ISOLATION, IDENTIFICATION AND PROPERTIES OF PYRANOANTHOCYANINS AND ANTHOCYANIN FORMS

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Dissertation for the degree of Philosophiae Doctor (PhD)

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2007

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Contents

Preface

This thesis is submitted for the degree of Philosophiae Doctor (PhD) in Chemistry at the University of Bergen, Norway. The work has been carried out at Department of Chemistry, University of Bergen, during the period 2003-2007. The thesis consists of 8 papers preceded by an abstract.

One aim of the present work was to isolate and determine the chemical structures of anthocyanins in different European and African plants, for documentation and chemotaxonomic considerations with respect to these pigments. The findings of new anthocyanins will expand the diversity of structures, which may through further examinations display other chemical and biological properties than previously known.

Another purpose was to enhance knowledge about the chemical properties of anthocyanins, 5-carboxypyranoanthocyanins in particular. The pyranoanthocyanins have interestingly been reported to display under weakly acidic to neutral conditions other properties (colours, higher stability etc.) than the common anthocyanins. After preparation of pyranoanthocyanins in a semi-preparative scale, the aim was to focus on comparative studies of various forms and specific structural positions of pyranoanthocyanins and common anthocyanins using advanced NMR instrumentation.

A third aim was to present accurate information about the reducing potential of various 5-carboxypyranoanthocyanins and anthocyanins. The value of anthocyanin literature with respect to antioxidant measurements on single anthocyanins are in many cases reduced due to limited considerations concerning the purity state of examined anthocyanin samples. Literature in the field has contradictory nature, and the relationship between anthocyanin structure and antioxidant capacity is not completely understood.

Chapter 1 gives an introduction to the thesis, chapter 2 presents the methods used in this work and chapter 3 gives the results covered by the papers I-VIII. The Appendix section includes presentation of the pigments (A) involved in the thesis and their structures (B). Appendix C and D present the ^{1}H and ^{13}C NMR data obtained in this work, respectively.

Acknowledgements

I will first of all thank my two supervisors; Professor Øyvind M. Andersen and Dr. Torgils Fossen for excellent supervision during my study. You have shared your wisdom with me and always been there for professional discussions. Especially I want to thank my main supervisor, Professor Øyvind M. Andersen, who also supervised me during my Master thesis. Thank you for your belief in me and thank you for always encouraging me to have belief in myself. I will always appreciate and be thankful for your enormous generosity and catching engagement.

I want to thank The Norwegian Research Council who provided the financial resources to make the project possible.

I wish to thank Dr. Nils Åge Frøystein and Principal Engineer Atle Aaberg for helping me with the NMR facilities, Unni Hauge and Terje Lygre for technical support and my co-authors for collaboration. General Manager of Tekna-Bergen, Ellen Hauge, for conversations and words of wisdom. Jarl Underhaug for 'oracle service' concerning constant computer and NMR questions.

I am grateful to my colleagues and friends for their support and their friendship; especially I want to thank Anna and Kay, Anne-Gro and Eirik, Lone, Silje, Kristin and Ørjan, you are enriching my days at and outside the department ☺.

*

My deepest gratitude goes to my family, my brother, Morten, who I'm so proud of, my father who has taught me the importance of having dreams and setting goals, my mother who has shown me the art of hard work and taking one step at a time. Thank you all for constant support and interest.

Finally I want to thank Rune, my husband, my best friend, for your enormous patience, encouragement and your love.

Bergen, 2007

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Abstract

This dissertation focuses on isolation and structural elucidation of anthocyanins, and their chemical properties.

The characterization of anthocyanins from castor (*Ricinus communis*), fourteen cultivars of European gooseberry (*Ribes grosssularia*), three other *Ribes* spp., two cultivars of Jostaberry (*R.* × *nidigrolaria*), seven *Hippeastrum* (Amaryllis) *hybridum* cultivars, and nineteen species belonging to Caprifoliaceae (genera *Sambucus*, *Lonicera* and *Viburnum*) are described. New anthocyanins include: Cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside (**11**), cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-(6'''-malonylglucopyranoside) (**19**) and the methyl esterified product (**18**), cyanidin 3-*O*-*β*-(6''-*E*-caffeoylglucopyranoside) (**15**), and cyanidin 3-*O*-*β*-(6''-*α*-arabinopyranosylglucopyranoside) (**6**), which is the first complete identification of the disaccharide vicianose. Pigment **15** together with cyanidin 3-*O*-*β*-(6''-*E*-caffeoylglucopyranoside) (**17**) constitute the major anthocyanin content in the cultivars; 'Samsø', 'Hinnomäki Red', 'Taastrup', 'Lofthus' and 'Glendal'. These cultivars may thus be good candidates for consumption, colorant and breeding programmes, because no other commercial available berries have been reported to contain as high proportions of aromatic acylated anthocyanins.

Anthocyanins from black beans (*Phaseolus vulgaris*) are for the first time hemisynthesized directly from a partly purified anthocyanin extract. The three mother anthocyanins (delphinidin 3-*O*-*β*-glucopyranoside (**22**), petunidin 3-*O*-*β*-glucopyranoside (**26**), malvidin 3-*O*-*β*-glucopyranoside (**27**)) and their hemisynthesis products, 5-carboxypyranoanthocyanins (**32**-**34**), were isolated in a preparative scale using Sephadex LH-20 column chromatography. The individual pigments (**22**, **26**, **27**, **32**–**34)** were characterized by NMR; the structures of **32** and **33** have previously only been tentatively identified.

The 3-*O*-*β*-glucopyranosides of delphinidin, petunidin and malvidin (*Phaseolus vulgaris*) (**22**, **26**, **27**) and cyanidin 3-*O*-*β*-galactopyranoside (from *Aronia melanocarpa*) (**4**) have been dissolved in deuterated methanolic solutions without and with acid (5%, CF_3COOD). Their hemiacetal (hemiketal) forms were characterized by NMR as two epimeric 2-hydroxy-hemiacetals. This is the first report of ^{13}C NMR assignments regarding two epimeric anthocyanin hemiacetal forms.

Displacement of nuclear hydrogen by deuterium at various sites on the aglycone part of delphinidin 3-*O*-*β*-glucopyranoside (**22**), petunidin 3-*O*-*β*-glucopyranoside (**26**), and

malvidin 3-*O*-*β*-glucopyranoside (**27**) in their flavylium cationic and hemiketal forms were examined based on integration data obtained by ^{1}H NMR spectroscopy. Similar measurements were performed on the three corresponding pyranoanthocyanins (**32**–**34**), and the flavonol rutin (quercetin 3-*O*-*β*-(6"-*α*-rhamnopyranosylglucopyranoside), **35**. H→D exchanges were observed for the nuclear protons of the A-rings (H-6 and H-8) of **22**, **26**, **27** and **35**. No similar H→D exchanges were observed for **32**–**34**. This is explained with a generalized mechanism including a positively charged σ–complex. Since the oxygen (6-O) included in the pyrano-ring of **32**–**34**, has not the same electron donating effect as the 5-OH group of **22**, **26** and **27**, the positively charged σ–complexes of **32**–**34** can not stabilize themselves to the same level as the corresponding complexes of **22**, **26** and **27**. The H→D exchange reactions appeared to be independent upon the concentration of the substrates and concentration of D^{+}, in accordance with a first order reaction.

Most antioxidant measurements are concentration dependent, and purity determination of examined compounds are crucial. To improve correctness in determination of anthocyanin purity, ^{1}H and ^{13}C NMR spectroscopy have been combined with HPLC-DAD and UV-Vis spectroscopy in analysis of anthocyanidin 3-glycosides and 5-carboxypyranoanthocyanidin 3-glycosides. The molar absorptivity (ε) values were found to be relatively similar, in contrast to previously reported literature values. The ε-values for both anthocyanidin 3-monoglycosides and 5-carboxypyranoanthocyanidin 3-glycosides were proposed to be 21800 and 22700 in acidified aqueous and methanolic solutions respectively. To assess the influence of structure on the potential antioxidant capacity of anthocyanins, the 3-glucosides of pelargonidin (**1**), cyanidin (**5**), peonidin (**24**), delphinidin (**22**), petunidin (**26**), malvidin (**27**), 5-carboxypyranopelargonidin (**28**), 5-carboxypyranocyanidin (**30**), 5-carboxypyranodelphinidin **(32**), 5-carboxypyranopetunidin (**33**), and 5-carboxypyranomalvidin (**34**) were examined by ferric ion reducing antioxidant power assay, FRAP. The reducing capacities of the individual anthocyanins were in the range of 0.9 to 5.2 Trolox equivalents. The two 5-carboxypyranoanthocyanins **30** and **32**, possessing pyrogallol- or catechol-type of B-rings, showed the highest potential antioxidant capacity measured by FRAP for any anthocyanin. The relative order of the reducing capacity of the various 5-carboxypyranoanthocyanidin 3-glucosides and anthocyanidin 3-glucosides were nearly alike whether determined by coulometric array detection or FRAP. The inclusion of the 5-hydroxyl in the D-ring and just one oxygen substituent on the B-ring as in **28**, diminished the reducing capacity considerably.

List of publications

I. Byamukama, R.; Jordheim, M.; Kiremire, B.; Andersen, Ø. M. New anthocyanins from stem bark of castor, *Ricinus communis*. *Phytochemistry* **2007**, submitted.

II. Jordheim, M.; Måge, F.; Andersen, Ø. M. Anthocyanins in berries of *Ribes* including Gooseberry cultivars with high content of acylated pigments. *Journal of Agricultural Food and Chemistry* **2007**, accepted.

III. Jordheim, M.; Giske, N. H.; Andersen, Ø. M. Anthocyanins in Caprifoliaceae. *Biochemical Systematics and Ecology* **2007**, *35*, 153–159.

IV. Byamukama, R.; Jordheim, M.; Kiremire, B.; Namukobe, J.; Andersen, Ø. M. Anthocyanins from flowers of *Hippeastrum* cultivars. *Scientia Horticulturae* **2006**, *109*, 262–266.

V. Jordheim, M.; Fossen, T.; Andersen, Ø. M. Preparative isolation and NMR characterization of carboxypyranoanthocyanins. *Journal of Agricultural Food and Chemistry* **2006**, *54*, 3572–3577.

VI. Jordheim, M.; Fossen, T.; Andersen, Ø. M. Characterization of hemiacetal forms of anthocyanidin 3-*O*-*β*-glycopyranosides. *Journal of Agricultural Food and Chemistry* **2006**, *54*, 9340–9346.

VII. Jordheim, M.; Fossen, T.; Songstad, J.; Andersen, Ø. M. Reactivity of anthocyanins and pyranoanthocyanins; studies on aromatic hydrogen-deuterium exchange reactions in methanol. *Journal of Agricultural Food and Chemistry* **2007**, submitted.

VIII. Jordheim, M.; Aaby, K.; Fossen, T.; Skrede, G.; Andersen, Ø. M. Molar absorptivities and reducing capacity of pyranoanthocyanins and other anthocyanins. *Journal of Agricultural Food and Chemistry* **2007**, submitted.

Chapter 1

INTRODUCTION

1.1 Flavonoids

Flavonoids are phenolic substances isolated from a wide range of vascular plants, and more than 8150 different flavonoids have been reported (Andersen and Markham, 2006). They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellents, and for light screening (Pieatta, 2000). Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating effects.

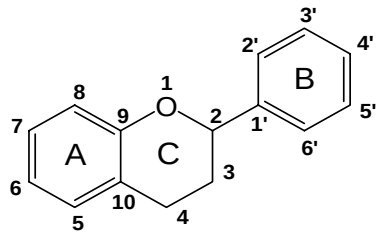


Figure 1. Basic flavonoid structure including the numbering system.

The basic flavonoid structure contains the flavan nucleus, which consists of 15 carbon atoms derived from a C_6-C_3-C_6 skeleton (Figure 1).

The 12 main classes of flavonoids differ in the level of oxidation and the substitution pattern on the C ring, while individual compounds within a class differ in the substitution pattern on the A and B rings.

1.2 Anthocyanins

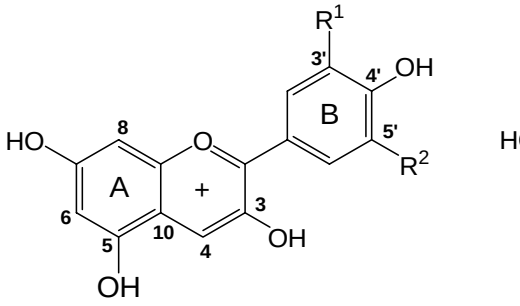
The word anthocyanin, derived from the Greek words *anthos* (flower) and *kyanos* (blue) was originally used to describe the blue pigments of the cornflower, *Centaurea cyanus* (Marquart, 1835). Anthocyanins are polyphenolic compounds responsible for cyanic colours ranging from salmon pink through red and violet to dark blue of most flowers, fruits, leaves and stems. They comprise the largest group of the water-soluble pigments in the plant kingdom (Strack and Wray, 1994), and during the last then years it has been an exponential increase in the report of new anthocyanin structures (Andersen and Jordheim, 2006). This can partly be explained by the use of improved analytical techniques, but the potential use of anthocyanins as health beneficial compounds is another reason for the increased scientific interest in these pigments. At the moment the actual number of anthocyanins reported with complete structure elucidation is 575 (Andersen and Jordheim, 2006; Andersen, 2007).

1.2.1 Structures

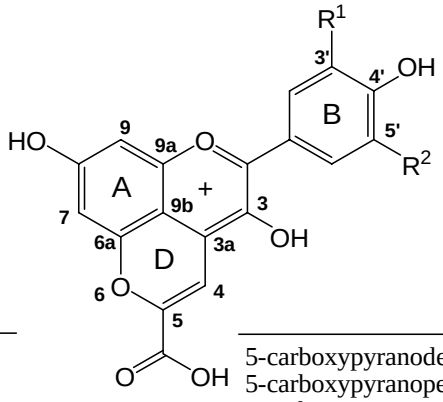
The anthocyanins consist of an aglycone (anthocyanidin), sugar(s), and, in many cases, acyl group(s). The classical anthocyanin aglycone is based on a C_{15} skeleton (C_6–C_3–C_6 skeleton) while the pyranoanthocyanins discussed in this thesis have an additional C_3 unit (Figure 2) (Andersen and Jordheim, 2006). Anthocyanins are positively charged at acidic pH (see 1.2.3), and this equilibrium form is called flavylium cation (2-phenylbenzopyrylium). Even though there are around 30 different anthocyanidins, approximately 90% of all anthocyanins are based on the six most common anthocyanidins; pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin, which only differ by the hydroxylation and methoxylation pattern on their B-rings (Figure 2, left). The anthocyanins will differ with respect to glycoslyation of hydroxyl groups, nature of glycosyl units, substitution pattern, and potential aliphatic and aromatic acylation (Andersen and Jordheim, 2006). The 3-deoxyanthocyanidins found in Sorghum; spagnorubins and rosacyanin B are the only anthocyanidins (aglycones) found in their nonglycosidated form in plants (Andersen and Jordheim, 2006) until Macz-Pop et al. (2006) indicated the presence of cyanidin, peonidin

and pelargonidin in black dried beans (*Phaseolus vulgaris* L.).

Pyranoanthocyanins (Figure 2, right) have been discovered in small amounts in wines and grape pomace (Bakker et al., 1997; Bakker and Timberlake et al., 1997; Fulcrand et al., 1998; Mateus et al., 2004; Cheynier, 2006), petals of *Rosa hybrida* cv. 'M'me Violet (Fukui et al., 2002, 2006), black carrot (*Daucus carota*) juice (Schwarz et al., 2004), and blood orange (*Citrus sinensis*) juice (Hillebrand et al., 2004). Among the carboxypyranoanthocyanins, vitisin A and acetylvitisin A were identified as the 3-glucoside and the 3-acetylglucoside of malvidin containing an additional $C_3H_2O_2$ unit linking the C-4 and the C-5 hydroxyl group. More recently, glucosides of carboxypyranocyanidin have been isolated from red onion (Fossen and Andersen, 2003), and carboxypyranopelargonidin 3-glucoside from strawberry (Andersen et al., 2004) extracts.



	R^1	R^2
pelargonidin	H	H
cyanidin	OH	H
peonidin	OCH_3	H
delphinidin	OH	OH
petunidin	OCH_3	OH
malvidin	OCH_3	OCH_3



	R^1	R^2
5-carboxypyranodelphinidin	OH	OH
5-carboxypyranopetunidin	OCH_3	OH
5-carboxypyranomalvidin	OCH_3	OCH_3

Figure 2. Left: Structures of the most common anthocyanidins occurring in nature.
Right: Structures of some 5-carboxypyranoanthocyanidins.

Analogous delphinidin and petunidin derivatives have been indicated in various pigment mixtures (Fulcrand et al., 1998; Benabdeljalil et al., 2000; Vivar-Quintana et al., 2002; Hayasaka et al., 2002; Wang et al., 2003; Alcalde-Eon et al., 2004; Villiers de et al., 2004; Calvo et al., 2004; Salas et al., 2005; Mazzuca et al., 2005; Faria et al., 2005).

Four reported methylpyranoanthocyanins from black currant seeds (Lu et al., 2000) were shown to be the oxidative cycloaddition products of the acetone extraction solvent and the natural anthocyanins (Lu et al., 2001). Pyranocyanin C and D and pyranodelphinidin C and D, were also isolated by the same group from an extract of black currant seeds (Lu et al.,

2002). These pigments were absent in fresh extracts, and their levels increased gradually with time. Their formation was likely to be from the reaction of the anthocyanins and *p*-coumaric acid in the extracts. Recently, analogous pigments have been isolated from strawberry and raspberry juices after addition of cinnamic acids (Rein et al., 2005).

Sugar moieties

Most anthocyanins are mono-, di-, or tri-glycosylated at the C-3 hydroxyl. Ternatin A1 (isolated from *Clitoria ternatea*) (Terahara et al., 1990) and cyanodelphin (isolated from *Delphinium hybridum*) (Kondo et al., 1991) are two impressing exceptions with seven glucosyl units. Beside the 3-position, anthocyanins can also be glycosylated at 5, 7, 3', 5' and more rarely at the 4' position (Brouillard 1988; Fossen et al., 2003b, Williams and Grayer, 2004, Bjorøy et al., 2007). The sugar moieties are found connected to the anthocyanidins through *O*-linkages, but in the purple flowers of *Tricyrtis formosana* 8-*C*-glucosylcyanidin 3-[6-(malonyl)glucoside] and 8-C-(6-*O*-*E*-sinapoyl)-glucosylcyanidin 3-[6-(malonyl)glucoside] have been reported (Saito et al., 2003; Tatsuzawa et al., 2004).

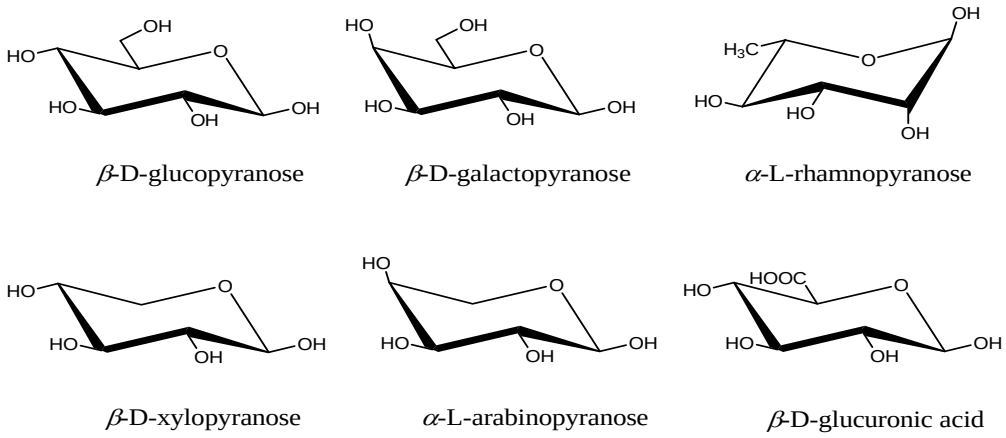


Figure 3. Structures of the monosaccharides found in anthocyanin structures.

The most common monosaccharide is glucose (90%), followed by rhamnose, galactose, xylose and arabinose (Figure 3) (Andersen and Jordheim, 2006). Glucuronic acid is the rarest monosaccharide found in anthocyanins.

Acyl moieties

More than 65% of the reported anthocyanins with properly identified structures are acylated, and anthocyanin diversity is highly associated with the nature, number, and linkage positions of the acyl groups (Andersen and Jordheim, 2006). The sugar units of anthocyanins may be acylated with aliphatic and/or aromatic acyl groups (Figure 4). The aromatic acyl groups include various hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic, and 3,5-dihydroxycinnamic acids) and two hydroxybenzoic acids (*p*-hydroxybenzoic- and gallic acid). Acylation with aliphatic acids includes malonic acid, which is the most frequent aliphatic acyl group, acetic, malic, succinic, tartaric and oxalic acids (Andersen and Jordheim, 2006). Because of the labile nature of the ester bond in case of aliphatic acylation, hydrolysis might occur during the workup procedure or storage in acidified solution. Anthocyanins acylated with dicarboxylic acids are subjected to both hydrolysis and esterification of the free carboxyl group in acidified alcoholic solutions (Fossen et al., 2001; Takeoka and Dao, 2002; Andersen and Francis, 2004).

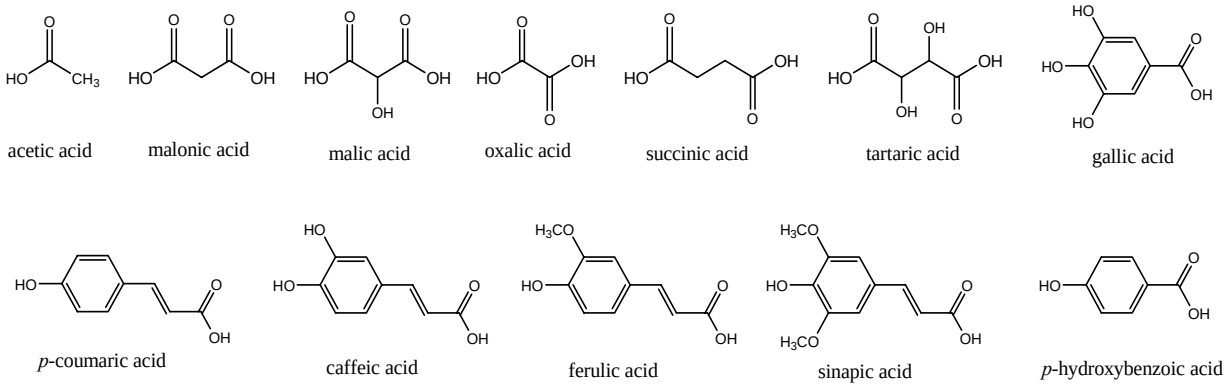


Figure 4. Structures of the aromatic and aliphatic acyl substituents found in anthocyanins.

1.2.2 Equilibrium forms

Anthocyanins are considered to occur in several equilibrium forms. Thermodynamic and kinetic studies have led to a generally accepted scheme with respect to the different transformations (proton transfer, isomerisation and tautomerization) of the flavylium cation of simple anthocyanins under various pH conditions (Sondheimer, 1953; Jurd, 1963a, 1963b; McClelland, 1980; Brouillard 1977a, 1977b; Brouillard and Dangles, 1994; Pina, 1998).

