Tokushima, Japan.*

Eeva A. Mustonen, *Department of Production Animal Medicine, University of Helsinki, Paroninkuja 20, 04920 Saarentaus, Finland.*

Yoko Nakamura, *Department of Chemistry, Faculty of Science, Tohoku University, Aramaki-aza Aoba, Aoba-ku, Sendai 980-8578, Japan.*

Patrick Pardon, *Université de Bordeaux, Institut des Sciences Moléculaires (CNRS-UMR 5255), Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, 33607 Pessac cedex, France.*

Stéphane Quideau, *Université de Bordeaux, Institut des Sciences Moléculaires (CNRS-UMR 5255), Institut Européen*

de Chimie et Biologie, 2 rue Robert Escarpit, 33607 Pessac cedex, France.

Ilkka Saastamoinen, *Department of Production Animal Medicine, University of Helsinki, Paroninkuja 20, 04920 Saarentaus, Finland.*

Hannu Saloniemi, *Department of Production Animal Medicine, University of Helsinki, Paroninkuja 20, 04920 Saarentaus, Finland.*

Cédric Saucier, *Institut des Sciences de la Vigne et du Vin, Bordeaux-Aquitaine (UMR-INRA 1219), Université de Bordeaux, 210 Chemin de Leysotte CS 50008, 33882 Villenave d'Ornon, France.*

Barbara Shukitt-Hale, *USDA-ARS, Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111, USA.*

Juhani Taponen, *Department of Production Animal Medicine, University of Helsinki, Paroninkuja 20, 04920 Saarentaus, Finland.*

Pierre-Louis Teissedre, *Institut des Sciences de la Vigne et du Vin, Bordeaux-Aquitaine (UMR-INRA 1219), Université de Bordeaux, 210 Chemin de Leysotte CS 50008, 33882 Villenave d'Ornon, France.*

Junji Terao, *Department of Food Science, Graduate School of Nutrition and Bioscience, The University of Tokushima, Kuramoto-cho 3 Tokushima, Japan.*

Minoru Ueda, *Department of Chemistry, Faculty of Science, Tohoku University, Aramaki-aza Aoba, Aoba-ku, Sendai 980-8578, Japan.*

Aila Vanhatalo, *Department of Animal Science, University of Helsinki, P.O. Box 28, 00014 Helsinki, Finland.*

Nigel C. Veitch, *Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK.*

Kristiina Wähälä, *Department of Chemistry, Organic Chemistry Laboratory, University of Helsinki, P.O. Box 55, 00014 Helsinki, Finland.*

Lauren M. Willis, *USDA-ARS, Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111, USA.*

Raul Zamora-Ros, *Nutrition and Food Science Department, XaRTA, INSA, Pharmacy Faculty, University of Barcelona, Av. Joan XXIII, s/n. 08028, Barcelona, Spain.*

Preface

Plant phenolics are secondary metabolites that constitute one of the most common and widespread group of substances in plants and that have been considered for a long time waste products of primary metabolism. Nowadays, plant phenols and polyphenols are considered to have a large and diverse array of beneficial effects on both plants and humans. The ability to synthesize secondary compounds has been selected throughout the course of evolution in different plant lineages when such compounds addressed specific needs. Secondary metabolites apparently act as defence (against herbivores, microbes, viruses, or competing plants) and signal compounds (to attract pollinating or seed-dispersing animals), as well as protect the plant from ultraviolet radiation and oxidants. Therefore, they represent adaptive characters that have been subjected to natural selection during evolution. In addition, biomedical research has revealed that dietary phenolics, because of their antioxidant and free radical scavenging properties, play important roles in the prevention of many of the major contemporary chronic diseases.

The diversity of structure and activity of phenolic compounds resulted in the multiplicity of research areas such as chemistry, biotechnology, ecology, physiology, nutrition, medicine, and cosmetics. The International Conference on Polyphenols, organized under the auspices of *Groupe Polyphénols*, is a unique opportunity for scientists in these and other fields to get together every other year and exchange their ideas and new findings.