In strongly acidic solutions (below pH 2) the flavylium cation is predominant (Figure 5, structure 1) giving rise to anthocyanin solutions which are red in colour. In slightly acidic or

neutral aqueous solutions anthocyanins are believed to exist as neutral and/or ionized quinonoidal bases (Figure 5, structure 2–4 and 5–7, respectively) after deprotonation. By hydration in weakly acidic solutions the flavylium cation form is more or less rapidly changed to the more stable colourless hemiketal (alternatively hemiacetal or carbinol pseudobase) and chalcone form. The hydration at the flavylium cation seems to occur mainly at the 2-position to give the hemiketal 2-adduct, but the possibility of a 4-adduct is also present (Figure 5, structure 8 and 9). The chalcone form is a result of a ring opening of the hemiketal form, and is considered to be an equilibrium form of the hemiketal (Figure 5, structure 10 (*Z*, *E*) and 11 (*Z*, *E*)). The different equilibrium structures have been proposed with different methods including pH-jump method, UV-visible and fluorescence spectroscopy (e.g., Pina, 1998) and in a few cases NMR spectroscopy (Cheminat and Brouillard, 1986; Mistry et al., 1991; Santos et al., 1993; Terahara et al., 1993; Bakker et al., 1997; Jordheim et al., 2006b; Fossen et al., 2007).

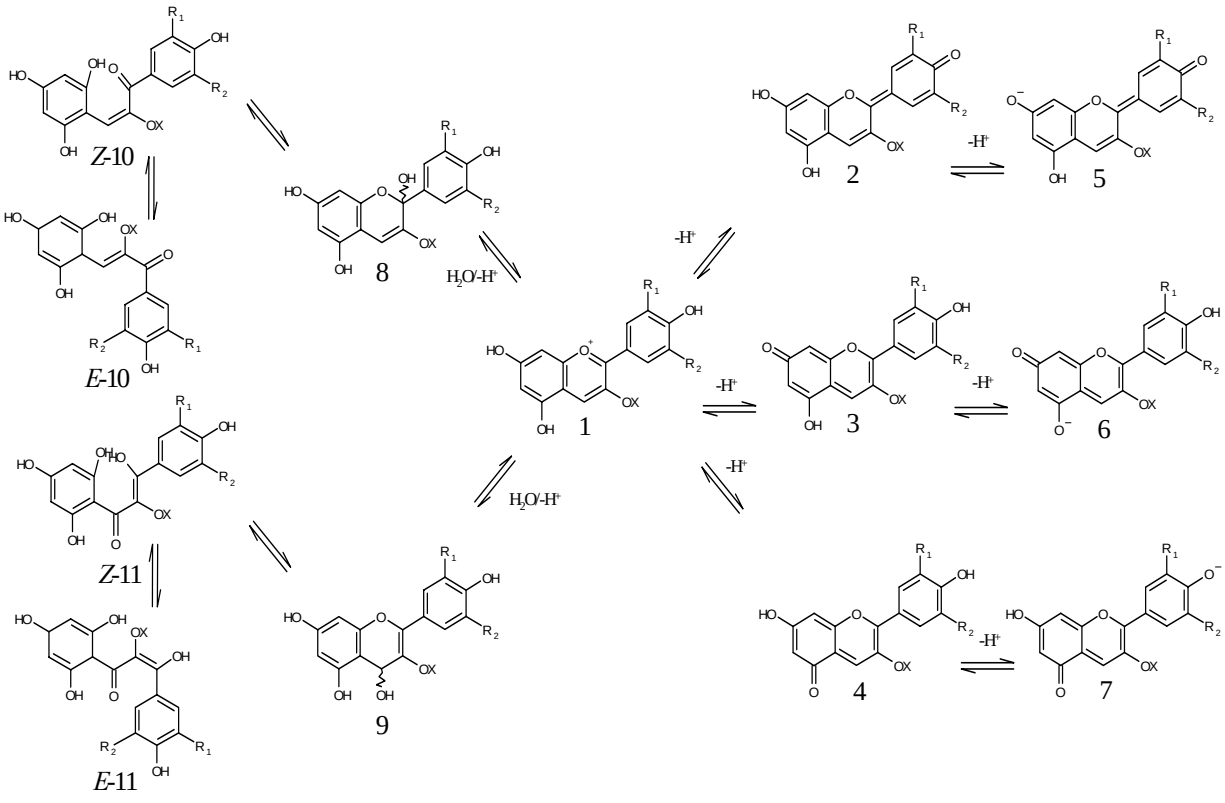


Figure 5. General scheme showing some possible anthocyanin transformations in aqueous solution. X = glycoside, R_1 and R_2 can be hydroxyl and/or methoxyl groups, depending on the type of aglycone. Other reactions may be involved (see references above).

The structural transformation reactions are mainly responsible for the fact that NMR and MS spectral methods previously were of limited value in the structural investigations of the equilibrium forms of anthocyanins (Hrazdina, 1982.).

1.2.3 Biosynthesis and anthocyanin cell accumulation

The initial step in biosynthesis of all flavonoids is the condensation of 4-coumarate coenzyme A (shikimate derived, B ring) with three malonyl coenzyme A molecules (polyketid origin, A ring) to give 2', 4', 6', 4-tetrahydroxychalcone, which is catalysed by the enzyme chalcone synthase (Strack and Wray, 1994). The chalcone is then isomerised to the flavanone naringenin, a key indermediate, which can be converted to several end-products including anthocyanins (Figure 6). (Strack and Wray, 1994; Cooper-Driver, 2001).

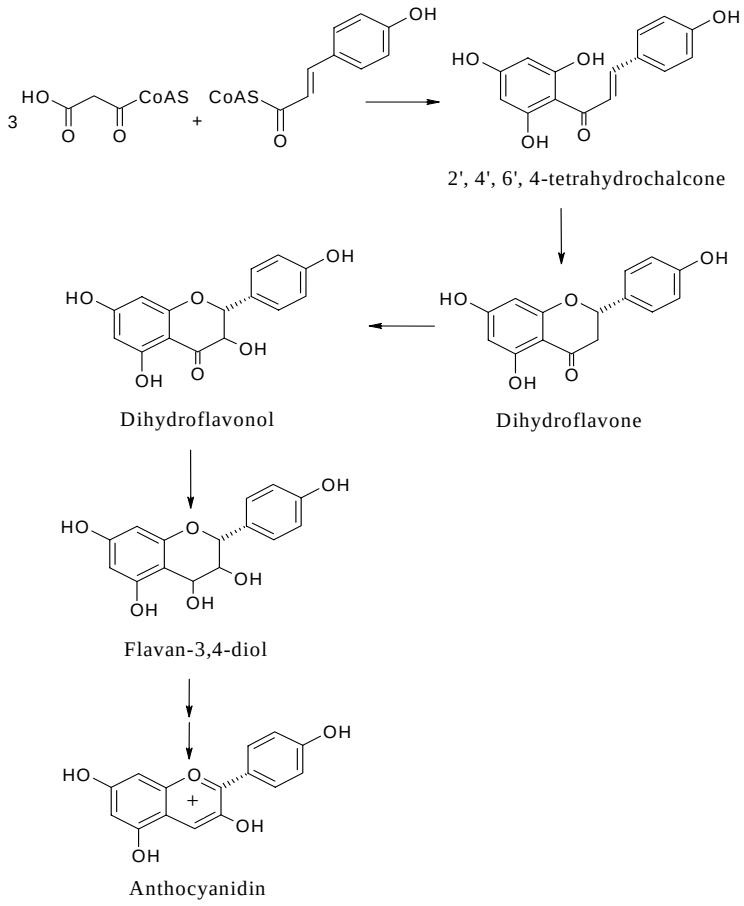


Figure 6. Schematic representation of the biosynthetic pathway of anthocyanins.

Based on the known biosynthetic pathways of flavonoids, it is assumed that different flavonoid groups have appeared sequentially during plant evolution. This assumption

presumes that the simple structural compounds or groups of compounds (e.g. flavanones) which appear early in the biosynthetic pathway, evolved in the first photosynthetic plants, whereas compounds synthesized later in the biosynthetic pathway (e.g. anthocyanins) would occur in the most recently evolved plants or extant plant taxa (Cooper-Driver and Bhattachary, 1998 and references therein; Swain, 1986; Stafford, 1991). But these assumptions have been questioned, and new data have become available. Anthocyanins are for example found in liverworts and ferns, so the ability to synthesize anthocyanins is an ancient one, and over the course of evolution the anthocyanins have developed varied functions in the biology of plants.

Although the biosynthetic pathways for anthocyanins and their regulation have been well studied, the mechanism of anthocyanin accumulation in the cell is poorly understood. In most plants anthocyanins are normally found dissolved uniformly in the vacuolar solution of epidermal cells. However, in certain species, the anthocyanins are localised in discrete regions of the cell vacuole. Markham and co-workers (2000) described these regions as intensely coloured intravacuolar bodies and defined them as anthocyanic vacuolar inclusions, AVIs, based on observations in blue-grey carnation and in purple lisianthus. Here AVIs occurred predominantly in the adaxial epidermal cells and their presence was shown to have a major influence on flower colour by enhancing both intensity and bluish hue. Electron microscopy studies on lisianthus epidermal tissue failed to detect a membrane boundary in AVI bodies, and AVIs isolated from lisianthus cells were shown to have a protein matrix with anthocyanins bound. Flavonol glycosides were not bound, showing AVIs specificity as vacuolar anthocyanin traps. Zhang et al. (2006) have recently used light and electron microscopy to investigate AVIs in different regions in petals of lisianthus. They observed three different forms of the AVIs; vesicle-like, rod-like and irregular shaped. No membrane was encompassing the AVI, which was in accordance with previous observations (Markham et al. 2000). Further analysis demonstrated the accumulation of anthocyanins in vesicle-like bodies in the cytoplasm, which themselves were contained in prevacuolar compartments, PVCs (Zhang et al., 2006). The vesicle-like bodies seemed to be transported into the central vacuole through the merging of the PVCs and the central vacuole in the epidermal cells. These results suggest the existence of mass transport of anthocyanins from the biosynthetic sites in the cytoplasm to the central vacuole where the PVCs play a major role.

1.2.4 Colour

Considerable effort has been made to give explanations for the colour variations expressed by anthocyanins in plants, and especially the blue colours (Brouillard and Dangles, 1994; Andersen and Jordheim, 2006). Four mechanisms, namely self-association, intramolecular co-pigmentation, intermolecular co-pigmentation between different molecules and complexation of anthocyanins with metal ions, have been suggested to stabilize the anthocyanins in the cell sap (Nerdal and Andersen, 1991). In addition various factors including sample concentration and nature of anthocyanin, anthocyanin equilibrium forms, the extent of anthocyanin glycosidation and acylation, and influence of external factors like pH, solvent, temperature, etc. may interact with previously mentioned stabilization mechanisms. Co-pigmentation is supposed to be the most common mechanism in the formation of blue flower colours, and together with pH probably the most important factor influencing the flower colour (Goto and Kondo, 1991; Brouillard and Dangles, 1994; Harborne and Williams, 2000).

The co-pigments of anthocyanins may include other flavonoids such as flavonols or flavones, with delphinidin as the most commonly described anthocyanidin and flavones as the co-pigments with most pronounced effects (Harborne and Williams, 2000). Intra- or intermolecular association may exist between the chromophore (anthocyanin) and the co-pigment when the two units are covalently linked through a dicarboxylixc acid (*Eichhornia crassipes*, Toki et al., 1994, 2004; *Allium schoenoprasum*, Fossen et al., 2000; lupins, Takeda et al., 1993 and orchids, Strack et al., 1989; Uphoff, 1982), or when the anthocyanin moiety is covalently linked directly to a flavanol unit (strawberry, Fossen et al., 2004).

The stability of and shift to blue colours for polyacylated anthocyanins have also been explained by intra- or intermolecular co-pigmentation involving stacking between anthocyanidin and aromatic acyl moieties (Dangles et al., 1993, 1994, 1997; Redus et al., 1999; Honda et al., 2001). The bathochromic effects have been shown to depend on the number of aromatic acyl groups present and their linkage positions. The proposed “sandwich” configuration with the 3'-acylglycosyl chain folded “over” and the 7-acylglycosyl chain folded “under” the chromophore, constituted the minimum energy conformation, providing effective protection against nucleophilic attack of the pyrylium ring by the solvent (water) (Figueiredo et al., 1999; Honda et al., 2001).

In a few cases anthocyanin complexation with metal ions has shown to be efficient in influencing anthocyanin colour. Kondo et al. presented in 1992 the extraordinary macro-molecule Commelinin found in flowers of *Commelina communis*. Here, two magnesium

molecules were central in a macro-molecule with six self-associated anthocyanin units and six flavone units. The stability and the intense blue flower colour were explained by intermolecular hydrophobic association. In 1998 Kondo et al. proposed a new molecular mechanism for blue colour expression based on protocyanin from cornflower, *Centaurea cyanus*. The blue colour was found to be caused by ligand to metal charge transfer (LMCT). However, recently it has been shown that additional presence of two Ca^{2+}-ions was essential for the formation of protocyanin (Shiono et al., 2005; Takeda et al., 2005). Another metalloanthocyanin, protodelphin, similar to commelinin and protocyanin, has been isolated from flowers of *Salvia patens* (Takeda et al., 1994). Recently an anthocyanin complex containing a cyanidin derivative, two or more equivalents of kampferol derivatives, 1/6 equivalents of Fe^{3+} and excess of Mg^{2+}-ions has been proposed to constitute the blue petal colour of Himalayan blue poppy (*Meconopsis grandis*) (Yoshida et al., 2006). It is also known that anthocyanins with hydroxyl groups in *ortho*-position to each other form complexes with triple charged metal ions leading to bathochromic and hyperchromic shifts in their absorption spectra (Dangles et al., 1994; Elhabiri et al., 1997). For example, the colour change of hydrangea (*Hydrangea macrophylla*) has been suggested to be caused by free Al^{3+} complexation, where the complex responds to slight vacuolar pH change (Kondo et al., 1999; Yoshida et al., 2004).

The different mechanisms described above are most probably influenced by the *in vivo* equilibrium form(s) of anthocyanin(s) (section 1.2.2), which occur in plants. Under physicochemical conditions close to those prevailing in the vacuoles of floral cells, the common anthocyanins have been shown to exist essentially in their colourless forms (Brouillard et al., 1977a, 1977b).

1.2.5 Stability

Considerations about anthocyanin stability are related to colour, equilibrium forms and co-pigmentation (section 1.2.2 and 1.2.4). These factors are again affected by pH, temperature, oxygen, light, ascorbic acid, nucleophilic agents, free sugars, sulphur dioxide and enzymes present (Iacobucci and Sweeny, 1983; Jackman et al., 1987; Francis, 1989; Cabrita, 1999).

Brouillard (1982) found that starting from the flavylium cationic form each of the reactions in the equilibrium scheme (Figure 5) were endothermic (Iacobucci and Sweeny, 1983). By heating an anthocyanin solution, the equilibrium was driven towards the chalcone form giving decreased quantities of the coloured flavylium cation form. Cooling reversed the

change. Iacobucci and Sweeny (1983) have reported cyanidin 3-rutinoside to be more stable than cyanidin aglycone, and the hydroxyl groups at the B-ring increased the stability of the anthocyanin compared to analogues methoxyl groups. More recent studies on the stability of various acylated anthocyanins in weak acidic or neutral aqueous solutions (mostly di- or poly-acylated pigments) have shown these anthocyanins to be more resistant to hydration, and hence possess a higher colour stability in weakly acid or neutral solutions compared to non-acylated anthocyanins (Goto el a., 1983, 1984; Saito et al., 1985, 1995; Idaka et al., 1987; Yoshida et al., 1991, 1992). With respect to the alkaline pH area the aromatic acylated petanin (petunidin 3-O-*β*-(6"-*O*-(4"'-*O*-*E*-*p*-coumaroyl-*O*-*α*-rhamnosyl)glucopyranoside)-5-*O*-*β*-glucopyranoside) afforded a higher colour intensity and higher or similar stability throughout the whole pH range compared to the simple cyanidin 3-glucoside (Fossen et al., 1998). The increased stability of aromatically acylated anthocyanins in alkaline solutions was also reported by Torskangerpoll and Andersen (2005). In addition, they experienced that cyanidin 3-(2"-glucosylglucoside)-5-glucoside was more unstable than cyanidin 3-glucoside at most pH values.

1.2.6 Functions in plants

Anthocyanins are involved in attraction of insects and animals for pollination and seed dispersal purposes as they constitute the chemical basis of flower colour in angiosperms (Strack and Wray, 1994; Harborne and Williams, 1995). Their presence in young leaves, seedlings, roots and stems are not that obvious. There is increasing evidence that anthocyanins, particularly when they are located at the upper surface of the leaf or in the epidermal cells, also have a role in the physiological survival of plants. It has been outlined that foliar anthocyanins accumulate in young, expanding foliage, in autumnal foliage of deciduous species, in response to nutrient deficiency, temperature changes or ultraviolet (UV) radiation exposure, and in association with damage or defense against browsing herbivores or pathogenic fungal infections (Harborne and Williams, 2000; Gould and Lee, 2002; Simmonds, 2003; Close and Beadle, 2003). The functions of anthocyanins have in this context mainly been hypothesized as a compatible solute contributing to osmotic adjustment to drought and frost stress, as antioxidant and as UV and visible light protectant.

1.2.7 Potential health effects

Anthocyanins have received increasing attention during the last fifteen years related to potential health effects, and they are nowadays regarded as important nutraceuticals. This is mainly due to their possible antioxidant effects, and they have been given a potential therapeutic role related to cardiovascular diseases, cancer treatment, inhibition of certain types of virus including the human immunodeficiency virus type 1 (HIV-1), and improvement of visual acuity (Talavera et al., 2006; Stintzing et al., 2002; Moyer et al., 2002; Sandvik, 2004; Rechner and Kroner, 2005; Cecchini et al., 2005; Kamei et al., 1995; Cooke et al., 2005; Beattie et al., 2005; Andersen et al., 1997; Jang et al., 2005; Nakaishi et al., 2000; Wrolstad et al., 2002). The extent of the anthocyanin antioxidant potential in humans, and other observed positive health effects studied *in vitro*, are of course *in vivo* dependent on the absorption, metabolism, distribution, and excretion of these compounds within the body after ingestion (Rice-Evans, 2003).

1.2.8 Bioavailability

To reveal the potential health effects of anthocyanins it is essential to understand their *in vivo* bioavailability and functions. Bioavailability studies involving anthocyanins are often performed with HPLC equipped with UV-Vis spectroscopy detector, and the detection is mainly based on the coloured flavylium cation form. In a study by McGhie et al. (2003) rats were fed with boysenberry extract. The stomach of the rats was thereafter coloured, indicating the presence of the flavylium cation. The small intestine did not show any traces of coloured anthocyanins, but after acidifying the intestinal tissue turned red. By acidifying plasma, urine and liver tissue colourless anthocyanins were transformed to the coloured flavylium cation forms and detected by UV-Vis spectroscopy (McGhie et al., 2003; Tsuda et al., 1999; Miyazawa et al., 1999; Matsumoto et al., 2001; Cao et al., 2001; Felgines et al., 2002; Felgines et al., 2003; Cooney et al., 2004; Passamonti et al., 2005; Talavera et al., 2004, 2006).

During the passage of anthocyanins through the gastrointestinal tract (GIT), they are exposed to different pH environments and might therefore exist at different forms (section 1.2.2). The anthocyanin forms present in the different regions and tissues of the GIT, and eventually during absorption, are not known with certainty (McGhie et al., 2003). It is likely that the flavylium cation will exist only in the lumen of the stomach due to low pH, and the

other forms will predominate lower down the GIT. McDougall and co-workers (2007) assessed the stability of red cabbage anthocyanins to simulated gastrointestinal digestion. They found that the anthocyanins were effectively stable under acidic gastric digestion conditions, but the total recovery after simulated pancreatic digestion was around 25% compared to around 100% recovery of phenol content. Acylated anthocyanins showed higher stability against pancreatic digestion than non-acylated forms, and anthocyanins with sinapic acid reduced the stability compared to the other hydroxycinnamic acids. They concluded that it is unlikely for the anthocyanins to reach the serum or survive long under serum conditions, and they attributed the biological activities of anthocyanins to be carried out by their metabolites.

Metabolites of anthocyanins are found in urine, kidney and liver tissue (Tsuda et al., 1999; Wu et al., 2002; Talavera et al., 2004, 2006). For example were methylated cyanidin 3-glucoside (peonidin 3-glc) and their glucuronidated derivatives identified in urine and plasma from aorta and mesenteric vein together with native cyanidin 3-glucoside. Native cyanidin 3-glucoside and its methylated derivatives appeared in the bile after as little as 25 minutes (Talavera et al., 2005). This supports the findings of Miyazawa et al. (1999), who reported a high concentration of methylated cyanidin 3-glucoside in rat liver and a low concentration in the plasma, indicating that these metabolites were excreted from the liver directly into the bile. Even sulfoconjugated cyanidin derivatives have been identified in the urine (Felgins et al., 2005). Anthocyanidin sulfoconjugate formation requires hydrolysis of the anthocyanin to the aglycone followed by sulfoconjugation of the aglycone by sulfotransferases present in numerous tissues, including intestine and liver. The metabolic fate of anthocyanins may also differ according to their aglycone structure and the main metabolites of blackberry anthocyanins found in human urine were anthocyanidin monoglucuronides (Felgins et al., 2005).

Anthocyanin concentration in plasma results from a balance between absorption and elimination (Passamonti et al., 2005). Both parameters are complex, because absorption may depend on gastrointestinal motility, blood flow, and the activity of membrane carriers. The removal rate of anthocyanins from the plasma depends on their uptake and metabolism in peripheral tissues, including excretion into bile and/or urine, and on the conversion between the different equilibrium forms of the anthocyanins. The occurrence of anthocyanins in the plasma could be related to their binding to proteins, which ensures their chemical stability. The variable levels of anthocyanins in the plasma may reflect individual differences with respect to the content of endogenous and exogenous competitors for protein-binding sites in

the blood. The rate of anthocyanin breakdown in the plasma is also influenced by different active redox compounds present. The mean concentration of anthocyanins in plasma, although low, seems to be adequate for antioxidant effect (Passamonti et al., 2005).

The fact that cyanidin 3-glucosides and cyanidin 3,5-diglucosides are found in rats and human (low concentration) plasma, strongly confirms the ability of glycosides to cross the small intestine (Tsuda et al., 1999; Miyazawa et al., 1999). Matuschek et al. (2006) found that cyanidin 3-glucoside was mainly absorbed in the jejunum of the small intestine, which suggests involvement of an active transport mechanism. Tsuda et al. (1999) studied cyanidin 3-glucoside and its metabolites in the jejunal tissue of rats after direct stomach incubation. The cyanidin 3-glucoside, the cyanidin aglycone and the oxidation product of cyanidin 3-glucoside, protocatechuicacid/3,4-dihydroxybenzoic acid, were all detected in the jejunal tissue. Cyanidin 3-glucoside was then rapidly detected in the plasma and its oxidation product was detected at concentrations eight times higher. The cyanidin aglycone was not found in the plasma. Whether anthocyanins can be transported by SGLT1 (Na–dependent glucose transporter in small intestine and kidney), as quercetin 3-glucoside, or they are liable to be attacked by the glycosidases has not yet been confirmed. It can be the case that anthocyanins and anthocyanidins have the same transport system as quercetin 3-glucoside, but the fast degradation of the unstable anthocyanidins prevents their detection.

Other studies have demonstrated the possibility of anthocyanin absorption from the stomach (Passamonti et al., 2002, 2003). Bilitranslocase, an organic anion membrane carrier expressed in epithelial cells of the gastric mucosa, is suggested to be involved. Anthocyanins are rapidly absorbed following oral administration; the absorption through the gastric wall may provide an explanation (Matuschek et al., 2006). Interestingly Passamonti et al. (2005) found anthocyanins intact in rat brains just a few minutes after administration into the stomach. This is unexpected because of the presence of the blood-brain barrier which is thought to be impermeable to >98% of small, polar molecules occurring in the blood. The area of penetration has not been detected. Another study done by Talavera et al. (2005) reports anthocyanins in rat brains after intake of anthocyanin rich diet, though with a different timing of the experiments.

Elimination of anthocyanins (mixed anthocyanins from berries) is quite rapid ($t_{1/2}$ = 1.5-3 h compared to; quercetin $t_{1/2}$ = 11-28 h), and accumulation is not likely to occur to any significant extent following normal dietary consumption (Kay, 2006). Studies identifying anthocyanins exclusively as unmetabolized parent compounds may result from either saturation of metabolic pathways following mega-dose interventions, insufficient extraction

procedures, and misidentification as a result of insufficient detection methods (i.e. using UV-Visible HPLC exclusively for identification) (Kay, 2006). He et al. (2006) have performed a long term (3 months) trial on rats, with a chokeberry-, bilberry-, and grape-enriched diet. In this study they observed a larger urinary excretion of methylated anthocyanins than in several shorter (less than 8-day adaptation) previously reported studies, suggesting the possible accumulation of anthocyanins in tissues or induction of methyltransferase. For the first time the occurrence of intact acylated anthocyanins in plasma and urine was demonstrated. However, this study supports the finding by numerous researchers that anthocyanins have very low absorption, and it was suggested that anthocyanins in the gut content may influence GIT health without being delivered by the blood circulation system. He et al. (2006) observed high concentrations of possible metabolites in plasma, which emphasises the importance of further investigation of the significance of the accumulation of colonic metabolites and aglycone breakdown products.

The pyranoanthocyanins have received increasing interest during the last years (Jordheim et al., 2006a). 5-carboxypyranomalvidin 3-glucoside (**34**) has been reported to be more stable at gastrointestinal condition, and may be more serum-available and exert biological effects at a cellular level (McDougall et al., 2005). No data on the serum uptake of pyranoanthocyanins have been reported, although there have been a number of studies on the bioavailability of red wine anthocyanins (Lapidot et al., 1998; Bub et al., 2001; Frank et al., 2003). Initial *in vitro* experiments have suggested that 5-carboxypyranomalvidin 3-glucoside derivatives are slightly less biologically effective than their parent anthocyanins (Garcia-Alonso et al., 2004), but their enhanced stability compared to the more common anthocyanins makes further research interesting.

1.2.9 Various applications

The almost universal distribution of anthocyanins in flowering plants, makes them also suitable for chemotaxonomic considerations both at the family and genus level (Cooper-Driver, 2001). With respect to flower colour breeding the flavonoid pathway which leads to anthocyanin biosynthesis, is well characterised (Tanaka et al., 2005). The genes encoding for the pathway enzymes have been cloned from many plants and can be easily extracted from public DNA data bases. Metabolic engineering of the flavonoid pathway has generally been the focus with respect to modification of flower colour, but the final visible colour of a flower is also a function of other factors like co-pigmentation and vacuolar pH (section 1.2.4). These

factors are again regulated by a number of genes, many of which have now been cloned and characterised. The application and commercialization in this context is today limited by the lack of efficient transformation systems for floricultural species.

In the human diet, anthocyanins are found in red wine, certain varieties of cereals and certain leafy and root vegetables (aubergines, cabbage, beans, onions, radishes), but they are most abundant in fruits. Overall cyanidin is the most common anthocyanidin found in foods (Manach et al., 2004). The anthocyanin content in foods (fruits, berries etc.) is generally proportional to colour intensity, and reach values up to 2–4 g/kg fresh wt in blackcurrants and blackberries. These values increase as the fruit ripens. Wine contains approximately 200–350 mg anthocyanins/L, and involved anthocyanins are transformed into various complex structures as the wine ages. As an example showing the difficulties of estimating the daily intake of anthocyanins one can compare the intakes of anthocyanins tabulated by Clifford and Brown (2006) (5–9 mg/daily), with the estimated daily intake of anthocyanins in USA reported by Kühnau (1976), which was estimated to be 215 mg during the summer and 180 mg during the winter.

There is a worldwide interest in increased use of food colorants from natural sources as a consequence of consumer preferences as well as legislative action in connection with synthetic dyes. The principal commercially available anthocyanin food colorants are derived from grapes (*Vitis* spp.), elderberry (*Sambucus nigra*), red cabbage (*Brassica oleracea*) and roselle (*Hibiscus sabdariffa*). Other commercial anthocyanin extracts can be obtained from blood orange (*Citrus sinensis*), black chokeberry (*Aronia melanocarpa*) and sweet potato (*Ipomoea batatas*) (Bridle and Timberlake, 1997). However a major problem with most anthocyanins has been insufficient stability in aqueous solutions at pHs above 3, and today the use of anthocyanins as food colorants is mainly limited to beverages and candies.

Chapter 2

EXPERIMENTAL METHODS USED

The isolation, purification and structure determination of pure anthocyanins are relatively time consuming processes, and because most anthocyanins are prone to be relatively unstable these processes must be handled with care. During the workup procedure the anthocyanins may also become more fragile because of the removal of stabilizing factors like free sugars and other phenolic compounds. Storage of anthocyanins in the dark, at low temperature and in the dry state, to reduce hydration and degradation, is therefore preferable. Typical procedures for isolation and characterization of pure anthocyanins consist of several steps: 1) extraction of the plant material, followed by a preliminary purification, step 2) fractionation of the mixture followed by isolation of pure pigments, and finally step 3) characterization and identification of pure anthocyanins (Strack and Wray, 1989).

In this chapter follows some details regarding experimental procedures and principles. See the individual papers for further details concerning exact procedures.

2.1 Extraction and purification

Extraction. Anthocyanins were normally extracted with methanol containing 0.5% trifluoracetic acid (TFA) (v/v). The black beans (*Phaseolus vulgaris*) were also pre-soaked in water containing 0.5% TFA (paper **V–VIII**). In this case this was done to improve the

extraction yields of anthocyanins because direct methanolic extractions provide very poor yield. The extraction was performed in refrigerator (5ºC) at low temperatures to avoid hydrolysis of potential acyl groups in the anthocyanin structure, and degradation. After extraction the extract was filtered, and the methanol was removed by evaporation under reduced pressure at relatively low temperatures (<30ºC).

In all papers (**I**–**VIII**) the following purification procedures with ethyl acetate and Amberlite XAD-7 column chromatography were performed.

Liquid-liquid partition. The combined aqueous concentrates after evaporation were purified by partition against ethyl acetate to remove chlorophylls, stilbenoids, less polar flavonoids and other non polar compounds from the mixture.
Amberlite XAD-7 (adsorption chromatography). The aqueous extracts obtained after the liquid-liquid partition step will also contain other water soluble compounds than anthocyanins, like free sugars and aliphatic acids. These non-aromatic compounds were removed with the use of Amberlite XAD-7 column chromatography. Amberlite XAD-7 adsorbs the aromatic compounds including anthocyanins and other flavonoids in aqueous solutions, whereas free sugars and other polar non-aromatic compounds were removed by washing with distilled water until the eluted water has a neutral pH. Then the adsorbed anthocyanins and other flavonoids were eluted using methanol containing 0.5% TFA (v/v) as mobile phase (Andersen, 1988a).

2.2 Sample fractionation and isolation of pure pigments

Size-exclusion chromatography and preparative HPLC have been used in fractionation and isolation of pure pigments.

Gel filtration column chromatography. In this work both Sephadex LH-20 (paper **I**, **IV**–**VIII**) and Toyopearl HW-40F (paper **I**–**VIII**) were used as column material. Both theses materials separate with respect to molecule size. The Toyopearl HW-40F material has smaller particle sizes than Sephadex LH-20, which implies a slower elution, higher degree of gel filtration and exclusion (Frøytlog et al., 1998). This makes Toyopearl HW-40F material suitable for separation of structurally similar pigments. But both techniques can provide excellent separation (Andersen and Francis 1996, Frøytlog et al., 1998; Andersen and Francis 2004;

Jordheim et al., 2006a). The purified XAD-7 extracts were dissolved in a small amount of the initial mobile phase (MeOH/H_2O/TFA; 20:80:0.5, v/v) followed by application. Separation was achieved by isocratic or gradient elution using increasing amounts of methanol. Since the main principle for the two types of column material used is size-exclusion, the anthocyanins may be mainly eluted in order of decreasing molecular mass. For example anthocyanidin tri-glycosides are eluted prior to anthocyanidin di-glycosides followed by anthocyanidin mono-glycosides. Acylated anthocyanins are usually more retarded than non-acylated anthocyanins because of increased adsorption (Henke, 1995). The anthocyanin containing fractions were collected on the basis of observed visual band separation.

Preparative HPLC. This is a technique with high resolving power. The instruments used was a Gilson 305/305 pump equipped with C_{18} reversed-phase column (ODS-Hypersil column (25 × 2.2 cm, 5 μm)) coupled to a multidiode array detector (HP-1040 A) (paper **II**) and a Gilson 305/306 pump equipped with a UV 6000LP detector and an ODS Hypersil column (25 × 2.2 cm; i.d.; 5 μm) (paper **I** and **IV**). The latter instrument was operated by Robert Byamukama at Makerere University, Kampala, Uganda. Information about the pigment retention times, UV/Vis spectra and peak purities could be obtained. The polar mobile phase used was a gradient consisting of variable proportions of H_2O-HCOOH (WF) (9:1, v/v) and H_2O-HCOOH-CH_3OH (WFM) (4:1:5, v/v).

2.3 Quantitative determination

Prior to the quantitative determination of various berries described in paper **II**, the berries were freeze-dried and pulverized. 1 g of each pulverized sample was weighed accurately, placed into a 15 mL screw-cap glass and extracted with 5 mL acidified methanol (0.5 % TFA) with magnetic stirring for 2 h followed by centrifugation at 3000*g* for 5 min. The supernatant was removed and stored in a sealed glass tube in freezer at -20ºC. This procedure was repeated twice. The combined supernatants were transferred into a volumetric flask to determine the total volume followed by HPLC analysis.

The quantitative determination of anthocyanins of *Hippeastrum* cultivars described in paper **IV** was performed on an extract (10 mL) of 5 g of fresh plant material. After one extraction no remaining visible colour could be observed in the plant material.

Prior to injection the solutions were filtered through a 0.45 μm Millipore membrane filter and 15 μl of the extract(s) was injected on the HPLC. The quantitative amounts were determined from a HPLC calibration curve of pure cyanidin 3-*O*-*β*-galactoside (isolated from *Aronia melanocarpa*), without taking into account the variation of molar absorption coefficients for individual pigments. The calibration curve was based on HPLC chromatograms recorded at 520 ± 20 nm for four (**II**) and seven (**IV**) different pigment concentrations. Statistical significance of 5 % ($p < 0.05$) was chosen, and a Student's t-test (Minitab) was performed.

2.4 Hemisynthesis of carboxypyranoanthocyanins

The simplest procedure to obtain the flavylium ring system was provided by the 100 years old work of Bülow and Wagner (Harborne, 1982; reviewed by Iacobucci and Sweeny, 1983).

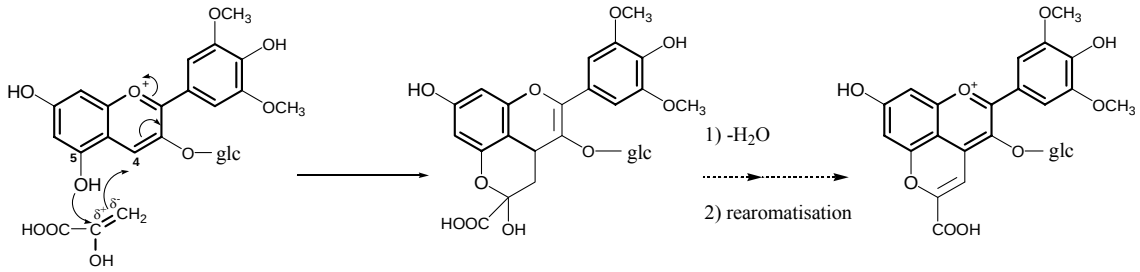


Figure 7. Mechanism postulated for the reaction between pyruvic acid and malvidin 3-glucoside (Fulcrand et al., 1998).

The procedures developed by Robinson and co-workers in the 1930s on synthesis of natural anthocyanin glycosides were epochal. An alternative approach to the synthesis of anthocyanidins and anthocyanins is provided by hemisynthetical procedures based on the reduction of flavanones, dihydroflavonols or flavonols to the corresponding anthocyanins.

In 1998 Fulcrand and co-workers identified carboxypyranoanthocyanins to be a class of stable pigments that could be derived from the reaction between pyruvic acid and grape anthocyanins. The formation of carboxypyranoanthocyanins are postulated to result from cyclisation between C-4 and the hydroxyl group at C-5 of the original flavylium moiety with the double bond of the enolic form of pyruvic acid, followed by dehydration and re-aromatisation steps (Figure 7). The reaction is thought to be an important route for conversion of grape anthocyanins into more stable pigments during maturation and ageing of wine.

In paper **V** carboxypyranoanthocyanins were produced by mixing the Amberlite XAD-7 purified anthocyanins (10 g), isolated from black beans (*Phaseolus vulgaris*), dissolved in ethanol (100 mL) containing 2 mL TFA and 2-oxopropanoic acid (pyruvic acid) (100 g) (Fluka, Germany) dissolved in distilled water (900 mL) (Fulcrand, et al., 1998; Jordheim et al., 2006a). The mixture was kept at 45ºC, and the synthesis was monitored by on-line HPLC (Agilent 1100 Series) after 5 min, 2 h, 5 h, 9 h, and 23 h. The reaction was terminated after 23 h by placing the reaction bottle in a refrigerator (4ºC). After termination of the synthesis, the ethanol in the reaction mixture was removed under reduced pressure, before the remaining aqueous concentrate was applied to an Amberlite XAD-7 column (70 × 5 cm). Excessive pyruvic acid was washed away with water (2 L), before the pigment mixture was eluted with methanol containing 0.5% TFA (v/v) (1 L).

2.5 Characterization and structure determination

For characterization and structure determination of individual pigments different chromatographic and spectroscopic techniques have been used; thin layer chromatography (TLC) (paper **I**, **III**, **IV** and **V**), colour measurements (paper **IV**), analytical HPLC, UV-Vis spectroscopy, mass spectrometry (MS) (paper **I-III**) and Nuclear Magnetic Resonance spectroscopy (NMR). TLC, HPLC and UV-Vis spectroscopy may give a lot of characteristic information about the type of anthocyanin, MS provides the molecular mass of the anthocyanin, but the application of a powerful NMR instrument is usually required for complete structure identification of anthocyanins. Structure elucidation of anthocyanins comprises 1) aglycone, 2) sugar units, and 3) acyl groups, as well as 4) determination of linkage positions between the different sub-groups.
In paper **VIII** coulometric determinations and FRAP were used to measure the reducing capacity of anthocyanins, these experimental methods were performed at Matforsk AS, Norwegian Food Research Institute, Oslo, Norway, by Kjersti Aaby.

Thin Layer Chromatography (TLC). TLC is considered to be one of the simplest of the chromatographic techniques. TLC facilitates short acquired time, and is a relatively inexpensive procedure. TLC was carried out on 0.1 mm cellulose F (Merck) plates (stationary phase) with the solvent FHW (HCO_2H – conc. HCl – H_2O; 25:24:51, v/v) (mobile phase). Authentic anthocyanins from the following sources were used as standards; strawberry (*Fragaria ananassa*) (Nerdal et al., 1992), black currant (*Ribes nigrum*) (Frøytlog et al.,

1998), lingonberry (*Vaccinium vites-idaea*) (Andersen, 1985), black elderberry (*Sambucus nigrum*) (Andersen et al., 1991) and American elderberry (*Sambucus canadensis*) (Johansen et al., 1991; Nakatani et al., 1995).

In the FHW solvent system anthocyanins with similar structure regarding sugars and acyl units will be separated with respect to the number of hydroxyl and methoxyl groups on the B-ring of the aglycone. Increasing hydroxylation and methoxylation will result in a decreasing retention factor (R_f). The hydroxyl groups have a greater impact than the methoxyl groups. The retention time will also increase with increasing number of sugars and acyl groups in the anthocyanin structure (Andersen, 1988b.).

Reversed phase analytical HPLC. HPLC is the method of choice for the accurate determination of both the composition and the concentration of anthocyanins in a given sample (Andersen and Francis, 2004 and reference therein; Merken and Beechner, 2000). In the papers (**I-VIII**) the HPLC equipped with a diode-array detector, analyses are performed using a C_{18} reverse phase column (250 × 4.6 mm, 5 μm particles). The elution system was binary, with an aqueous acidified solvent (A) and a less polar acidified acetonitrile (B) solvent.

The main chromatographical separation principle involved in reversed-phase HPLC is the partition of solutes between the polar mobile phase and the non-polar stationary phase. The overall polarity and the stereochemistry of the anthocyanins are the key factors for separation (Strack and Wray, 1989, 1994; Andersen and Francis, 2004). The elution of anthocyanins in reversed-phase HPLC columns depends on the pattern of hydroxylation/methoxylation of the aglycone, the degree of glycoslyation and acyl substitution, as well as on the mobile phase composition and solvent gradient steepness. The nature of the aglycone contribute to anthocyanin retention in the order; delphinidin < cyanidin < pelargonidin < petunidin < peonidin < malvidin. Anthocyanin glycosides elute in the following order: 3, 7-diglucosides < 3, 5-diglucosides < 3-sophorosides < 3-galactosides < 3-lathyrosides < 3-sambubioscides < 3-glucosides < 3-arabinosides < 3-rutinosides < 3-rahmnosides. The presence of aromatic or aliphatic acylation increases retention times compared to the corresponding non-acylated derivatives.

UV-Visible spectroscopy (UV-Vis). UV-Vis spectra of the compounds discussed in this work were obtained online during the various analytical HPLC analyses or with a Cary 3 UV-instrument. The most important spectral parameters derived from the UV-Vis spectra of

anthocyanins are; $\lambda_{Vis\text{-}max}$, the absorption (A) at λ = 440 nm compared to A at $\lambda_{Vis\text{-}max}$ ($A_{440}/A_{Vis\text{-}max}$), and A at $\lambda_{Uv\text{-}max}$ compared to A at $\lambda_{Vis\text{-}max}$ ($A_{Uv\text{-}max}/A_{Vis\text{-}max}$). The absorption maxima in the visible region are mainly dependent of the nature of the aglycone, the position of sugar substituents on the aglycone, and the presence of aromatic acyl groups. In general, for anthocyanin 3-*O*-glycosides having a free 5-hydroxyl, the value of $A_{440}/A_{Vis\text{-}max}$ is in the range of 0.2-0.3. When the 5-position is glycosylated, $A_{440}/A_{Vis\text{-}max}$ is in the range of 0.1-0.2 (Harborne, 1958). The presence of aromatic acylation can be determined using the ratio $A_{Uv\text{-}max}/A_{Vis\text{-}max}$. Typical mono-aromatic acylation of anthocyanins may give a $A_{Uv\text{-}max}/A_{Vis\text{-}max}$ ratio of ~ 0.6-1.3. A higher ratio may indicate several aromatic acyl residues (Ando et al., 1999). Since aliphatic acyl groups are lacking significant UV-Vis absorption, their presence can not be directly detected by UV-Vis spectroscopy.

Colour measurements (CIELab system). Colour measurements were performed with an Ultra Scan XE Hunter Colorimeter and the colours were described with the basis in the CIEL*C*h_{ab} system (Figure 8). Colour measurements were performed in paper **IV**.

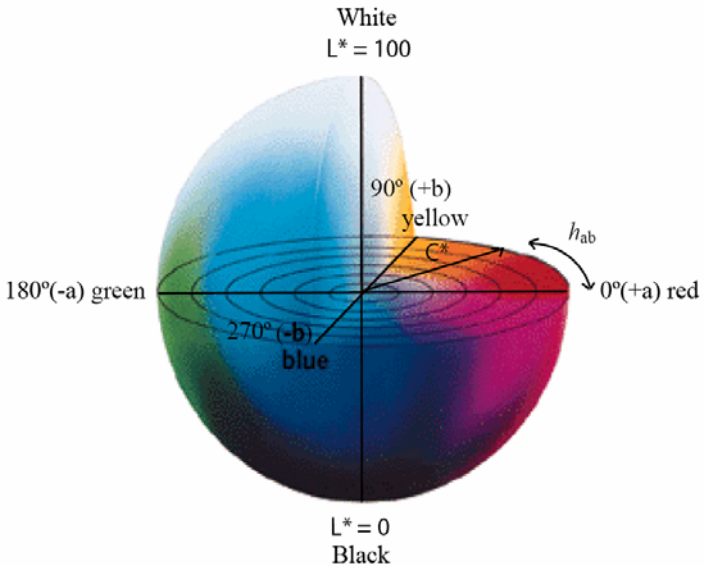


Figure 8. The three-dimensional CIELab colour space, showing h_{ab} (hue angel), C* (chroma) and L* (lightness). (Modified figure from http://www.linocolor.com)

The colour measurements were performed on anthocyanins dissolved in acidified methanol (0.5 % TFA). The L* describes the lightness of the colour, going from black (L* = 0) to white (L* = 100). The C* parameter describes the chroma or saturation of the colour, a measure of

how far from the grey tone the colour is. The higher the C* value, the more saturated is the colour. The hue angle (h_{ab}) describes the colour tonalities (red (0º), green (180º/-180º), blue (270º/-90º) and yellow (90º)). The hue angel is based on the CIEL*a*b* system, where a and b are Cartesian coordinates, and these Cartesian coordinates are based on the tristimulus values X, Y, and Z (Gonnet, 1998).

Coulometric detection. Electrochemical (EC) detectors measure chemical properties of a compound, and not a physical property such as UV absorption (Manach, 2003). Different electrochemically active substituents on similar compounds can lead to characteristic voltammetric behaviour. Coulometric detection has been used fragmentary for characterisation and quantification of flavonoids (Gamache et al., 1993; Milbury, 2001; Manach, 2003). A positive, linear correlation between antioxidant activity of fruits and vegetables measured as ORAC values and the total electrochemical responses obtained by HPLC coupled to a coulometric array detector, has been reported by Guo et al. (1997). Yang and co-workers (2001) have suggested that electrochemical properties of related flavonoids may be used as indexes of their antioxidant activities in biological systems. With respect to anthocyanins, Kozminski and Brett (2006) have recently showed that HPLC with electrochemical detection is more sensitive than using a photodiode array detector for separation and determination of six common anthocyanins. Aaby et al. (2004) have studied what parameters in the coulometric analysis, which best describe the antioxidant activities of anthocyanins and other phenolic compounds.