The last edition of the conference (the 24th edition) was hosted by the University of Salamanca, Spain, from July 8 to 11, 2008, and covered five topics:

1. *Chemistry*: Structure, reactivity, physicochemical properties, analytical methods, synthesis

2. *Biosynthesis and metabolic engineering*: Molecular biology, omics, enzymology, gene expression and regulation, biotechnology

3. *Roles in Plant Ecophysiology and Environment*: Plant growth and development, biotic and abiotic stress, resistance, sustainable development, by-products valorization

4. *Food and Beverages*: Composition, organoleptic properties, impact of processing and storage, functional foods, nutraceuticals

5. *Health and Disease*: Medicinal properties, mode of action, bioavailability and metabolism, cosmetics

Some 450 participants from 41 countries attended Salamanca's Conference, where over 370 presentations were made, including 330 posters, 31 selected oral communications, and 12 invited lectures made by acknowledged experts. The present second volume in the series includes chapters from the guest speakers and some invited contributors.

The 24th International Conference on Polyphenols would not have been possible without the generous support of public and private donors such as the Spanish *Ministerio de Ciencia e Innovación*, *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA), *Junta de Castilla y León*, and *Caja Duero*. Furthermore, we are also indebted to the Nutraceutical Group, Indena, "Viñas del Jaro" wine cellars, and Phytolab that also sponsored the conference. Our sincere thanks to all of them.

Celestino Santos-Buelga,

Maria Teresa Escribano-Bailon,

Vincenzo Lattanzio

Chapter 1

The Visible Flavonoids or Anthocyanins: From Research to Applications

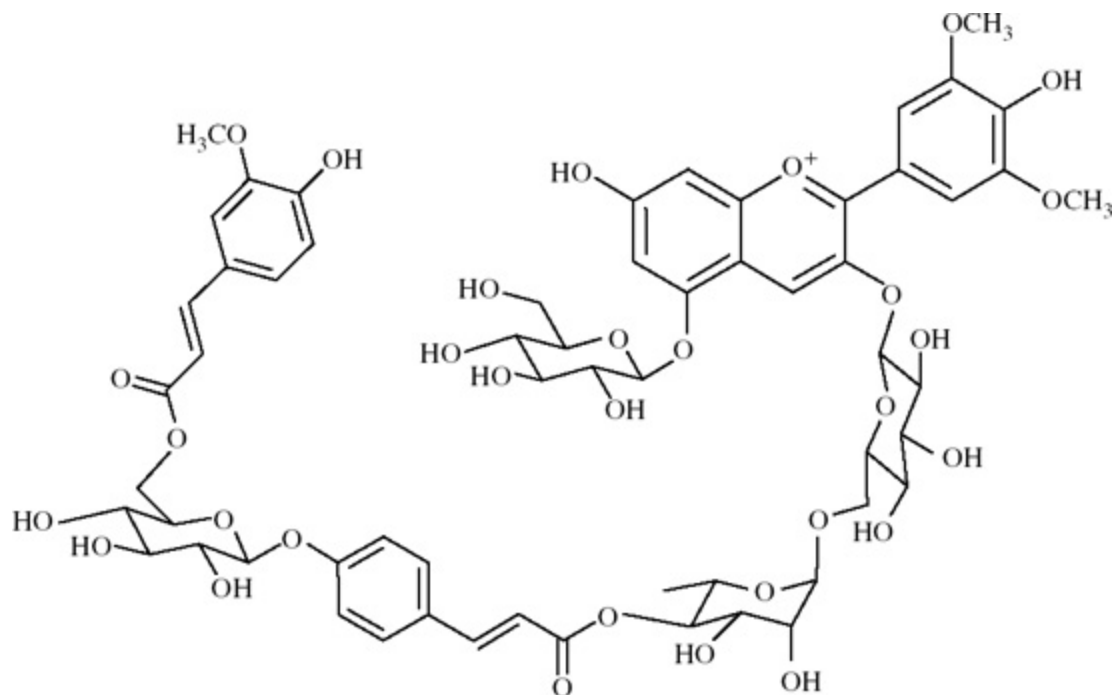
Raymond Brouillard, Stefan Chassaing, Géraldine Isorez, Marie Kueny-Stotz, and Paulo Figueiredo

1.1 Introduction

Anthocyanins are polyphenolic pigments responsible for most of the color diversity found in plants. Here the *in vivo* color expression and the stability of anthocyanins are interpreted by extrapolation of the results acquired *in vitro* with model solutions of pigments obtained through plant extraction or laboratorial synthesis. Behavior of anthocyanins is explained in terms of molecular interactions of the chromophore units with parts of the pigments themselves and/or with some constituents of the plant cell. These include, among others, diverse polyphenols, metal cations, and inorganic salts. Attention is also given to the biophysicochemical environment found in plant vacuoles that plays a fundamental role on the intermolecular and intramolecular associations displayed by anthocyanins. For example, anthocyanin Z-chalcones (retrochalcones) provide an unexpected open cavity for the ferric cation. Medicinal, nutritional, and industrial applications of anthocyanins are proposed.