Nuclear Magnetic Resonance (NMR). The establishment of the detailed structure of anthocyanins usually requires information from several different techniques, including analytical HPLC and UV-Vis spectroscopy, NMR and mass spectrometry (MS). Recent technological advances in development of high-field magnets and cryoprobe technology have further improved the resolution and sensitivity of the powerful NMR techniques. With a combination of various 1D and 2D NMR experiments the assignment of all ^{1}H and ^{13}C resonances in an anthocyanin structure is possible (Pedersen, 1996; Andersen and Fossen, 2003; Fossen and Andersen, 2006; Jordheim et al., 2006b). Different NMR experiments used in this work are commented below.

1D ^{1}H NMR. The 1D proton spectra of anthocyanins provide quantitative information about proton chemical shifts and their coupling constants (J_{HH}) and give quantitative information by

integrating baseline-separated signals or selected spectral regions. Information about the nature of the aglycone, type and number of sugar and acyl substituents can also be provided. The chemical shift values also indicate linkage positions between different sub-units of the anthocyanin.

1D ^{13}C NMR. Spin Echo Fourier Transform (SEFT) and Compensated Attached Proton Test (CAPT) have been used along with different 2D techniques to obtain the accurate carbon chemical shifts. The SEFT sequence suffers from the use of a 90º-excitation pulse, which requires long repetition times. This feature has been significantly improved with CAPT.

Due to low abundance (1.1%) and the lesser favourable magnetogyric ratio of ^{13}C compared to ^{1}H, the ^{13}C-spectra have lower signal to noise levels than the corresponding ^{1}H-spectra. In addition will the ^{13}C signal normally decrease in intensity because of the J_{CH}-couplings, which will split the signals into multiplets. But the last problem is eliminated with proton decoupling where multiplets collapse into singlets. The signals (C, CH, CH_2, CH_3) are differentiated with the aims of different delays in the pulse-program. The C and CH_2 signals will be distinguish from the CH and CH_3 signals by having opposite phases.

2D ^{1}H-^{1}H TOtal Correlation SpectroscopY (2D TOCSY). This is a two dimensional homo-nuclear NMR technique which has diagonal peaks, and identical proton chemical shift axes as the COSY technique. TOCSY is used to find the proton chemical shifts for all protons which belong to the same spin system, even if the protons are not directly *J*-coupled. Thus, TOCSY is very useful for determination of individual ^{1}H chemical shifts of the various sugar units linked to the anthocyanidin. This is particularly relevant when the anthocyanin contains more than one sugar unit.

2D ^{1}H-^{1}H gradient selected, Double Quantum Filter Correlation SpectroscopY (gs-DQF-COSY). This technique is used to assign the different proton signals based on the couplings through bonds (*J*-coupling). COSY is a 2D homo-nuclear technique where the diagonal peaks represent the actual proton spectrum and the crosspeaks show which protons are *J*-coupled to each other (Figure 9).

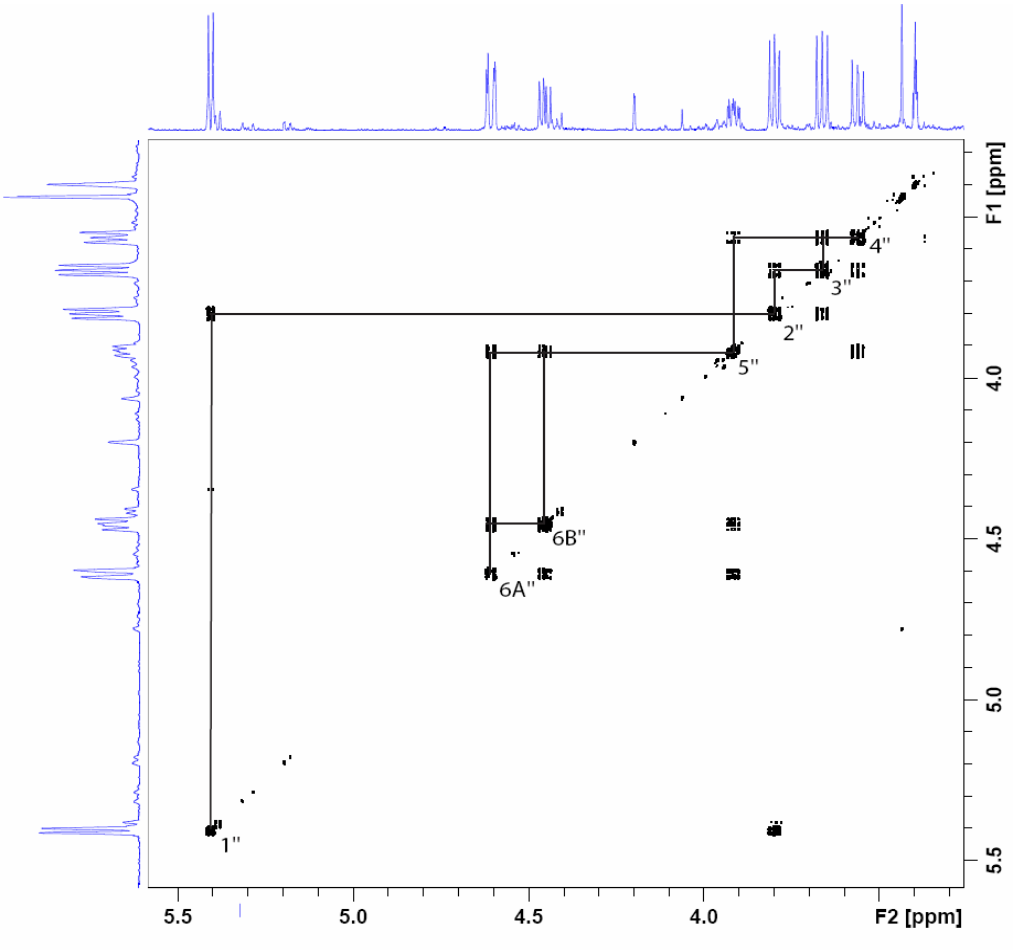


Figure 9. ^{1}H-^{1}H COSY NMR spectrum (600.13 MHz) of the sugar region of cyanidin 3-*O*-*β*-(6"-*E*-caffeoylglucopyranoside) (**15**) in CF_3CO_2D–CD_3OD (5:95, v/v) recorded at 25ºC, isolated from gooseberries (*Ribes grosssularia* L.).

2D ^{1}H-^{1}H Nuclear Overhauser and Exchange Effect SpectroscopY (2D NOESY). This is a 2D homo-nuclear technique which is based on coupling through space. The method can provide information about the molecular geometry, conformation and linkage between anthocyanin sub-units. Exchange cross peaks between analogous protons of species that are in equilibrium with each other may be observed in NOESY spectra, which will result in positive cross peaks (Figure 10). A cross peak due to NOE correlation will be negative (Santos et al., 1993; paper **VI**: Jordheim et al., 2006b).

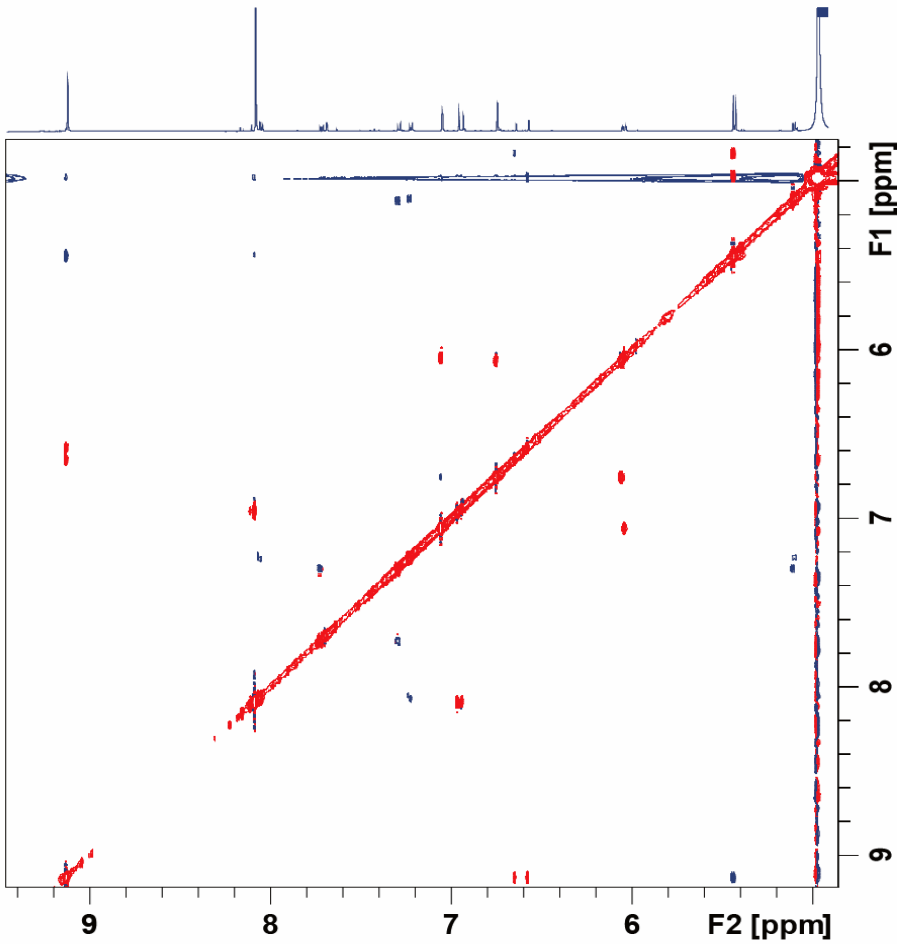


Figure 10. ^{1}H-^{1}H NOESY NMR spectrum (600.13 MHz) of the expanded aromatic region of malvidin 3-*O*-*β*-glucopyranoside (**27**) in pure CD_3OD recorded at 25ºC, isolated from black beans (*Phaseolus vulgaris*). Negative cross-peaks due to NOE correlation are blue. Positive cross-peaks due to chemical exchange are red.

2D ^{1}H-^{13}C gradient-selected Heteronuclear Single Quantum Coherence (gs-HSQC). The inverse-detected 2D-heteronuclear experiment correlates ^{1}H and ^{13}C chemical shifts through single-bond heteronuclear couplings $^{1}J_{CH.}$ The HSQC spectrum shows only protons that are directly attached to a carbon atom and vice versa.

2D ^{1}H-^{13}C gradient-selected Heteronuclear Multiple Bond Correlation (gs-HMBC). The HMBC correlates ^{1}H and ^{13}C chemical shifts through multiple-bond heteronuclear couplings. The most important ones are $^{2}J_{CH}$ and $^{3}J_{CH}$, for which the strongest cross-peaks are observed. In addition some $^{1}J_{CH}$ correlations and some long distance correlations may be observed. In the spectra recorded for the anthocyanins the $^{1}J_{CH}$ large doublet may be observed in the

HBMC spectra because of incomplete suppression. In the heteronuclear multiple-bond correlation spectra, most quaternary carbon resonances may be assigned.

Mass Spectrometry (MS). Mass Spectrometry has in the present work been applied to measure the molecular mass, and only in some cases fragment ions, with the purpose of verification of the various structure determinations. Mass spectral measurements were obtained by electrospray ionization in positive (ESP+) mode using a JMS-T100LC with an AccuTOF LP mass separator in paper **I** and **II** performed by the Jeol Company (Paris). In paper **III** the electrospray mass spectrometry analysis was performed on a Quattro II MS/MS (Micromass, UK) with API source and flow injection at Polyphenols Laboratory AS (Sandnes, Norway).

The Ferric Reducing Ability of Plasma (FRAP assay). The FRAP assay is a simple, automated test measuring the ferric reducing ability of plasma, and the assay is presented as a method which measures the "antioxidant power" (Benzie and Strain, 1996). This test was used in connection with paper **VIII** following the procedure described by Benzie and Strain (1996) with modifications (Aaby et al., 2004). The assays were carried out on a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany) using the 595 nm absorbance filter. Anthocyanin solution (250 μM, 10 μL) was added manually to the plate, and mixed with freshly prepared FRAP reagent (190 μL) added by the plate reader. The reaction was conducted at 27 °C, and absorbance measured every 2 min for 60 min.

Chapter 3

RESULTS AND DISCUSSION

3.1 New anthocyanin sources (I-IV)

Documentation of the anthocyanin content of different botanical sources is important for determination of structural diversity as well as for food and health perspectives. In this work different part of plants have been examined for anthocyanins including stem bark (**I**), berries (**II**, **III**) and flowers (**IV**). Novel compounds have been identified, and new information about anthocyanin composition in plants has been revealed.

3.1.1 New anthocyanins from stem bark of castor, *Ricinus communis* (I)

Ricinus communis L. (Euphorbiaceae) is a soft-wooded small tree widespread throughout the tropic and sub-tropic regions of the world (Ivan, 1998). It is an important oilseed crop that produces an oil rich in ricinoleic acid, which confers unique properties to the oil (Velasco *et al*., 2005; Rojas-Barros *et al*., 2004; Zhang *et al*., 2005). The structure of the anthocyanins in the castor plant has previously not been reported.

The HPLC chromatogram of the fresh acidified methanolic extract of the stem bark of *Ricinus communis* L. detected in the visible spectral region revealed two anthocyanins (**11** and **18)**. After storage in the extraction solvent, the HPLC chromatogram showed three

anthocyanins (**11, 18, 19**) (Figure 11). The relatively amounts of **11** and **18** in the initial extract were 21 and 79 %, respectively. After storage in the extraction solvent for weeks, the relative amounts of **11, 18** and **19** were 51, 12 and 37% respectively. The UV–Vis spectra of the three anthocyanins recorded on-line during HPLC analysis showed visible maxima around 520 nm, and their $A_{440}/A_{\text{Vis-max}}$ were in the range of 15 to 20%, indicating a 3,5-diglycoside based on cyanidin or peonidin aglycones.

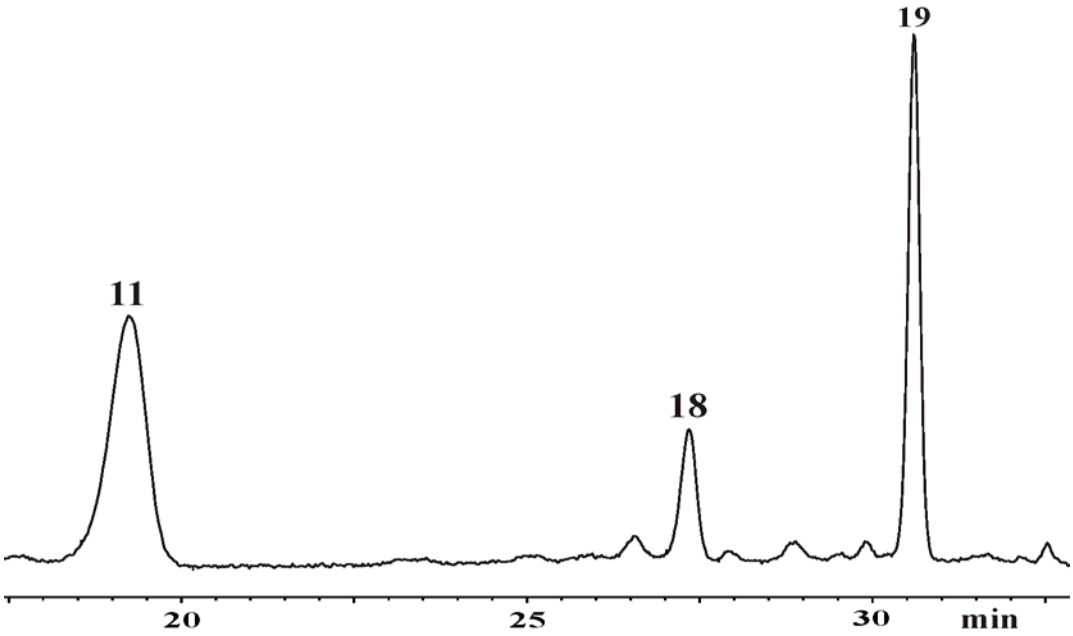


Figure 11. HPLC profile of the anthocyanins (**11**, **18** and **19**) in the *Ricinus communis* extract after storage.

*Structural elucidation of pigments (**11**, **18** and **19**)*

The downfield part of the 1D ^{1}H NMR spectrum of **11** showed a singlet at 9.04 ppm (H-4), a 3H AMX system at 8.42 ppm (*dd*, 8.8 Hz, 2.3 Hz; H-6'), 8.15 ppm (*d*, 2.3 Hz; H-2') and 7.12 ppm (*d*, 8.8 Hz; H-5') and an unresolved 2H AB system at 7.17 ppm (H-8) and 7.13 ppm (H-6), respectively, in accordance with the anthocyanin, cyanidin. The sugar region of the 1D ^{1}H NMR of **11** showed the presence of two sugar units revealed by two anomeric protons with a *β*-configuration (H-1": $^{3}J_{HH}$ = 7.0 Hz, H-1"': $^{3}J_{HH}$ = 7.9 Hz). The COSY and TOCSY spectra were in accordance with 13 sugar protons, which indicated that one of the sugar units was a pentose, and the other a hexose. Starting from H-1" at δ 5.49 (J =7.0 Hz), the observed cross-peak at 5.49/3.81 ppm in the COSY spectrum supported by corresponding crosspeak in the HSQC spectrum, permitted the assignments of H-2", H-3", H-4", H-5A" and H-5B". The

chemical shifts and the coupling constants of this glycosyl unit were in accordance with a *β*-xylopyranosyl. A crosspeak at δ 5.49/145.26 in the HMBC spectrum between H-1" and C-3 of the aglycone confirmed the connection point of the xylosyl unit to the 3-position of the aglycone. By using the doublet at δ 5.28 (J = 7.9 Hz) as the starting point in the COSY and TOCSY spectra, it was likewise possible to assign all the chemical shifts for the second monosaccharide moiety, *β*-glucopyranosyl. A cross peak at δ 5.28/156.95 in the HMBC spectrum confirmed the connection point of this unit to be in the 5-position of the aglycone. The molecular mass (m/z 581.1495) in the ESI+ high resolution mass spectrum of **11** corresponding to $C_{26}H_{29}O_{15}^{+}$, confirmed the structure to be cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside, which is a new anthocyanin in plants.

The NMR resonances of pigment **18** shared many similarities with the corresponding resonances of **11**, in accordance with a cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside derivative. However, the chemical shift values of H-6A''' (*δ* 4.62), H-6B''' (*δ* 4.42), H-5''' (*δ* 3.89) and C-6''' (*δ* 65.3), indicated the presence of acylation at the 6'''-hydroxyl. The crosspeaks at *δ* 4.62/168.7 (H-6A'''/M^{I}) and 4.42/168.7 (H-6B'''/M^{I}) in the HMBC spectrum confirmed that an acyl moiety was linked to this hydroxyl group. The molecular mass (m/z 667.1478) in the ESI+ high resolution mass spectrum of **18** corresponding to $C_{29}H_{31}O_{18}^{+}$, was in accordance with cyanidin 3-xylopyranoside-5-glucopyranoside with an additional malonyl unit. Thus, the identity of **18** was determined to be the new anthocyanin cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-(6'''-malonylglucopyranoside).

Pigment **19** was identified as the esterified form of pigment **18**. Methyl esterification of the terminal carboxyl group of malonyl units may occur easily in the acidified methanolic solvents normally used for extraction and isolation (Fossen et al., 2001; Bloor and Abrahams, 2002). The molecular ion at *m*/*z* 681.1478 in the positive ion ESI was in accordance with cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-(6'''-malonylglucopyranoside) with an additional mass of 14 amu. The crosspeak at *δ* 3.76/168.6 (H-M^{IV}/C-M^{III}), and the crosspeaks at *δ* 4.66/167.9 (H-6A'''/C-M^{I}) and 4.42/167.9 (H-6B'''/C-M^{I}) in the HMBC (Figure 12) confirmed the identity of pigment **19** to be cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-(6'''-methylmalonateglucopyranoside).

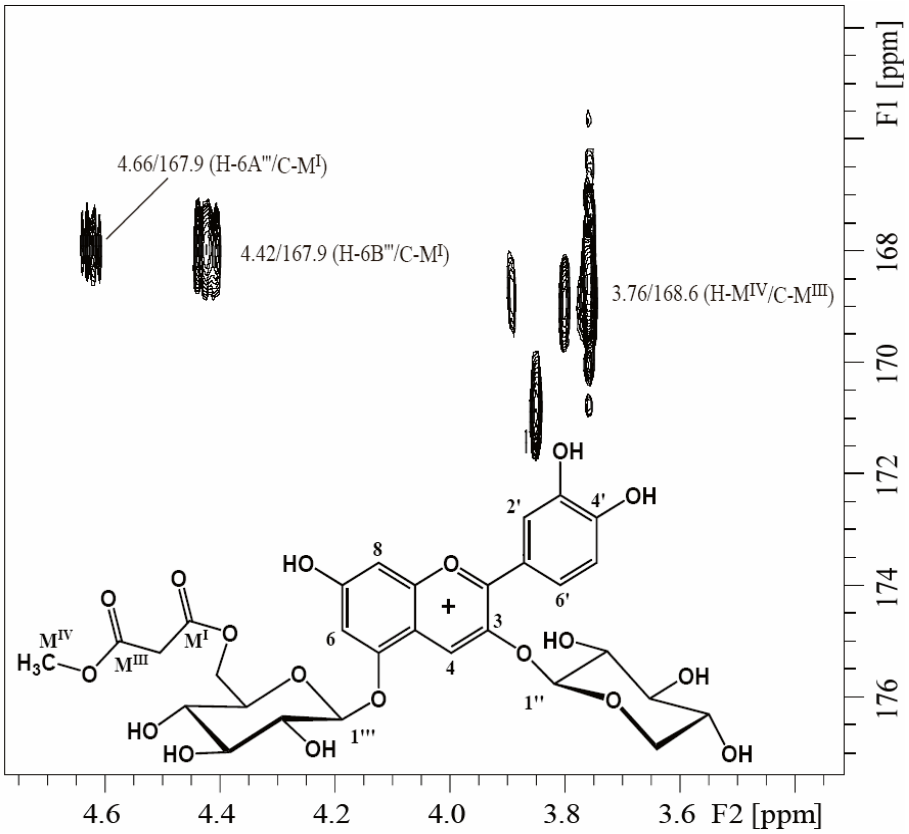


Figure 12. Expanded region of the HMBC spectrum of pigment **19** in CF_3CO_2D–CD_3OD (5:95, v/v) recorded at 25ºC.

3.1.2 Anthocyanins in berries of *Ribes* including gooseberry cultivars with high content of acylated pigments (II)

In slightly acidic to neutral aqueous solutions most anthocyanins without aromatic acylation occur on their most instable equilibrium forms (Cabrita et al., 2000). Some acylated anthocyanins have been reported to have unique physiological functions (Matsui et al., 2001; Noda et al., 2000), however, the absorption and bioavailability of anthocyanins with aromatic acylation in humans are controversial (Suda et al., 2002; Harada et al., 2004; Giusti and Wrolstad, 2003; Kurilich et al., 2005; Karakaya 2004; Fleschhut et al., 2006; Ichiyanagi et al., 2006). Several species belonging to the genus *Ribes*, such as black currants (*R. nigrum* L.) and red currants (*R. rubrum* L.), are among the most commonly consumed berries in the Western diet. The gooseberries are cultivated in many private gardens; however, in recent years they have been of limited commercial value.

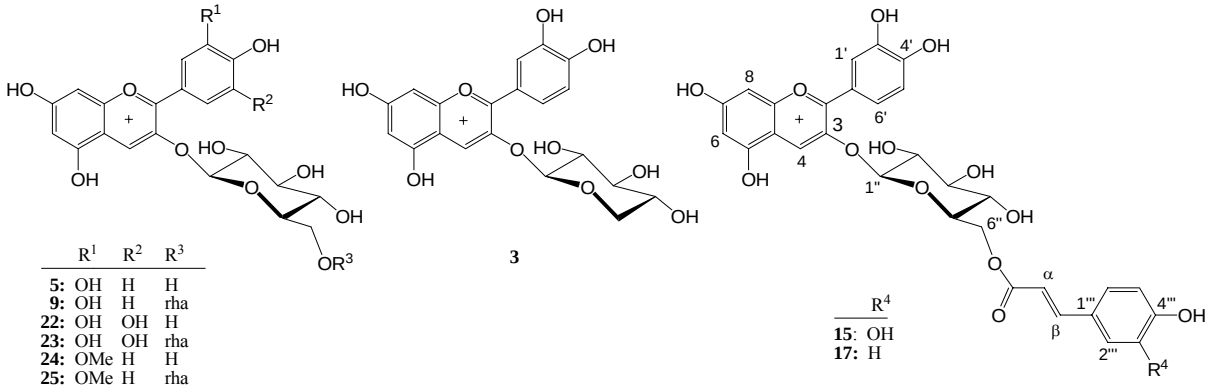


Figure 13. Structures of the anthocyanins identified in the examined *Ribes* species. **3**: Cy 3-xyl, **5**: Cy 3-glc, **9**: Cy 3-[6-(rha)glc], **15**: Cy 3-[6-*E*-(caf)]glc, **17**: Cy 3-[6-*E*-*p*-(cum)glc], **22**: Dp 3-glc, **23**: Dp 3-[6-(rha)glc], **24**: Pn 3-glc, **25**: Pn 3-[6-(rha)glc], pigment **16** is similar to **17**, but the *p*-coumaroyl has *cis* configuration. Cy = cyanidin; Dp = delphinidin; Pn = peonidin; xyl = xyloside; rha = rhamnoside; glc = glucoside; cum = coumaroyl; caf = caffeoyl.

In fourteen cultivars of European gooseberry (*R. grossularia* var. *uva crispa*), the alpine currant (*R. alpinum* L.), golden currant (*R. aureum* Pursh), red flowering currant (*R. sanguineum* Pursh) and the two cultivars of Jostaberries (*R.* × *nidigrolaria* Bauer) altogether

nine (**3**, **5**, **9**, **15**, **17**, **22-25**) different anthocyanins where identified (Figure 13), with the aims of different chromatographic and spectroscopic techniques.

*Structural elucidation of pigments (**15** and **17**)*

The 1D ^{1}H NMR spectra of both **15** (Figure 14) and **17** revealed the existence of a cyanidin aglycone, one monosaccharide, and one *E*-hydroxycinnamic acyl group.

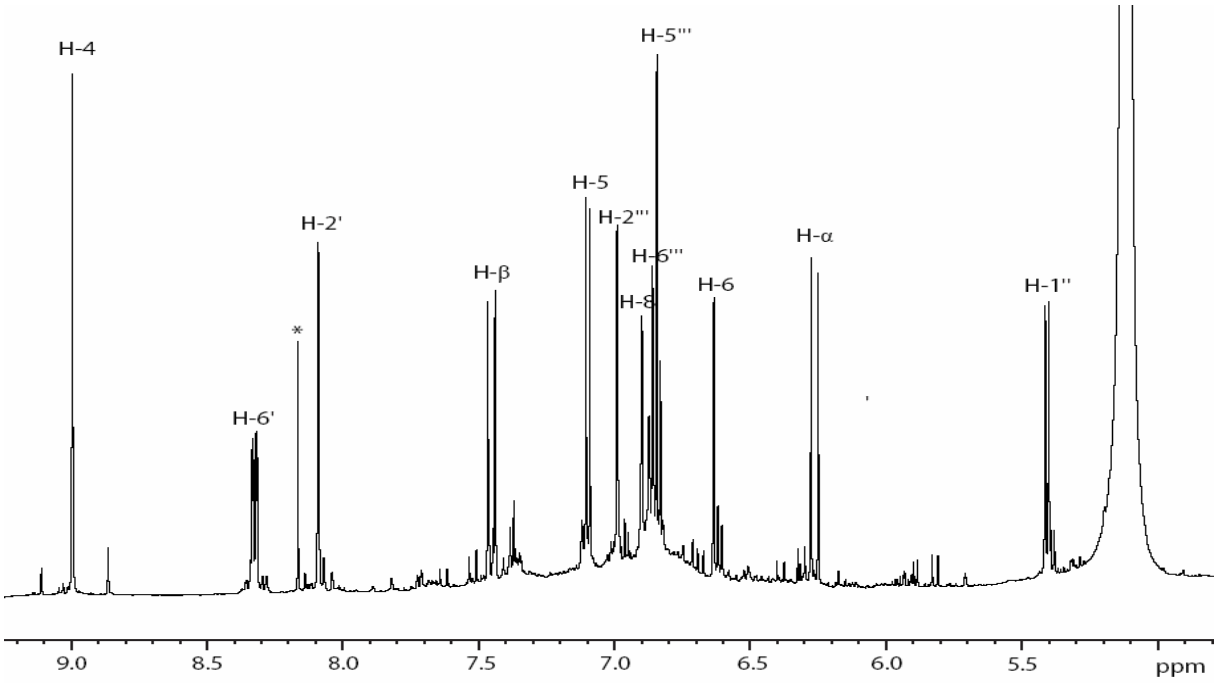


Figure 14. ^{1}H NMR spectra (600.13 MHz) of cyanidin 3-*O*-*β*-(6"-*E*-caffeoylglucopyranoside) (**15**) in CF_3CO_2D–CD_3OD (5:95, v/v) recorded at 25ºC.

HMBC spectra were used to assign C-8, C-10, C-3, C-9, C-5, C-2, C-4 and the carbons belonging to the anthocyanidin B-ring. Exact ^{13}C chemical shift values were obtained from a ^{13}C CAPT spectrum (Figure 15). The ^{1}H and ^{13}C resonances of the monosaccharides of **15** and **17** were assigned by a combination of 1D ^{1}H NMR, DQF-COSY, TOCSY, and HSQC experiments, in accordance with *β*-glucopyranose. The crosspeaks at 5.41/145.1 (**15**: H-1"/C-3) and 5.39/144.7 (**17**: H-1"/C-3) in the HMBC spectra confirmed the linkage between the aglycone and the sugar unit to be at the 3-hydroxyl.

The doublets at *δ* 6.99 (*d*, 1.8 Hz; H-2'''), 6.26 (*d*, 15.9 Hz; H-*α*) and 7.45 (*d*, 15.9 Hz; H-β), together with the multiplets at 6.84 (H-5''') and 6.86 (H-6''') in the 1D ^{1}H NMR spectrum of **15** where in accordance with a caffeoyl unit.

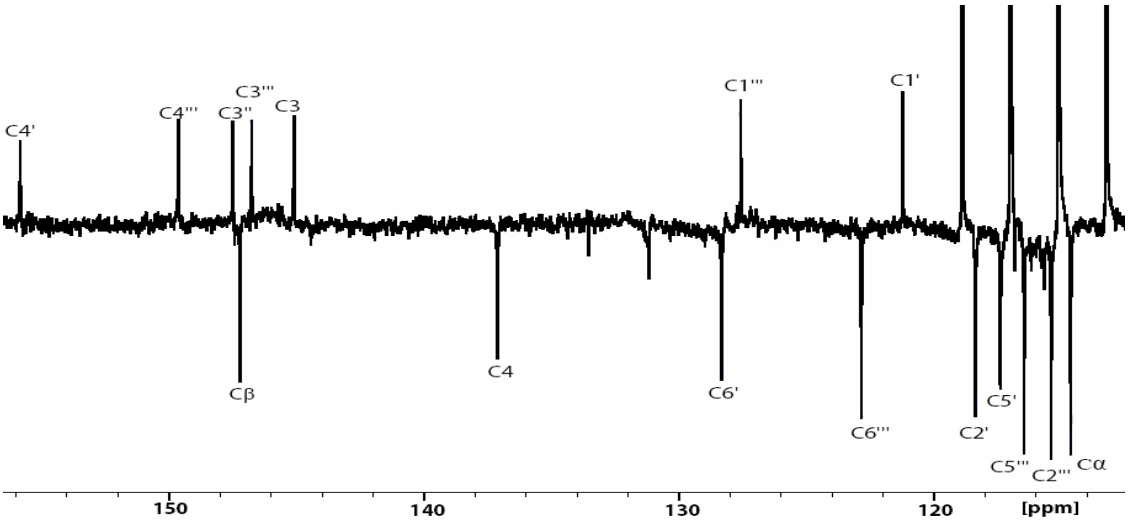


Figure 15. Expanded region of the ^{13}C CAPT NMR (600.13 MHz) spectrum of cyanidin 3-*O*-*β*-(6"-*E*-caffeoylglucopyranoside) (**15**) in CF_3CO_2D–CD_3OD (5:95, v/v) recorded at 25ºC.

The crosspeaks at *δ* 4.61/168.9 (H-6A"/C=O caffeoyl) and *δ* 4.45/168.9 (H-6B"/C=O caffeoyl) confirmed the linkage between the 3-glucose and the caffeoyl moiety to be at the 6"-hydroxyl, and the molecular ion at *m/z* 611.1379 in the high-resolution MS spectrum, were in accordance with cyanidin 3-*O*-*β*-(6"-*E*-caffeoylglucopyranoside). Pigment **15** has been tentatively identified in extracts of several plant species. However, this is the first examination of this pigment by NMR revealing the anomeric configuration and ring size of the *β*-glucopyranosyl, and the 6"-linkage to the caffeoyl moiety.

Most of the chemical shift values in the 1D ^{1}H NMR spectrum of **17** were similar to those of **16**. However, the shifts at *δ* 7.36 (*d*, 8.6 Hz; H-2"',6"'), 6.86 (*d*, 6.9 Hz; H-3"',5"'), 6.31 (*d*, 15.9 Hz; H-*α*) and 7.51 (*d*, 15.9 Hz; H-β), were in accordance with a *p*-coumaroyl unit. Figure 16 shows the HSQC spectrum of the sugar region of pigment **17**. The large downfield shift effects observed for 6A" and 6B", respectively (Table D-2), are due to the substitution by the *p*-coumaroyl unit. The ^{13}C NMR data supported the determination of the pyranose-form of the sugar moiety, and the identity of **17** was confirmed to be cyanidin 3-*O*-*β*-(6"-*E*-*p*-coumaroylglucopyranoside).

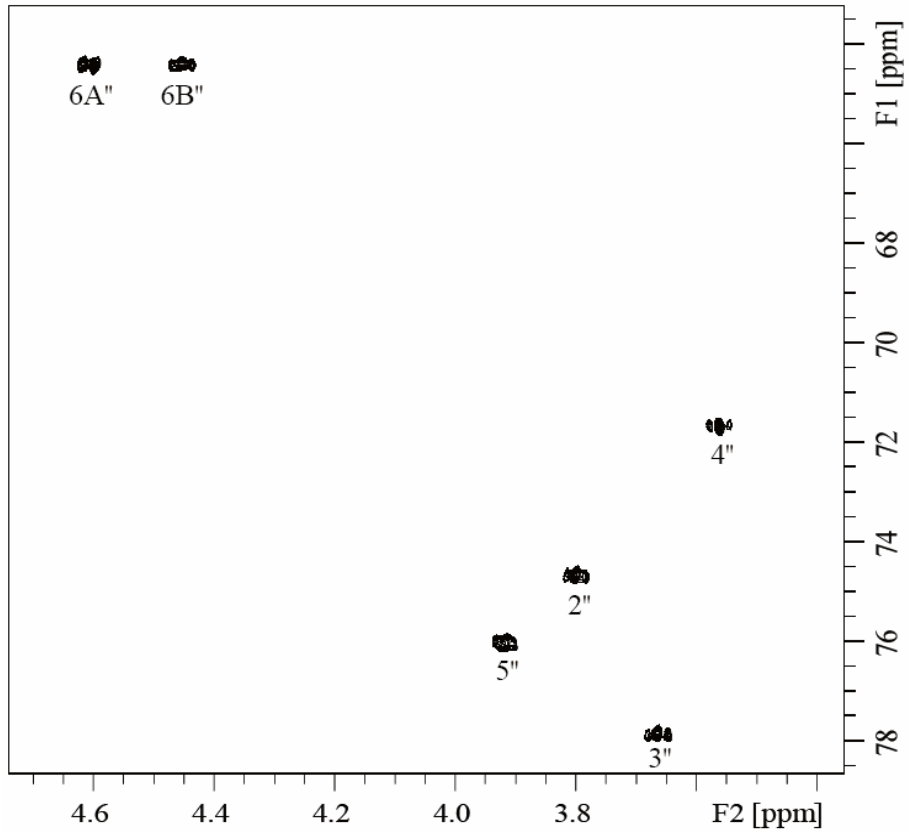


Figure 16. ^{1}H-^{13}C HSQC NMR spectrum of the sugar region of pigment **17** in CF_3CO_2D–CD_3OD (5:95, v/v) recorded at 25ºC.

Figure 17 shows the UV-Vis spectrum of the two pigments, **15** and **17**, with Vis_{max} at 523 nm, (local UV_{max} 283 nm, 329 nm) and Vis_{max} at 522 nm (local UV_{max} 283 nm, 314 nm), respectively.

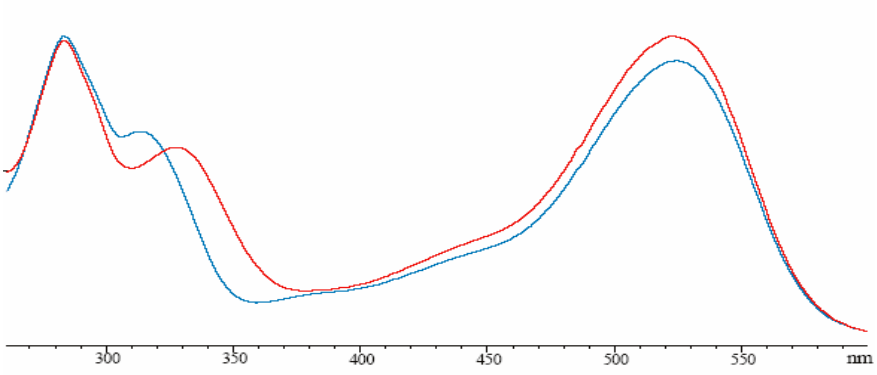


Figure 17. UV-Vis spectrum of cyanidin 3-(6"-*E*-caffeoylglucoside) (**15**) (red) and cyanidin 3-(6"-*E*-*p*-coumaroylglucoside) (**17**) (blue) recorded on-line during HPLC analysis.

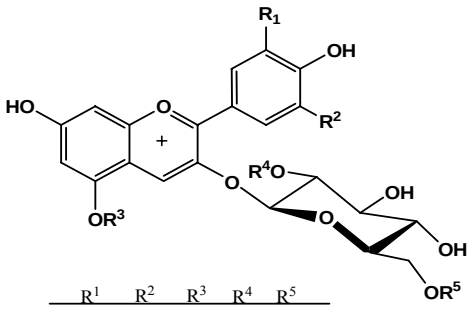
Qualitative and quantitative content

All the examined European gooseberry (*R. grossularia*) cultivars showed nearly the same qualitative anthocyanin content including pigments **3**, **5**, **9**, **15**-**17**, **24** and **25** (Figure 12) Peonidin 3-glucoside (**24**), cyanidin 3-(6"-*E*-caffeoylglucoside) (**15**) and cyanidin 3-(6"-*E*-*p*-coumaroylglucoside) (**17)** have only tentatively been assigned in gooseberries before (Määttä-Riihinen et al., 2004; Wu et al., 2004). Pigment **16** was tentatively identified as cyanidin 3-(6"-*Z*-*p*-coumaroylglucoside) based on high-resolution MS data (*m/z* 595.1405), and online-HPLC. The major anthocyanins of the two jostaberry (*Ribes x nidigrolaria*) cultivars 'Josta' and 'Jostine', (**5**, **9**, **15**, **17, 22**, **23**) reflected the major anthocyanins of both of its original parents, gooseberry and blackcurrant (Slimestad and Solheim, 2002; Frøytlog et al., 1998).

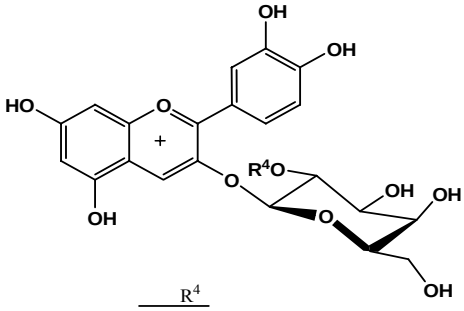
The total anthocyanin content in the examined gooseberry cultivars varied from 0.30 mg/g dry weight in 'Pax' to 2.23 mg/g dry weight in 'Glendale'. When assuming the water content of the fresh berries to be 88% (Wu et al., 2006), the anthocyanin amounts in 'Pax' and 'Glendale' corresponded to 3.60 and 26.76 mg/100 g fresh weight (FW), respectively. The two jostaberry cultivars, which originally are hybrids between gooseberry and black currant, had higher total anthocyanin content (40.0 and 45.7 mg/100 g FW) than the gooseberries, however, considerably lower content than reported for black currant (Clifford 2000; Määttä et al., 2001, Wu et al., 2006). The anthocyanin content in commercial available berries such as strawberry, red currant and black currant have been reported to be 15-41 mg, 13-18 mg and 130-500 mg/100 g FW, respectively (Clifford 2000; Määttä et al., 2001; Wu et al., 2006). The gooseberry cultivars with the highest anthocyanin content ('Glendal', 'Samsø', 'Rolanda') are thus in the range of red currant and strawberry. Several of the gooseberry cultivars contained relative high amounts of the aromatic acylated pigments, **15** and **17**. In 'Lofthus', 'Samsø', 'Martlet', 'Hinnonmäki Red' and 'Taastrup' these pigments constituted together as much as 57, 52, 52, 49 and 49% of the total anthocyanin content, respectively. When the total amount of acylated anthocyanins in the various cultivars are considered, 'Samsø', 'Hinnomäki Red', 'Taastrup', 'Lofthus' and 'Glendal' are the most obvious candidates for consumption, colorant and breeding programmes. As far as we know there exists no report on cultivated berries of commercial value used in the human diet containing anthocyanins acylated with aromatic acyl groups as the major pigments.

3.1.3 Anthocyanins in Caprifoliaceae (III)

Plants in the family Caprifoliaceae are perennial and mostly woody plants that include vines, shrubs, and small trees with berries taking colours from orange to black. Fruit characters are found to be particularly important in the classification of individual genera (Manchester and Donoghue, 1995). Caused by potential health benefits, the anthocyanin content in berries of some Caprifoliaceae species has received attention, in particular juices and extracts from elderberry, *Sambucus nigra*, which have been used in clinical studies (Abuja et al., 1998; Netzel et al., 2002; Wu et al., 2002; Bitsch et al., 2004). In a detailed study of floral anatomy and morphology it is suggested that the genus *Lonicera* has a different origin than *Sambucus* and *Viburnum* (Wilkinson, 1949). These studies indicated that *Sambucus* and *Viburnum* shared several characteristics not found in the rest of the family, and a segregation of these genera into two or more families have been discussed.



	R^1	R^2	R^3	R^4	R^5
1:	H	H	H	H	H
5:	OH	H	H	H	H
6:	OH	H	H	H	ara
8:	OH	H	H	xyl	H
9:	OH	H	H	H	rha
13:	OH	H	H	xyl	rha
14:	OH	H	glc	xyl	H
20:	OH	H	glc	xyl	*Z*-cum
21:	OH	H	glc	xyl	*E*-cum
22:	OH	OH	H	H	H



	R^4
4:	H
7:	xyl

Figure 18. 1 = Pg 3-glc, **4** = Cy 3-gal, **5** = Cy 3-glc, **6** = Cy3-[6-(ara)glc], **7 =** Cy 3-[2-(xyl)gal], **8** = Cy 3-[2-(xyl)glc], **9** = Cy 3-[6-(rha)glc], **13** = Cy 3-[2-(xyl)-6-(rha)glc], **14** = Cy 3-[2-(xyl)glc]-5-glc, **20** = Cy 3-[2-(xyl)-6-*Z*-*p*-(cum)glc]-5-glc, **21** = Cy 3-[2-(xyl)-6-*E*-*p*-(cum)glc]-5-glc, **22** = Dp 3-glc. Pg = pelargonidin; Cy = cyanidin; Dp = delphinidin; Pn = peonidin; ara = arabinoside; rha = rhamnoside; xyl = xyloside; gal = galactoside; glc = glucoside; cum = coumaroyl.

Twelve (**1**, **4-9**, **13**, **14**, **20-22**) (Figure 18) anthocyanins where identified by NMR and co-chromatography with authentic anthocyanins in the analysis of nineteen species belonging to

the *Sambucus*, *Lonicera* and *Viburnum* genera (Caprifoliacea). The authentic anthocyanins were from the following sources: *Fragaria ananassa*: **1** (Nerdal et al, 1992); *Ribes nigrum*: **5**, **9** and **22** (Frøytlog et al., 1998); *Vaccinium vitis-idaea*: **4** (Andersen, 1985); *Sambucus nigrum*: **13** (Andersen et al., 1991); *Sambucus canadensis*: **14**, **20** and **21** (Johansen et al., 1991; Nakatani et al., 1995). NMR elucidation of pigment **6** (cyanidin 3-*O*-*β*-(6"-*α*-arabinopyranosylglucopyranoside) is the first complete identification of the disaccharide vicianose (6"-*α*-arabinopyranosyl-*β*-glucopyranose), linked to an anthocyanidin.