Colors are conferred to plants by chlorophylls, carotenoids, and flavonoids (Britton, 1983). Chlorophylls are responsible for the green colors displayed by the leaves, whereas carotenoids provide some of the red-orange hues often found in fruits, flowers, and other plant constituents. Flavonoids belong to a larger family, the polyphenols, and can be found in most flowers and fruits (Brouillard & Dangles, 1993; Andersen & Jordheim, 2006). They include the principal elements responsible for the color diversity found in the plant world, the anthocyanins ([Fig. 1.1](#)). In fact, these pigments are the only polyphenols that possess the ability to absorb light both in the ultra-violet and in all the visible range (from yellow-orange to bluish-green) (Goto & Kondo, 1991). It is well known that anthocyanins are at the origin of plants' most brilliant colors, a phenomenon particularly visible from flowers. Nevertheless, there exists only one chromophore - the flavylum nucleus - whose subtle interactions with vacuole biochemicals, including water, are capable of providing all kind of colors.

[Fig. 1.1](#) Structure of one of the numerous anthocyanins isolated from violet petals of *Petunia hybrida* cv. Festival (Gonzalez *et al.*, 2001).



Anthocyanins are stored in an organized aqueous medium in the cell vacuoles. A slightly acidic environment (pH 3-5; Stewart *et al.*, 1975) rich in inorganic ions and other polyphenols is essential for the transformations in these pigments that enable the formation of molecular complexes and subsequent color changes and stabilization (Brouillard & Dangles, 1993). The basic structure common to almost all anthocyanins is a 2-phenylbenzopyrylium (flavylium) heterocyclic skeleton bearing at least one sugar residue. Aliphatic or aromatic organic acids may esterify the sugar hydroxyls. Furthermore, OH and OCH₃ groups that bestow the characteristic names of the six basic anthocyanic structures ([Table 1.1](#)) typically substitute the B-ring of the aglycone moiety of these pigments. The existence of at least one free OH group is needed to produce the structural changes, described later, conducing to color variation. The structure presented in [Fig. 1.2](#) depicts the positively charged flavylium cation, which is the dominant equilibrium form in strongly acidic aqueous solutions. The positive charge is delocalized through all the pyrylium moieties, although carbons 2 and 4 are the more positively charged atoms (Ami

6 *et al.*, 1990). The relative ease of deprotonation of the two OH groups at positions 4' and 7 contributes to the color changes of the anthocyanin. One of these hydroxyls loses a proton at pH ~ 4, producing the quinonoid bases AH (Fig. 1.3) that exhibit a chromatic deviation toward longer wavelengths relative to the flavylum cation (AH₂⁺). At pH close to neutrality, a second deprotonation occurs leading to the formation of the anionic quinonoid bases (A⁻), with another blue shift in the absorption spectrum. Moreover, the flavylum cation is susceptible to nucleophilic attack at the charge-defective positions 2 and/or 4, as evident from the strong electronic density calculated for the frontier lowest unoccupied molecular orbital (LUMO). When in an aqueous environment, the water molecules, available in large quantity, add to the flavylum form at pH values above 1.5-2.0, resulting in a loss of color owing to the formation of the colorless hemiketal adduct (BH₂) through a slow pseudo acid-base equilibrium. This may eventually be followed by a ring opening that leads to the formation of the retrochalcones (C_E and C_Z), which are also almost colorless. This loss of color can be reversed by a simple reacidification with complete recovery of the colored flavylum cation.

Table 1.1 Anthocyanins are glycosylated polyphenols with a basic C-15 skeleton hydroxylated at positions 4' and 7 that can be divided in six basic structures according to the pattern of the substituents at positions 3' and 5'.