Structure elucidation of cyanidin 3-O-β-*(6"*-α-*arabinopyranosylglucopyranoside) (**6**)*

The UV-Vis spectrum of **6** showed visible maximum at 528 nm with A_{440}/A_{528} of 27% in agreement with a cyanidin or peonidin 3-glycoside. A molecular ion at m/z 581 in the ESI-MS spectrum of **6** was in accordance with cyanidin connected to one hexose and one pentose unit. On the basis of the signals in the 1D ^{1}H NMR, ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{13}C SEFT, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectra, the chemical shifts (^{1}H and ^{13}C) of **6** were in agreement with cyanidin linked to one *β*-glucopyranose and one *α*-arabinopyranose unit. A possible way to determine the ring size of monosaccharides was to use the carbon shift values (Bock and Thøgersen, 1982). In arabinosides the ^{13}C shifts of C-2 and C-4 in arabinofuranoside were found to be 10-15 ppm downfield to the corresponding signals C-2 (72.9 ppm) and C-4 (69.6 ppm) in arabinopyranoside. The carbon shifts observed for the arabinoside unit in **6** were thus in agreement with the arabinopyranoside form. The cross-peak at *δ* 5.33/145.59 (H-1"/C-3) in the HMBC spectrum revealed that the glucose unit was attached to the aglycone 3-hydroxyl position. The high-field position of the anomeric proton of the arabinose unit (*d* 4.26) indicated a terminal sugar unit. The pronounced down-field shift of C-6" (*d* 69.51), and the cross-peak at d 4.26/69.51 in the HMBC spectrum between H-1'" and C-6" confirmed that the arabinose residue was connected to C-6" of the glucose ring, in accordance with cyanidin 3-*O*-*β*-(6"-*α*-arabinopyranosylglucopyranoside) (**6**).

Chemotaxonomy

The berries of species belonging to *Sambucus* vary from orange through red to black, but most of them are characterized by the same anthocyanins; cyanidin 3-sambubioside-5-glucoside (**14**) and its (*Z*)- and (*E*)-*p*-coumaroyl derivatives (**20** and **21**). When it comes to species in the genera *Lonicera* and *Viburnum*, the chemotaxonomic importance of their anthocyanin content is more limited. Simple anthocyanin 3-monoglucosides (mainly **5**) predominate in berries of *Lonicera* species, however, this is the most common anthocyanin

found in nature (Andersen and Jordheim, 2006). *L. maximowiczii* var. *sachalinensis* contained 86% pelargonidin 3-glucoside (**1**) in addition to cyanidin 3-rutinoside, **9**, (11%), while *L. maakii* contained delphinidin 3-glucoside, **22**, (25%) and cyanidin 3-sambubioside-5-glucoside, **14**, (75%). Three of the species (*L. maakii*, *L. henryi* and *L. caucasica*) contained **14**, including the disaccharide sambubiose, which has been found in all examined *Sambucus* species. The examined species of *Viburnum* contained one or more cyanidin 3-glycosides, however, with differences in their individual anthocyanin pattern. Berries of *V. opulus* were rather outstanding containing the novel pigment cyanidin 3-vicianoside (**6**).

The segregation of *Sambucus* and *Viburnum* from the rest of Caprifoliaceae, as suggested by Wilkinson (1949), is thus not supported by the anthocyanin content found in the examined species of this study.

3.1.4 Anthocyanins from flowers of *Hippeastrum* cultivars (IV)

The genus *Hippeastrum*, also referred to as Amaryllis (Hofmann et al., 2003), belongs to the family Amaryllidaceae. Many of the species in this genus have large and colourful flowers favourable for instance as Christmas and New Year ornamentals (Silberbush et al., 2003). In the family Amaryllidaceae the 3-glucoside and 3-xylosylglucoside of pelargonidin and cyanidin have previously been identified in *Lycoris* (Arisumi, 1971), and the 3,5-diglucoside of cyanidin, peonidin and pelargonidin, the 3-glucoside of cyanidin and pelargonidin, cyanidin 3-sophoroside and two partly identified anthocyanins have been detected in *Nerine* (Arisumi and Shioya, 1970). Furthermore the anthocyanin pelargonidin 3-glucoside has previously been identified in *Hippeastrum* petals as a minor component (Hrazdina, 1988). This tentative identification was based on HPLC of the petal extract hydrolysate and TLC examination of *Hippeastrum* petal extracts, which showed five major components.

Figure 19. Three of the six examined *Hippeastrum* cultivars. **A**. 'Royal velvet', **B**. 'La Paz', **C**. 'Magic Green'.

Flowers of *Hippeastrum* x *hybridum* cv. spp. collected from Makerere University campus in Kampala (Uganda) in August 2004 and flowers of six *Hippeastrum hybridum* cultivars ('Red Lion', 'Royal Velvet', 'La Paz', 'Jungle Star', 'Magic Green' and 'Liberty' (dark red)) purchased in Bergen (Norway) in November 2004, were examined. Pictures of 'Royal Velvet', 'La Paz' and 'Magic Green' are shown in Figure 19.

Qualitative and quantitative anthocyanin content

The anthocyanins, cyanidin 3-*O*-*β*-(6"-*α*-rhamnopyranosylglucopyranoside) (**9**) and pelargonidin 3-*O*-*β*-(6"-*α*-rhamnopyranosylglucopyranoside) (**2**), were isolated from the ornamental flowers of a Ugandan *Hippeastrum* cultivar by a combination of chromatographic techniques. Their structures were elucidated mainly by the use of homo- and heteronuclear NMR spectroscopy and electrospray mass spectrometry. The same two anthocyanins were found in six different *Hippeastrum* cultivars purchased in Norway. However, the absolute amount of the anthocyanins (0.08 to 1.79 mg/g, fresh weight) and their relative proportions varied considerably from cultivar to cultivar (13.2 to 96.5% of **9**).

In vivo petal colour versus anthocyanin content

The colours of fresh petals of the three *Hippeastrum* cultivars 'Red Lion', 'Royal Velvet' and 'Liberty' were described by the CIELab coordinates L* (lightness), C* (chroma) and hab (hue angles). The other cultivars ('Magic Green', 'Jungel Star' and 'La Paz') have not been analysed by these coordinates due to lack of uniform petal colours. The three former samples were described by having hue angles corresponding to scarlet nuances (hab = 22–358) with the highest numerical value for 'Red Lion'. This latter cultivar also revealed the highest L* and C* values. The hybrids 'Liberty' and 'Royal Velvet' showed similar quantitative anthocyanin content and similar L* values, while 'Red Lion' had the lowest anthocyanin content and highest L* value. With respect to a correlation between the CIELab parameters and the qualitative anthocyanin content in the three hybrids, both the C* and hab values increased with increasing proportions of **2** relative to **9**. The most reddish petals, which was expressed by 'Royal Velvet', contained the highest relative proportion of the anthocyanin (**9**) with the highest λ_{max}-value (510 nm). Flowers containing pelargonidin (**2**) as the major anthocyanidin revealed a more orange colour than those having the corresponding cyanidin derivative (**9**) as the major anthocyanin. Thus, the *in vivo* colours of *Hippeastrum* cultivars seem to be correlated with the type and proportions of anthocyanins present in their petals.

3.2 Anthocyanins – new structural characteristics (V-VII)

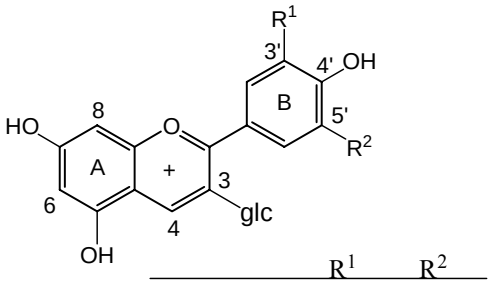
Carboxypyranoanthocyanins have been reported to have other properties (colour, higher stability etc.) than analogous anthocyanins under weakly acidic to neutral conditions, but the availability of isolated and synthetically prepared carboxypyranoanthocyanins has only been achieved in the low milligram scale. Hemisynthesis and isolation of carboxypyranoanthocyanins in a preparative scale (**V**) allowed comparative examinations of these pigments with analogous anthocyanins. Hemiacetal (hemiketal) forms of some anthocyanins have been structural elucidated (**VI**), and reactivity at specific structure sites of some anthocyanins and carboxypyranoanthocyanins in neutral and acidified CD_3OD have been revealed (**VII**).

3.2.1 Preparative isolation and NMR characterization of carboxypyranoanthocyanins (V)

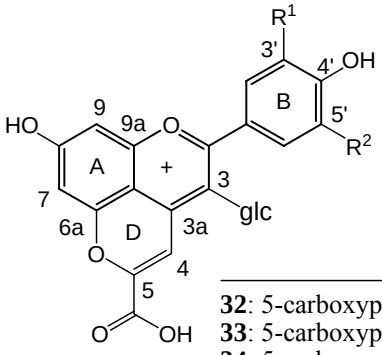
It has been shown that anthocyanins with 4-substituted aglycones like carboxypyranoanthocyanins have favourable properties such as higher resistance to bleaching by sulfur dioxide, higher colour intensity and restricted formation of the unstable colourless equilibrium forms under weakly acidic-neutral solution conditions compared to analogous anthocyanidin 3-glucosides (Bakker et al., 1997; Andersen et al., 2004; Romero and Bakker, 2000; Mateus and de Freitas, 2001). The carboxypyranoanthocyanins may thus be used as colour additives in food, or as antioxidants, etc. Although several methods have been developed for separating anthocyanins, even on a preparative scale (Andersen and Francis, 2004), no method has addressed isolation of individual pigments in mixtures of carboxypyranoanthocyanins.

Hemisynthesis and preparative isolation of carboxypyranoanthocyanins

Three carboxypyranoanthocyanins (**32-34**) (Figure 20) were produced by nucleophilic addition of pyruvic acid to a purified extract of black beans (*Phaseolus vulgaris*) containing a mixture of the 3-glucosides of delphinidin (**22)**, petunidin (**26**) and malvidin (**27**) (Figure 20). The reaction was monitored by on-line HPLC and terminated after 23 hours, when the original anthocyanins (**22**, **26** and **27**) and the synthesised carboxypyranoanthocyanins (**32**, **33** and **34**) occurred in considerable amounts.



	R^1	R^2
22: delphinidin	OH	OH
26: petunidin	OCH_3	OH
27: malvidin	OCH_3	OCH_3



	R^1	R^2
32: 5-carboxypyranodelphinidin	OH	OH
33: 5-carboxypyranopetunidin	OCH_3	OH
34: 5-carboxypyranomalvidin	OCH_3	OCH_3

Figure 20. The structures of the 3-glucosides of delphinidin (**22)**, petunidin (**26**) and malvidin (**27**), 5-carboxypyranodelphinidin (**32**), 5-carboxypyranopetunidin (**33**) and 5-carboxypyranomalvidin (**34**).

During separation of the pigment mixture on a Sephadex LH-20 column a total of seventeen fractions were collected manually on the basis of band colours. The pigment content of each fraction was analysed by analytical HPLC and TLC. UV-Vis spectra of pigments **26** and **33** are given in Figure 21.

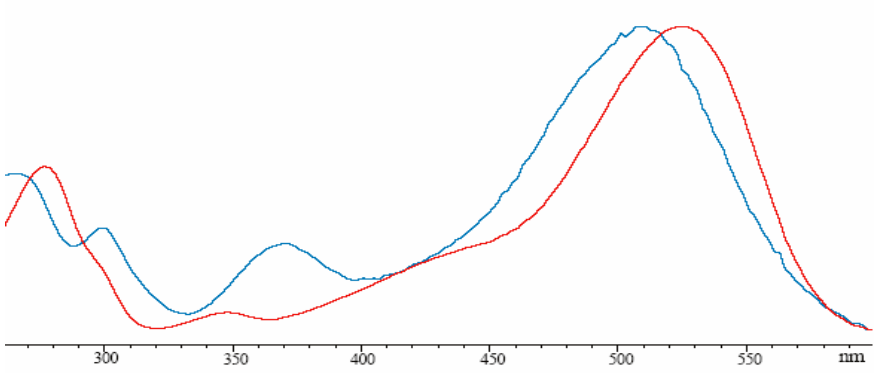


Figure 21. UV-Vis spectra of **26** (red) and **33** (blue) recorded on-line during HPLC analysis.

Altogether six bands with mauve to red colours were chromatographically separated (Figure 22). Both the carboxypyranoanthocyanins and the anthocyanins were separated according to their molecular masses. Thus, the 5-carboxypyranomalvidin 3-glucoside (**34**) was eluted prior to the corresponding pyruvic adducts of petunidin 3-glucoside (**33**) and delphinidin 3-glucoside (**32**) followed by the 3-glucosides of malvidin (**27**), petunidin (**26**) and delphinidin (**22**). The separation procedure applied to a 6.5 gram sample of the pigment mixture from the first synthesis yielded in a one-step separation 376, 325, 376, 165, 163, and 140 mg of **22**, **32** **26**, **33**, **27** and **34**, respectively, with purities of up to 98, 89, 99, 87, 55, and 81%. The low

purity of pigment **27** (55 %), which was eluted in band 4, was due to co-elution with another phenolic compound detected at 280 nm. This impurity was removed by chromatography on a Toyopearl HW-40F column.

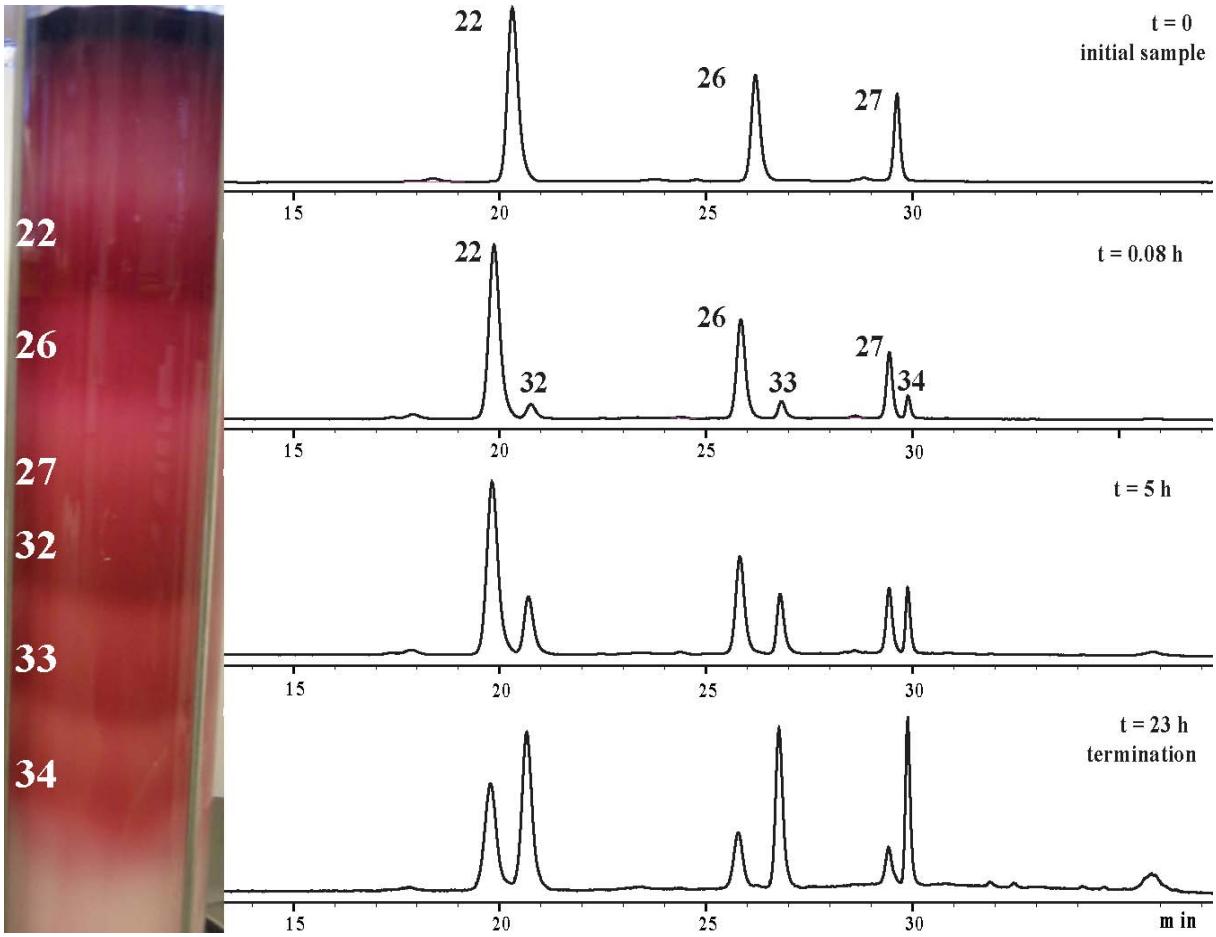


Figure 22. Left: Picture of the Sephadex LH-20 column during separation of carboxypyranoanthocyanins and anthocyanins. 5-carboxypyranomalvidin 3-glc (**34**) (band 1) was eluted prior to 5-carboxypyranopetunidin 3-glc (**33**) (band 2) and 5-carboxypyranodelphinidin 3-glc (**32**) (band 3) followed by malvidin 3-glc (**27**) in band 4, petunidin 3-glc (**26**) (band 5) and delphinidin 3-glc (**22**) (band 6). **Right:** HPLC chromatograms of the reaction mixture at 520 ± 20 nm after various time intervals. glc = glucoside.

The individual pigments (**22**, **26**, **27**, **32**–**34**) were subjected to NMR analysis. The structures of **32** and **33** have previously been tentatively identified mainly by mass spectrometric data acquired from complex mixtures in wine samples or from modified blueberry extract (Faria et al., 2005). In the next section follows a detailed structural NMR elucidation of 5-

carboxypyranopetunidin 3-glucoside (**33**). The structure of 5-carboxypyranodelphinidin 3-glucoside (**32**) was similarly assigned.

NMR elucidation of 5-carboxypyranopetunidin 3-O-β-*glucopyranoside (**33**)*

In Figure 23 the ^{1}H NMR spectrum of petunidin 3-glucoside (**26**) (A) is compared to the ^{1}H NMR spectrum of 5-carboxypyranopetunidin 3-glucoside (**33**) (B). For pigment **33** five signals were found in the aromatic region; namely a singlet at δ 8.08 (H-4), two *meta*-coupled hydrogens at δ 7.25 (*d*, 1.9 Hz; H-7) and δ 7.34 (*d*, 1.9 Hz; H-9), and a AX system at δ 7.62 (*d*, 2.2 Hz; H-6') and δ 7.82 (*d*, 2.2 Hz; H-2'), respectively, revealing a 4-substituted anthocyanin having an asymmetrically substituted B-ring. The assignments of H-9 and H-7 may be reversed. The crosspeaks at δ 8.08/161.1 (H-4/COOH), δ 8.08/136.2 (H-4/C-3), δ 8.08/155.7 (H-4/C-5), δ 8.08/110.9 (H-4/C-9b), and the $^{1}J_{CH}$ correlation at δ 8.08/107.4 (H-4/C-4) in the HMBC spectrum of **33** were used to assign COOH, C-3, C-5, C-9b and C-4. Furthermore, C-2 was identified by its long-range correlation with H-2' and H-6' (δ 7.82/166.2, and δ 7.62/166.2) respectively.

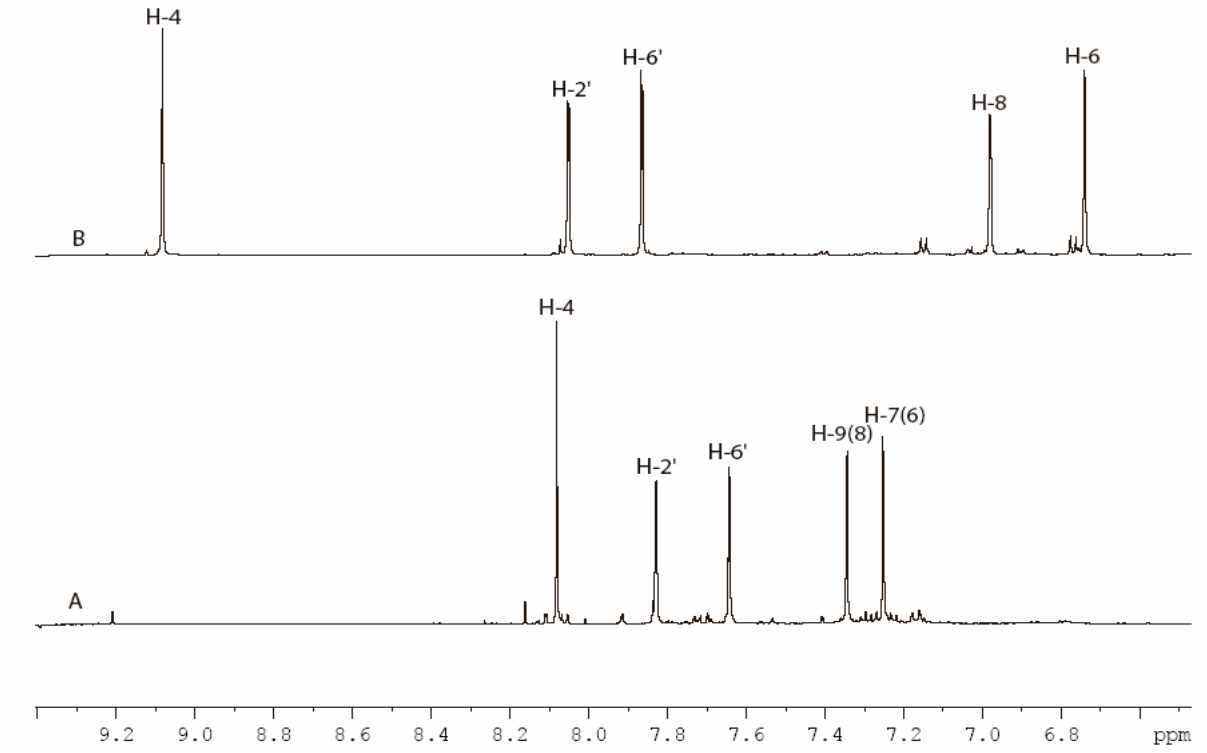


Figure 23. ^{1}H NMR spectra (600.13 MHz) of (A) petunidin 3-*O*-*β*-glucopyranoside (**26**) and (B) 5-carboxypyranopetunidin 3-*O*-*β*-glucopyranoside (**33**) in CF_3CO_2D–CD_3OD (5:95, v/v). The spectra were recorded at 25ºC. See Figure 20 for structures.

The anthocyanidin A-ring ^{13}C signals were assigned by the crosspeaks at δ 7.34/169.7 (H-9/C-8), δ 7.34/154.5 (H-9/C-9a), δ 7.34/110.9 (H-9/C-9b), δ 7.34/101.9 (H-9/C-7), δ 7.25/169.7 (H-7/C-8), δ 7.25/154.6 (H-7/C-6a), δ 7.25/110.9 (H-7/C-9b), and δ 7.25/101.6 (H-7/C-9), respectively, observed in the HMBC spectrum (Figure 24). The carbons belonging to the anthocyanidin B-ring were assigned by the crosspeaks at δ 3.99/149.9 (OCH_3/C-3'), δ 7.82/143.9 (H-2'/C-4'), δ 7.62/143.9 (H-6'/C-4'), δ 7.82/149.9 (H-2'/C-3'), δ 7.62/147.3 (H-6'/C-5'), δ 7.82/113.5 (H-2'/C-6'), δ 7.62/108.7 (H-6'/C-2') and δ 7.82/120.2 (H-2'/C-1'). There were no obvious crosspeaks in the HMBC spectrum involving C-3a, however, a resonance at 149.70 in the CAPT spectrum was assigned to this carbon. Thus, the aglycone of **33** was in agreement with 5-carboxy-2-(3,4-dihydroxy-5-methoxyphenyl)-3,8-dihydroxy-pyrano[4,3,2-*de*]-1-benzopyrylium, 5-carboxypyranopetunidin. The ^{1}H and ^{13}C signals of the sugar region of **33** were in accordance with β-glucopyranose (Pedersen et al., 1993). The crosspeak at 4.81/136.2 (H-1"/C-3) in the HMBC spectrum confirmed the linkage between the aglycone and the sugar unit to be at the 3-hydroxyl.

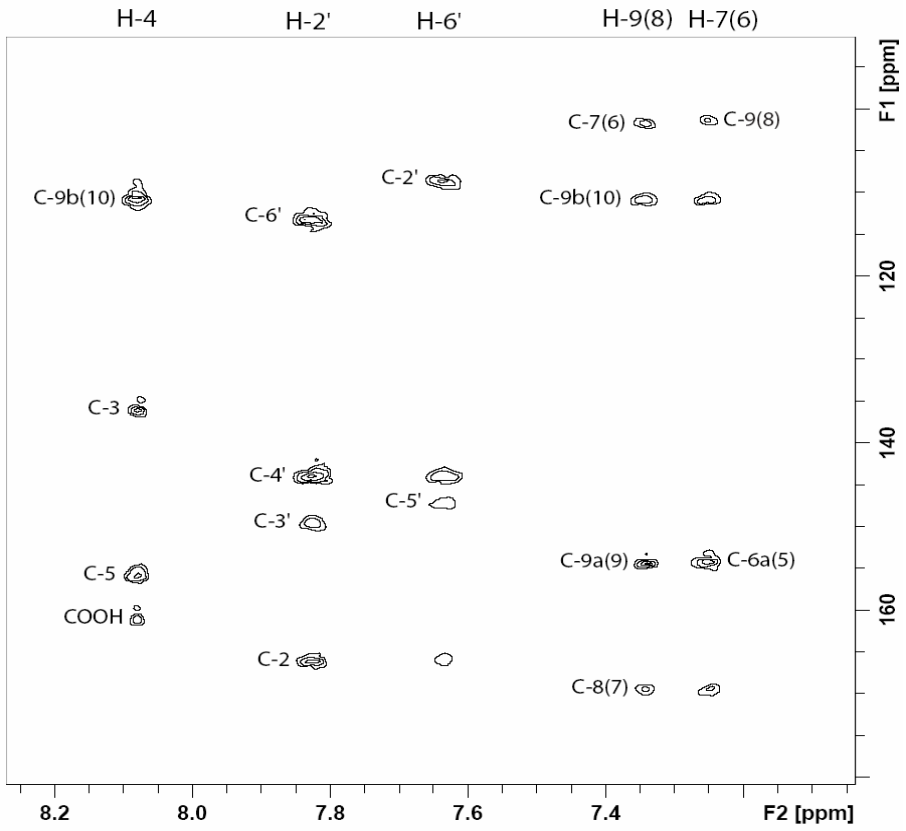
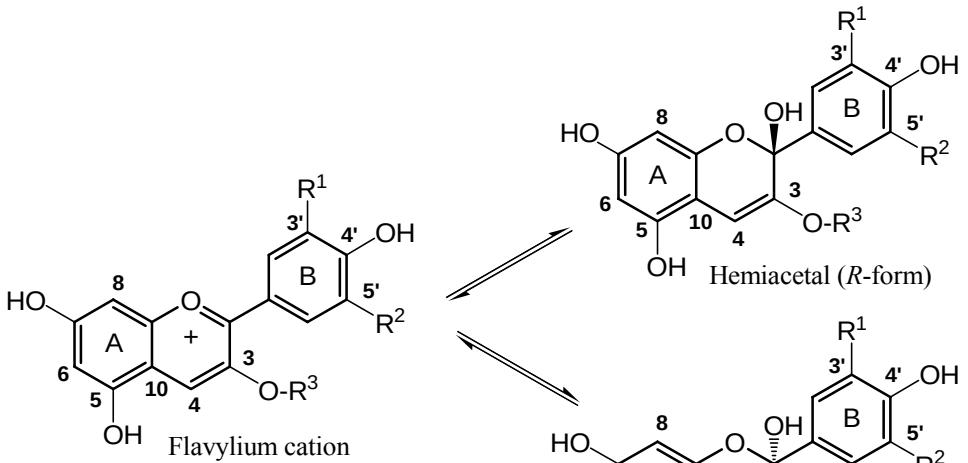


Figure 24. ^{1}H-^{13}C HMBC NMR spectrum of the aromatic region of pigment **33** in CF_3CO_2D–CD_3OD (5:95, v/v) recorded at 25ºC. Number in brackets corresponds to analogous positions in common anthocyanins.

3.2.2 Characterization of hemiacetal forms of anthocyanidin 3-*O*-*β*-glycopyranosides (VI)

The structures of the 3-*O*-*β*-glucopyranosides of delphinidin, petunidin and malvidin (**22**, **26** and **27**) and cyanidin 3-*O*-*β*-galactopyranoside (**4**) dissolved in deuterated methanolic solutions without and with acid (5%, CF_3COOD) were identified by homo- and heteronuclear NMR techniques. The hemiacetal forms of all the four anthocyanins (Figure 25) were characterized as two epimeric 2-hydroxy-hemiacetals based on assignments of both proton and carbon NMR signals together with chemical shift considerations. This is the first report of ^{13}C NMR assignments of two epimeric anthocyanin hemiacetal forms. Under slightly acidic to neutral conditions, which is a relevant pH range for *in vivo* conditions in plants and in the human gastrointestinal tract, this type of anthocyanins has previously been considered to occur predominantly as hemiacetals (Brouillard and Dangles, 1994; Markakis, 1982).

Traditionally the hydrated forms of the flavylium cationic form are named hemiacetals, however, with respect to their chemical nature these colourless forms are hemiketals and not hemiacetals. In this work we have used the traditionally term; hemiacetals.



	R^1	R^2	R^3
4: cyanidin	OH	H	gal
22: delphinidin	OH	OH	glc
26: petunidin	OCH_3	OH	glc
27: malvidin	OCH_3	OCH_3	glc

Figure 25. Equilibrium forms of **4**, **22**, **26** and **27** dissolved in non-acidified CD_3OD. Whether the hemiacetal *R*- or *S*-form constitute the major (**a**) or the minor (**b**) form is not known.

*Structural elucidation of major (**a**) and minor (**b**) hemiacetal forms of malvidin 3-*O*-β-glucopyranoside (**27**)*

In the downfield region of the ^{1}H NMR spectrum of pigment **27** dissolved in pure CD_3OD more than twelve aromatic proton signals were present (Figure 26, B). Four of these had similar chemical shift values and coupling constants as the four aromatic proton signals representing the flavylium cationic form of **27** in CF_3COOD–CD_3OD (5:95, v/v) (Figure 26, A). The relationship between the proton resonances of the flavylium cation and other signals in the downfield region were revealed by exchange crosspeaks in the 2D ^{1}H-^{1}H NOESY NMR spectrum of **27** dissolved in pure CD_3OD (Figure 27). Two more anthocyanidin forms (**27a** and **27b**) in addition to the flavylium cationic form were thus identified.

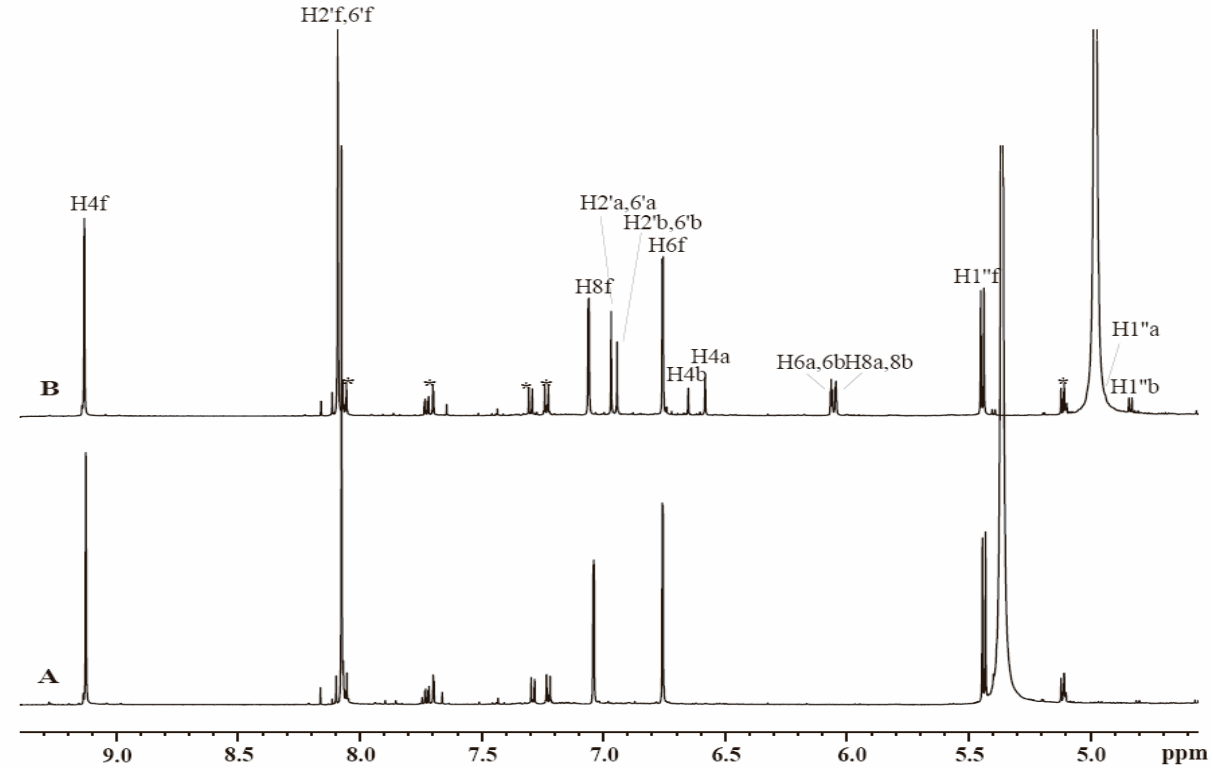


Figure 26. ^{1}H NMR spectra (600.13 MHz) of malvidin 3-*O*-*β*-glucopyranoside (**27**) in CF_3CO_2D–CD_3OD (5:95, v/v) (A) and in pure CD_3OD (B). Both samples are recorded at 25ºC. f = flavylium cation; a = hemiacetal **a** (major); b = hemiacetal **b** (minor); * = impurities.

Starting with the H-4f/H-4a exchange crosspeak at *δ* 9.13/6.58 in the NOESY spectrum (Figure 27), which is used to assign H-4a, the proton and carbon chemical shifts of **27a** (the major form) were thereafter assigned; crosspeaks at *δ* 6.58/103.1 (H-4a/C-2a), *δ*

6.58/154.6 (H-4a/C-5a), *δ* 6.58/152.9 (H-4a/C-9a), *δ* 6.58/145.4 (H-4a/C-3a) in the HMBC spectrum of **27** were used to assign C-2a, C-5a, C-9a and C-3a, respectively (Figure 28).

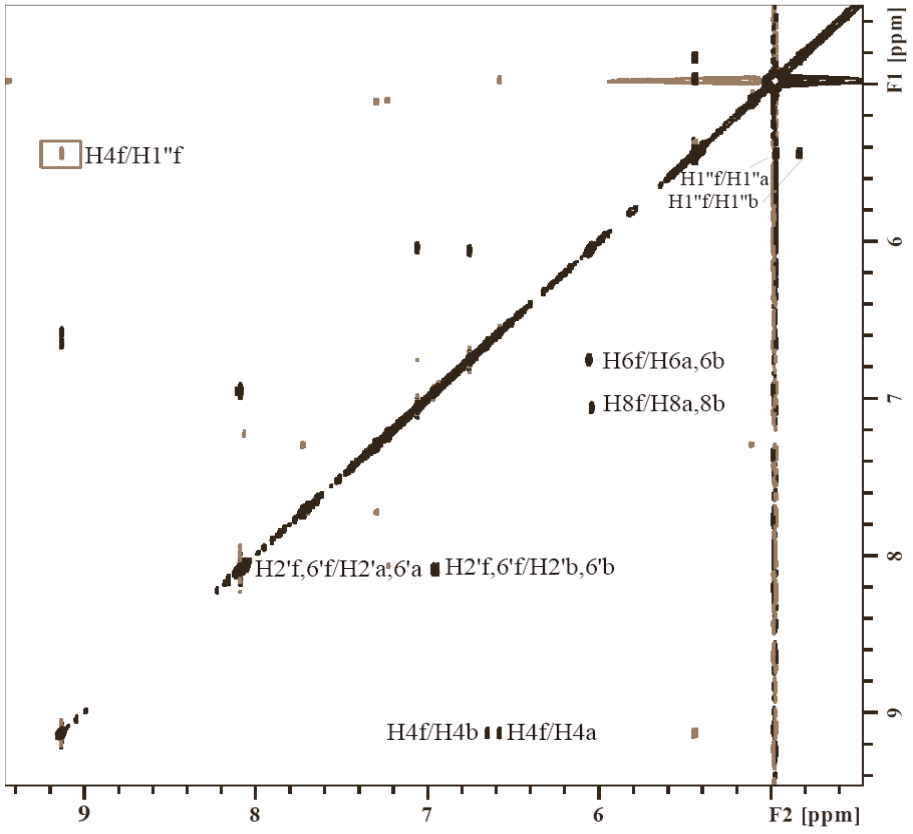


Figure 27. Expanded region of the NOESY spectrum (600.13 MHz) of malvidin 3-*O*-*β*-glucopyranoside (**27**) in pure CD_3OD recorded at 25ºC. A negative crosspeak due to NOE correlation between H-4 and H-1" of the flavylium cation is enclosed in a box. Other labelled crosspeaks are positive and caused by chemical exchanges between the flavylium cation (f) and its corresponding hemiacetal forms (a, major and b, minor) (**27a**, **27b**).

Similarly, the carbons belonging to the B-ring of **27a** were assigned from the crosspeaks at *δ* 6.97/103.1 (H-2'a,6'a/C-2a), *δ* 6.97/136.8 (H-2'a,6'a/C-4'a), *δ* 6.97/148.5 (H-2'a,6'a/C-3'a,5'a), *δ* 6.97/132.2 (H-2'a,6'a/C-1'a), *δ* 6.97/105.9 (H-2'a,6'a/C-2'a,6'a) and *δ* 3.91/148.5 (-OCH_3-a/C-3'a,5'a) (Figure 28), while the rest of the A-ring carbons were identified from crosspeaks at *δ* 6.046/158.4 (H-8a/C-7a), *δ* 6.046/101.8 (H-8a/C-10a), *δ* 6.046/97.2 (H-8a/C-6a), *δ* 6.065/158.4 (H-6a/C-7a), *δ* 6.065/154.6 (H-6a/C-5a), *δ* 6.065/101.8 (H-6a/C-10a), *δ* 6.065/95.1 (H-6a/C-8a).

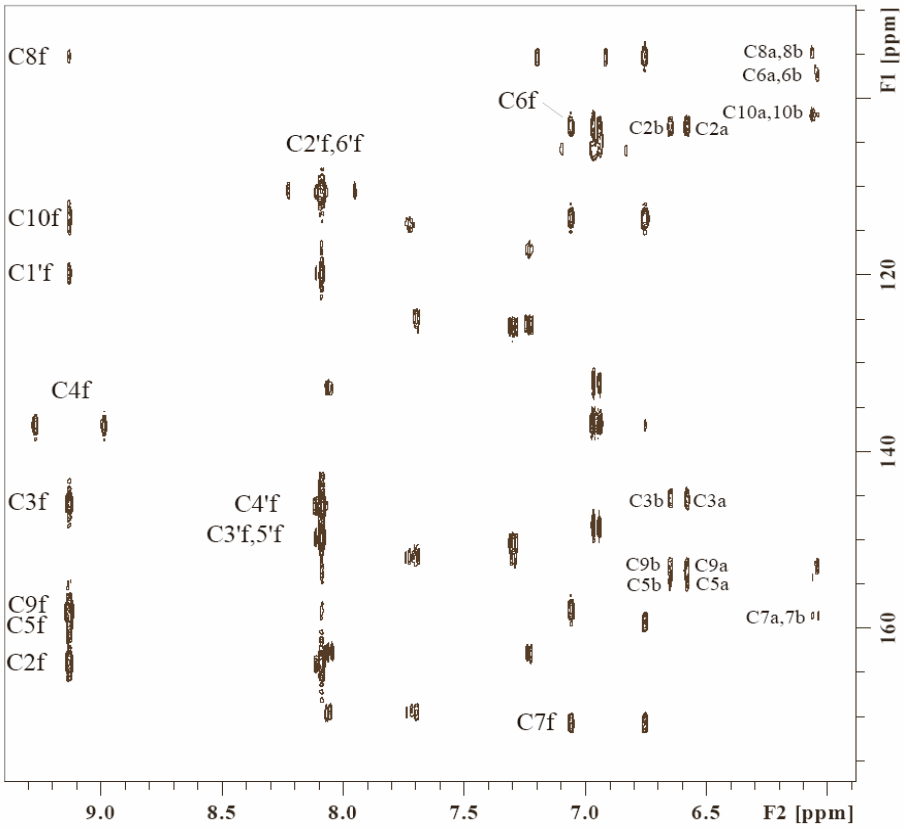


Figure 28. Expanded region of the HMBC spectrum (600.13 MHz) of malvidin 3-*O*-*β*-glucopyranoside (**27**) in pure CD_3OD recorded at 25ºC. f = flavylium cation; a = hemiacetal **a** (major) (**27a**); b = hemiacetal **b** (minor) (**27b**).

The chemical shift of C-4a was assigned by a crosspeak at δ 6.58/98.6 (H-4a/C-4a) in the HSQC spectrum. The remaining problem was to address the structural differences between the flavylium cation and anthocyanidin form **a**. The most apparent difference between the ^{1}H NMR spectra of these two forms consisted of the 2.55 ppm upfield shift of H-4 of anthocyanidin form **a** (Figure 26). This upfield shift of H-4 indicated a lower conjugation of anthocyanidin form **a** compared to the flavylium cation, in accordance with a hemiacetal. Likewise the outstanding 60.9 ppm and 38.5 ppm upfield shift of C-2 and C-4 of anthocyanidin form **a** compared to the flavylium cation were consistent with the 2-hydroxy hemiacetal form. In an analogous manner the ^{1}H and ^{13}C chemical shifts of anthocyanidin form **b** (minor) were assigned.

The sugar region of the ^{1}H NMR spectrum of **27** (Figure 26) revealed three anomeric signals (δ 5.45, δ 4.97 and δ 4.83) with similar anomeric coupling constants (7.8 Hz). The most downfield anomeric signal showed highest intensity and was assigned to the flavylium cation

(f) by a HMBC crosspeak at δ 5.44/145.3 (H-1"f/C-3f). The NOESY spectrum of **27** (Figure 27) revealed exchange crosspeaks at δ 4.97/5.44 (H-1"a/H-1"f) and δ 4.83/5.44 (H-1"b/H-1"f) between the anomeric signal of the flavylium cation and the other anomeric signals of the two hemiacetal forms respectively.

Proportions of hemiacetal forms

Limited information exists about elucidation of anthocyanin hemiacetal structures (Cheminat and Brouillard, 1986; Mistry et al., 1991; Santos et al., 1993; Terahara et al., 1993; Bakker et al., 1997). Under *in vivo* conditions simple anthocyanins like **4**, **22**, **26** and **27** may occur on several equilibrium forms, of which some are regarded as relatively unstable even at short storage intervals (Cabrita et al., 2000). Reduced solubility of most anthocyanins in aqueous solutions compared to alcoholic anthocyanin solutions may be another limiting factor. In the present studies, the anthocyanins (**4**, **22**, **26** and **27**) were dissolved in pure deuterated methanol, which facilitated full assignments of chemical shifts of both the hydrogens and carbons of two epimeric 2–hydroxy hemiacetals of each of these four anthocyanins. In the NOESY NMR spectra strong exchange crosspeaks between analogous signals of the hemiacetals and the flavylium cationic form were observed, showing that the two epimeric hemiacetal forms were in equilibrium with the flavylium cationic form. Similar exchange crosspeaks between the two epimeric hemiacetal forms were not detected for any of the four pigments. It is also interesting to note that the molar ratio between the flavylium cation, the hemiacetal **a** form and the hemiacetal **b** form for each anthocyanidin (Table 1) remained essentially unchanged for several weeks. The individual proportions of the flavylium cationic form and the two hemiacetal forms is hereby proposed to be nearly similar for anthocyanidin 3-monoglycosides, regardless of the nature of the anthocyanidin or monosaccharide, at least when dissolved in deuterated methanol.

Table 1. Proportions (%) of the flavylium cation/hemiacetal **a**/hemiacetal **b** recorded by integration of ^{1}H NMR spectra of cyanidin 3-galactoside (**4**), delphinidin 3-glucoside (**22**), petunidin 3-glucoside (**26**) and malvidin 3-glucoside (**27**) after 24 h storage in CD_3OD at 25ºC.

	flavylium cation	hemiacetal **a**	hemiacetal **b**
4	67	20	13
22	82	10	8
26	79	12	9
27	75	15	10

3.2.3 Reactivity of anthocyanins and pyranoanthocyanins; studies on aromatic hydrogen-deuterium exchange reactions in methanol (VII)

Anthocyanins are good candidates for studies of aromatic H→D exchange reactions, which may occur because of presence of aromatic hydroxyl groups, various resonance structures arising through three conjugated ring systems, and the occurrence of various equilibrium forms. It has previously been reported that H-6 and H-8 of the flavylium cationic form of pelargonidin (Pedersen et al., 1993) and malvidin (Santos et al., 1993) in anthocyanins are exchanged with deuterium in acidified D_2O and acidified CD_3OD. Detailed chemical studies with focus on potential H→D exchange reactions at specific sites of the various anthocyanidin structures, delphinidin 3-*O*-*β*-glucopyranoside (**22**), petunidin 3-*O*-*β*-glucopyranoside (**26**), and malvidin 3-*O*-*β*-glucopyranoside (**27**), in their flavylium cationic and hemiketal equilibrium forms, their three corresponding pyranoanthocyanins (**32**–**34**), and the flavonol rutin (quercetin 3-*O*-*β*-(6-*α*-rhamnopyranosylglucopyranoside), **35**, were in the present study based on integration data obtained by ^{1}H NMR spectroscopy. The aim was to aid the understanding of properties, metabolism and functions of the anthocyanin molecules commonly found in berries, fruits, vegetables and in derived products.

Deuterium exchange of aromatic hydrogens

The H→D exchange reactions of the hydrogens at various sites of the aglycones of **22**, **26**, **27**, **32**–**35** were measured during storage of these pigments (~ 10 mM) dissolved in CF_3CO_2D-CD_3OD (5:95, v/v) at room temperature. No exchange was found to occur for H-4 (**22**, **26**, **27**) or any of the B-ring hydrogens (H-2', H-5' and H-6') (**22**, **26**, **27**, **32**–**35**) even after storage for weeks.