Anthocyanidin common name	3' and 5' substituents
Pelargonidin	H/H
Cyanidin	OH/H
Peonidin	OCH ₃ /H
Delphinidin	OH/OH
Petunidin	OH/OCH ₃
Malvidin	OCH ₃ /OCH ₃

Fig. 1.2 The anthocyanin flavylum chromophore, a carboxonium cation stable in aqueous media. R is usually sugar or acylated sugar.

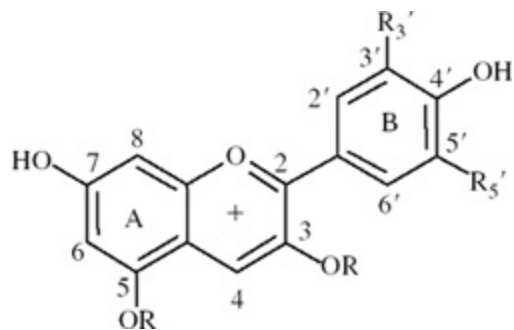
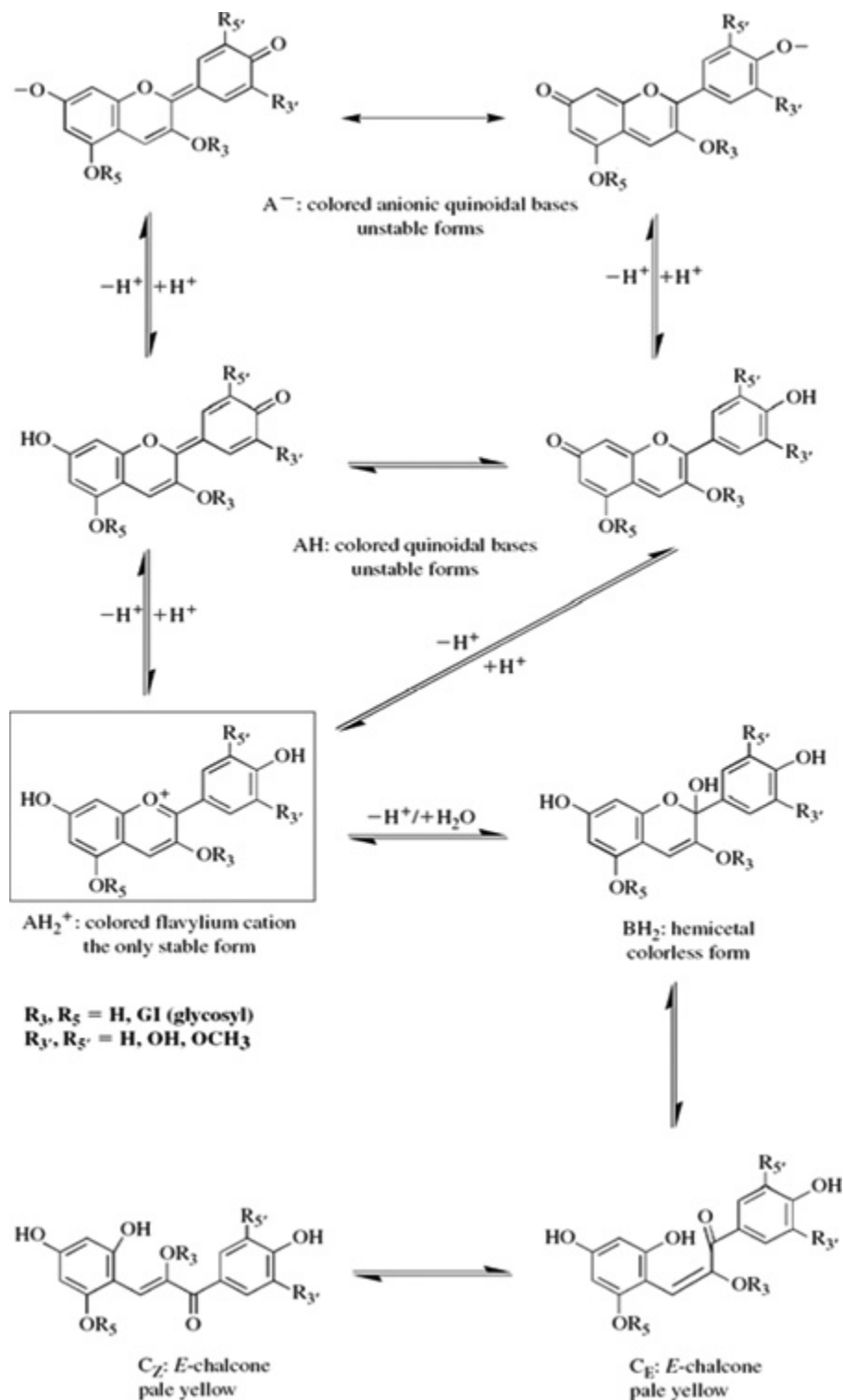


Fig. 1.3 Anthocyanin equilibria in aqueous solution and the corresponding structural transformations. AH_2^+ represents the flavylium cation that predominates at acidic pH values; AH represents the two tautomeric quinonoid bases; A^- depicts the anionic quinonoid bases that appears in alkaline solutions; BH_2 is the colorless hemiketal adduct; and C_E and C_Z are isomeric retrochalcones.



In the laboratory, aqueous solutions of anthocyanins, even kept under physicochemical conditions (temperature, pH, light, oxygen) similar to the ones found in plant vacuoles, tend to lose their bright colors either by formation of the

colorless species or by degradation leading to the irreversible cleavage of the molecule (Furtado *et al.*, 1993; Figueiredo, 1994). However, *in planta*, the colorless forms BH₂, C_{E'}, and C_Z are rarely found and the colors last for several days or even weeks, indicating the existence of vacuolar mechanisms that stabilize the colored species. Moreover, the same anthocyanin can be found in flowers of different tints, a fact that indicates the existence of diverse interactions of the pigment with the cellular environment. Among the stabilizing mechanisms found in the plant world, the most widespread are copigmentation and metal complexation or even combinations of the two (Goto & Kondo, 1991). The first one was found to be present in some flowers and its behavior in model solutions was thoroughly investigated (Robinson & Robinson, 1931; Brouillard, 1981, 1983; Brouillard *et al.*, 1989, 1991; Dangles & Brouillard, 1992a,b; Wigand *et al.*, 1992; Dangles *et al.*, 1993a,b; Dangles & Elhajji, 1994; Figueiredo *et al.*, 1996b), whereas the second is expected to occur between all anthocyanins possessing a catechol group in their B-ring and small divalent and trivalent metal cations (Dangles *et al.*, 1994a; Elhabiri *et al.*, 1997). In this chapter, we give more insight to these phenomena by means of an investigation on the interactions between several metals and a series of natural and synthetic anthocyanic pigments bearing different substitution patterns. New views on anthocyanin iron complexation, as well as some thoughts on possible applications, are also developed.

1.2 Copigmentation of anthocyanins

Copigmentation or anthocyanin color exaltation results from the presence of special molecules or copigments in an

aqueous environment. This phenomenon is known for long, but even today, nothing comparable has been uncovered from the rest of the huge polyphenol family or any other class of organic molecules.

Copigmentation can be defined as a hydrophobic π - π molecular interaction, through a vertical stacking, between a planar anthocyanin structure (flavylium cation or quinonoid base) and another planar molecule possessing no color by itself, which results in an enhancement, and generally a modification, in the original color of the pigment-containing solution. Most polyphenols can act as copigments, their efficiency depending on their chemical structures. However, other families of molecules were also found to include good copigments, for example, purines and alkaloids (Elhabiri *et al.*, 1997), and several more will probably be uncovered as further investigations are on the way.

This loose association between the copigment and one of the colored forms of the anthocyanin, generally the flavylium cation, produces, in electronic absorption terms, both hyperchromic and bathochromic shifts (Asen *et al.*, 1972). Such spectral changes can be explained by (1) a partial desolvation of pigment and copigment molecules when the water molecules rearrange around the newly formed complex, allowing a closer contact between both structures (copigmentation generally originates 1:1 complexes) with the consequent formation of more chromophores owing to a more difficult access of the solvent molecules to the electrophilic site C-2 (hyperchromism) and (2) the change in polarity in the immediate vicinity of the anthocyanin brought about by the displacement of some water molecules by the less polar organic copigment (bathochromism).

The color enhancement effect is more spectacular in mildly acidic solutions than in very acidic solutions owing to