After 24 hours the integrated area of the ^{1}H NMR signals of H-6 and H-8 on the A-rings of **22**, **26** and **27** were reduced with 23 to 36% compared to the corresponding signals measured 30 minutes after sample preparation (Figure 29). After 7 to 11 days the three pigments had experienced around 90% H→D exchange; after around five months the signals representing H-6 and H-8 were barely detectable. When **22**, **26** and **27** were dissolved in CD_3OD at room temperature; similar exchange patterns and rates were observed for the hemiketal forms as for the flavylium forms. Correspondingly, none of the carboxypyranoanthocyanins (**32**–**34**) experienced H→D exchange at their A-rings, even after

10 days of storage. On the other hand, even faster H→D exchange was observed for H-β (H-4) at their D-rings.

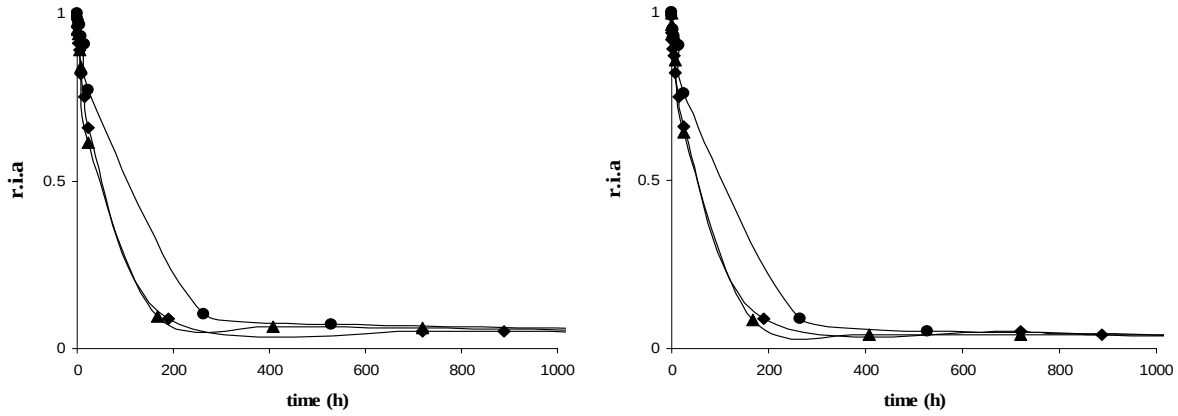


Figure 29. Relative integrated area (r.i.a) of H-6 (left) and H-8 (right) in the ^{1}H NMR spectra of the flavylium forms of delphinidin 3-glc (●) (**22**), petunidin 3-glc (♦) (**26**) and malvidin 3-glc (▲) (**27**) plotted against time (hours). The first 1000 hours (~42 days) after sample preparation are shown in the figure. glc = glucoside.

Impact of acidity and concentration

Exchange experiments performed with petunidin 3-glucoside (**26**) dissolved in CD_3OD without acid, and with 5% and 15% CF_3CO_2D (v/v), respectively, showed very similar H→D exchange rates for H-6 and H-8.

Kolar (1971) has examined H→D exchange reactions for H-6 and H-8 of some methylated flavanols in D_2O/dioxane (3:1) solutions after heating for 16 h at 95° in Pyrex glass. He concluded that the observed exchange reactions, following first order kinetics, were typical electrophilic aromatic substitution reactions being catalyzed by acid. When no acid was present the observed exchange reactions were suggested to be promoted by the Pyrex glass of the applied NMR–tube assisting in the exchange process. Our experiments with **26** in pure CD_3OD using both Wilmad Pyrex glass and Norell N-51A glass NMR tubes, the latter carefully cleansed with 0.2 M NaOH prior to use to remove possible surface acidity, led to no change of exchange rates of H-6 and H-8 during the first 24 h. This suggests that impurities may not be the cause of the relatively rapid H→D exchange reactions observed for **22**, **26** and **27** dissolved in pure CD_3OD. D^+ formation from CD_3OD, as H^+ formation from CH_3OH, will only take place at elevated pressure and temperature (Raveendran et al., 2005).

Studies of the potential influence of pigment concentration on the H→D exchange rates of H-6 and H-8 were performed with 10 mM, 20 mM and 40 mM samples of petunidin 3-*O*-*β*-glucopyranoside (**26**) dissolved in CF_3CO_2D–CD_3OD (5:95, v/v). Each of the individual samples of pigment **26** dissolved at these three different concentrations led to similar H→D exchange rates for both H-6 and H-8, showing the exchange mechanism not to be significantly affected by the anthocyanin concentration.

Impact of structure

The generalized scheme for H→D exchange reactions of the aromatic A-ring hydrogens of **22**, **26** and **27**, including a positively charged σ–complex, is shown in Figure 30. Contrary to the H→D exchanges observed in the A-rings of **22**, **26** and **27**, pigments **32**–**34** in their flavylium cationic forms showed in the present study no aromatic H→D exchange for any of their A-ring hydrogens (H-6 and H-8). Apparently, the oxygen atom (6-O) in the D-ring of **32**–**34** does not have the same electron donating effect as the 5-OH group in **22**, **26** and **27**, and the positively charged σ–complexes of **32**–**34** can not be stabilized to the same extent as the corresponding complexes of **22**, **26** and **27**.

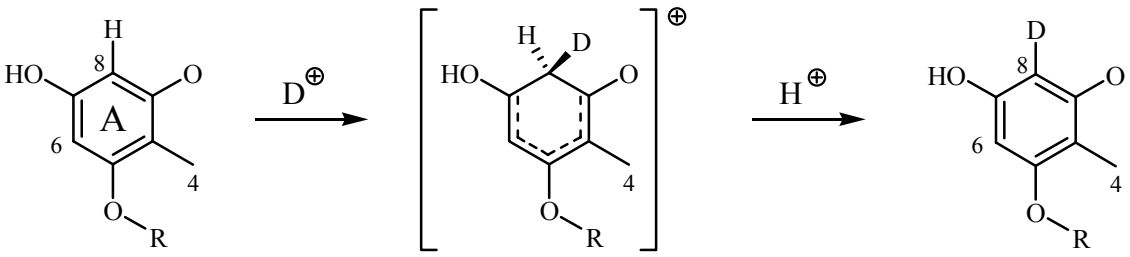


Figure 30. Generalized scheme showing the proposed mechanism for the deuterium exchange of H-8 of the A-ring of anthocyanins **22**, **26** and **27** and the flavonol rutin (**35**). Similar scheme may represent the corresponding exchange of H-6 in **22**, **26** and **27**.

For comparison, similar experiments were performed involving quercetin 3-rutinoside (**34**). When this flavonol was dissolved in $CF_3CO_2DCD_3OD$ (5:95, v/v), the rate of the H→D exchanges of the aromatic A-ring hydrogens were reduced compared to the corresponding reactions of anthocyanins **22**, **26** and **27**. However, the H→D exchange rate of H-8 in rutin was considerably higher than the corresponding rate of H-6; 12% and 2% reduction of signal responses in the NMR spectra after 24 h, respectively. Preferences for the exchange reaction

at C-8 (Figure 30) suggests that this position may have a more positive charge than C-6 in the intermediate σ–complex, leading to higher stability of the σ–complex involved in the exchange of H-8. This effect was not obvious in the study of anthocyanins **22**, **26** and **27**.

Reactivity of anthocyanins and pyranoanthocyanins

The compounds **22**, **26** and **27** exchange hydrogen with deuterium quite readily at position C-6 and C-8 in the A-ring with approximately equal rates. The reactions, as viewed by the decrease of the relative integrated area (r.i.a) *versus* time (Figure 29), appear to be of first order. Since attempts to apply second order rate equations on these reactions failed, the observed reactions may not appear to be initiated by any form of stacking of the compounds when they are dissolved in the applied solvent mixtures. This conclusion is substantiated by the fact that the observed H→D exchange rates are independent upon the concentration of the substrates.

No H→D exchange was observed for the B-ring hydrogens of **22**, **26** and **27**, despite the presence of OH and OCH_3 substituents in the C-3' and C-5' positions. These substituents, however, may seem to influence the exchange rates at C-6 and C-8 in the A-ring to some extent. From Figure 29 one may conclude that the exchange rates of H-6 and H-8 of **26** and **27** are twice as fast as that of **22**, with half-lives of ~50 h and ~100 h, respectively. In contrast to **26** and **27**, pigment **22** has only OH-groups as oxygen substituents on the B-ring.

One notable result in the present study is the observation that the H→D exchange rates at the C-6 and C-8 positions in **26** are essentially the same in pure CD_3OD and in CD_3OD containing 5 % or 15 % CF_3CO_2D (v/v), respectively, corresponding to CF_3CO_2D being 0.7 M or 2.1 M. Although $[D^+]$ will be significantly less than 0.7 M and 2.1 M for these high concentrations, particularly since acids are known to be less dissociated in methanol than in water (pK_a of CF_3COOH in H_2O is ~ 0.0), the present data seem to indicate that the H→D exchange reactions of these anthocyanins are independent upon the concentration of D^+. H→D exchange reactions in aromatic compounds dissolved in deuterated water and alcohols, even when substituted with donor substituents as in the 1,3,5–trimethoxybenzene, are known to proceed only extremely slowly at room temperature when no acids are present (Kresge and Chiang, 1961; Junk and Catallo, 1997; Bai et al., 2000). Generally, these reactions (when measurable) take place with an early transition state forming a Wheland (Pfeiffer) intermediate (Dewar and Dougherty, 1975), presumably through a non-planar aromatic ring with some sp^3- hybridization of the *ipso*-carbon atom. One may speculate whether such

intermediates may be possible allowing for H→D exchange of A-ring hydrogens in anthocyanins - without being influenced by acid being present.

3.3 The reducing capacity of anthocyanins and carboxypyranoanthocyanins (VIII)

Most antioxidant measurements are concentration dependent, and the purity determination of the examined compounds are crucial. Today many of the different antioxidant measurements have presented contradicting results. In paper **VIII** the importance of NMR techniques together with DAD–HPLC in purity determinations has been highlighted. The diversity of molar absorptivity values has been discussed, and new values have been proposed. After careful purity considerations, new information about the reducing capacity of pyranoanthocyanins and anthocyanins has been revealed.

3.3.1 Molar absorptivities and reducing capacity of pyranoanthocyanins and other anthocyanins (VIII)

A variety of methods used for determination of antioxidant capacity of anthocyanin samples have been described (Prior et al., 2005). In order to get comparable results the measurements for individual anthocyanins have usually been compared with similar measurements for ascorbic acid or Trolox; often expressed as Trolox equivalents (Garcia-Alonso et al., 2004). After surveying the literature, it is obvious that these results are strongly dependent on the antioxidant assays applied (Garcia-Alonso et al., 2004; Tsuda et al., 1994; Satué-Gracia et al., 1997; Miller et al., 1997; Wang et al., 1997; Pool-Zobel et al., 1999; Degenhardt et al., 2000; Stintzing et al., 2002; Seeram et al., 2002a, 2002b; Kim, M.-Y. et al., 2003; Chun et al., 2003; Kähkönen et al., 2003; Kim et al., 2004; Lapornik et al., 2004; Awika et al., 2004; Rahman et al., 2006), and most comparative studies conclude that each methodology gives different responses for the same compounds or samples (Arnao et al., 1999; Baderschneider et al., 1999; Perez et al., 2000; Schwarz et al., 2001). The relative antioxidant capacity order of various anthocyanins has even been altered just by changing the concentration of the examined compounds (Kähkönen et al., 2003).

Anthocyanin purity

The merit of antioxidant capacity values for individual anthocyanins depends on the precision in determination of pigment purity or sample concentration. Whether anthocyanins are purchased from commercial sources or isolated by the scientific groups, the purity of individual compounds has mainly been determined by DAD–HPLC, LC–MS or both methods (Santos-Buelga et al., 2003; Andersen and Francis, 2004; Giusti and Wrolstad, 2005).

However, as shown in Figure 31 and 32, anthocyanin purity values obtained by DAD–HPLC have their limitations. When comparing the HPLC chromatograms (Figure 31) recorded both at 520 ± 20 nm and 280 ± 10 nm for malvidin 3-glucoside (**27**) after purification by successive use of various types of column chromatography (Amberlite XAD-7, Sephadex LH-20 and Toyopearl HW-40F), the absence of additional peaks in the two HPLC chromatograms indicated indeed a very clean sample.

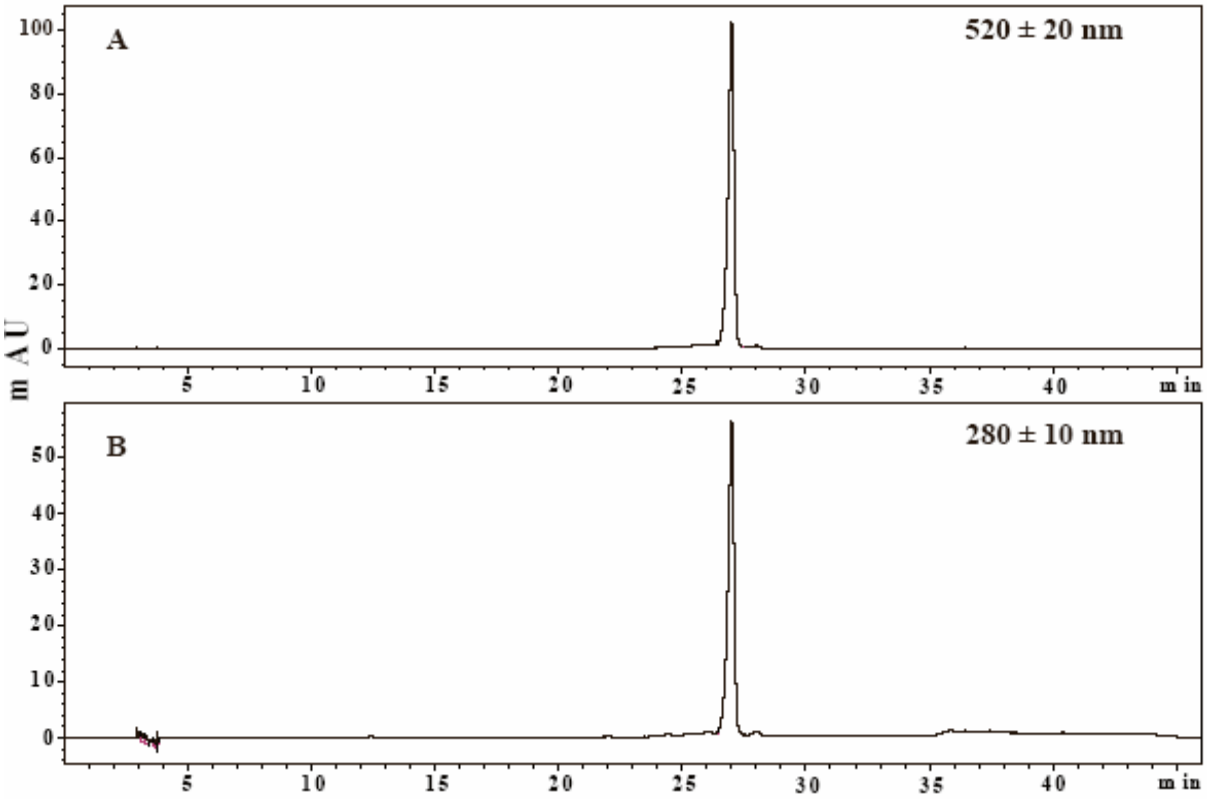


Figure 31. HPLC chromatograms (detected at 520 ± 20 nm and 280 ± 10 nm, respectively) of malvidin 3-glucoside (**27**) after purification by XAD-7, Sephadex LH-20 and Toyopearl HW-40F column chromatography.

However, when comparing the 1D ^{1}H NMR spectrum of the same sample (spectrum A in Figure 32), with a similar spectrum of pure **27** (spectrum B in Figure 32), it was clear that **27** in the former sample was not even the major aromatic compound.

Purity analyses based on DAD–HPLC chromatograms have to reflect the following considerations: Chromatograms recorded in the visible area (typical between 500 and 550 nm) fail to detect aromatic compounds absorbing at shorter wavelengths. When additional HPLC chromatograms recorded in the UV-visible region of the spectrum are included (typically around 280 nm), impurities lacking an UV-absorbing chromophore will still be invisible.

These impurities might be perceived by the use of NMR and MS. However, eventual water and inorganic salt content will normally not be determined by either of these methods. Furthermore, compounds with different chromatographic properties to those of anthocyanins might not show up in the HPLC chromatograms, independently of the HPLC detector, due to strong interaction with the stationary phase of the column.

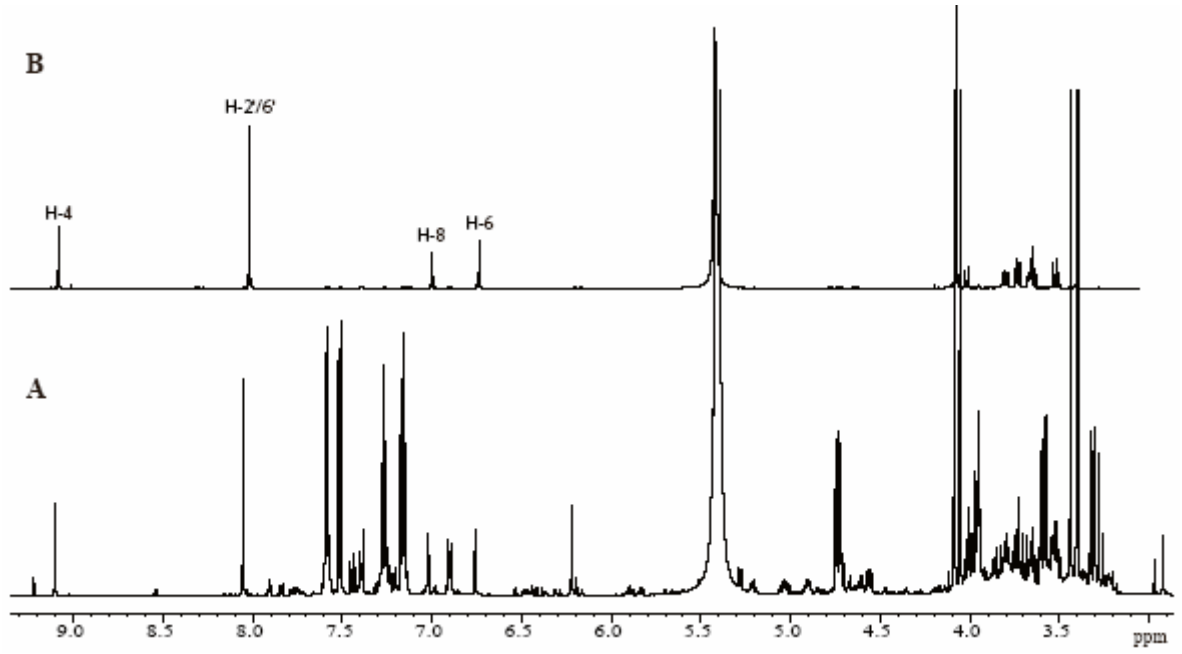


Figure 32. **A.** ^{1}H NMR spectra (600.13 MHz) of the same malvidin 3-*O*-*β*-glucopyranoside (**27**) sample as shown in the HPLC chromatograms (Figure 31). **B.** ^{1}H NMR spectra (600.13 MHz) of pure malvidin 3-*O*-*β*-glucopyranoside (**27**). Both NMR samples (conc. *ca* 11 mM) are dissolved in CF_3CO_2D–CD_3OD; 5:95, v/v and recorded at 25°C.

This latter case is most probably the reason for the discrepancy between the DAD–HPLC and ^{1}H NMR results obtained for the anthocyanin sample examined in Figure 31 and 32. The confidence of DAD–HPLC analysis for determination of anthocyanin purity may thus be improved considerably in combination with NMR analysis.

Another routinely used approach employed to define or measure purity/concentration of anthocyanin samples includes the utilization of molar absorptivity (ε) values. Major difficulties here with respect to exact mass determinations are reflected by the huge variations among the reported ε-values. For instance, the ε-value of malvidin 3-glucoside (**27**) dissolved in 0.1% HCl in methanol has been reported separately to be both 13900 and 29500 (L cm^{-1} mol^{-1}) (at $\lambda_{vis\text{-}max}$ 546) (Giusti et al., 1999). In addition to substantial variation between ε-

values given for the same anthocyanin, even in the same solvent, there exist inconsistent differences between structurally very similar anthocyanins. Other impurities than anthocyanins/other pigments results in the calculation of too low ε-values, which according to Lambert-Beer's law (A = εcl) gives too high anthocyanin concentrations. Consequently, impurities or selected ε-values with too low numbers will imply that the measured antioxidant capacities are presented to be lower than reality. Additionally, some reported ε-values and purity determinations are hampered by the lack of anthocyanin counterions in the calculations.

To improve the control procedure in estimations of anthocyanin purity we have combined ^{1}H and ^{13}C NMR spectroscopy with HPLC-DAD and UV-Vis spectroscopy in the purity analysis of various anthocyanins. Based on our measurements, we suggest the average ε-values for both anthocyanidin 3-monoglycosides and 5-carboxypyranoanthocyanidin 3-glycosides to be 21900 and 22700 (L cm^{-1} mol^{-1}) in acidified aqueous and methanolic solutions, respectively.

Reducing capacity of anthocyanins and pyranoanthocyanins

To assess the influence of structure on the potential antioxidant capacity of anthocyanins, the 3-glucosides of pelargonidin (**1**), cyanidin (**5**), peonidin (**24**), delphinidin (**22**), petunidin (**26**), malvidin (**27**), 5-carboxypyranopelargonidin (**28**), 5-carboxypyranocyanidin (**30**), 5-carboxypyranodelphinidin (**32**), 5-carboxypyranopetunidin (**33**), 5-carboxypyranomalvidin (**34**), and the 3-galactosides of cyanidin (**4**) and 5-carboxypyranocyanidin (**29**), were examined by the FRAP method. The concentration of each anthocyanin dissolved in acidified methanolic solutions was first determined by absorption spectroscopy using the reported molar absorptivity (ε) value of 22700 (L cm^{-1} mol^{-1}) at the visible absorption maxima for the thirteen anthocyanins. The reducing capacities of the individual pigments were expressed as μmol Trolox equivalents per μmol anthocyanin. The reducing capacities of the individual anthocyanins were in the range of 0.9 to 5.2 Trolox equivalents. The two 5-carboxypyranoanthocyanins **32** and **30** showed the highest potential antioxidant capacity ever measured by FRAP for any anthocyanin (5.2 and 4.8 Trolox equivalents, respectively). However, nearly similar values were obtained for **5** and **22**. These four pigments possess vicinal trihydroxyl (pyrogallol-type) or *o*-dihydroxyl (catechol-type) groups on their B-rings. Compounds **28** and **1**, with only one hydroxyl group on their B-rings, showed the lowest reducing capacities (0.9 and 2.7 Trolox equivalents, respectively). The large difference

between the latter two values indicates that the inclusion of the 5-hydroxyl in the D-ring has a very negative effect on the reducing capacity, when there is just one oxygen substituent on the B-ring.

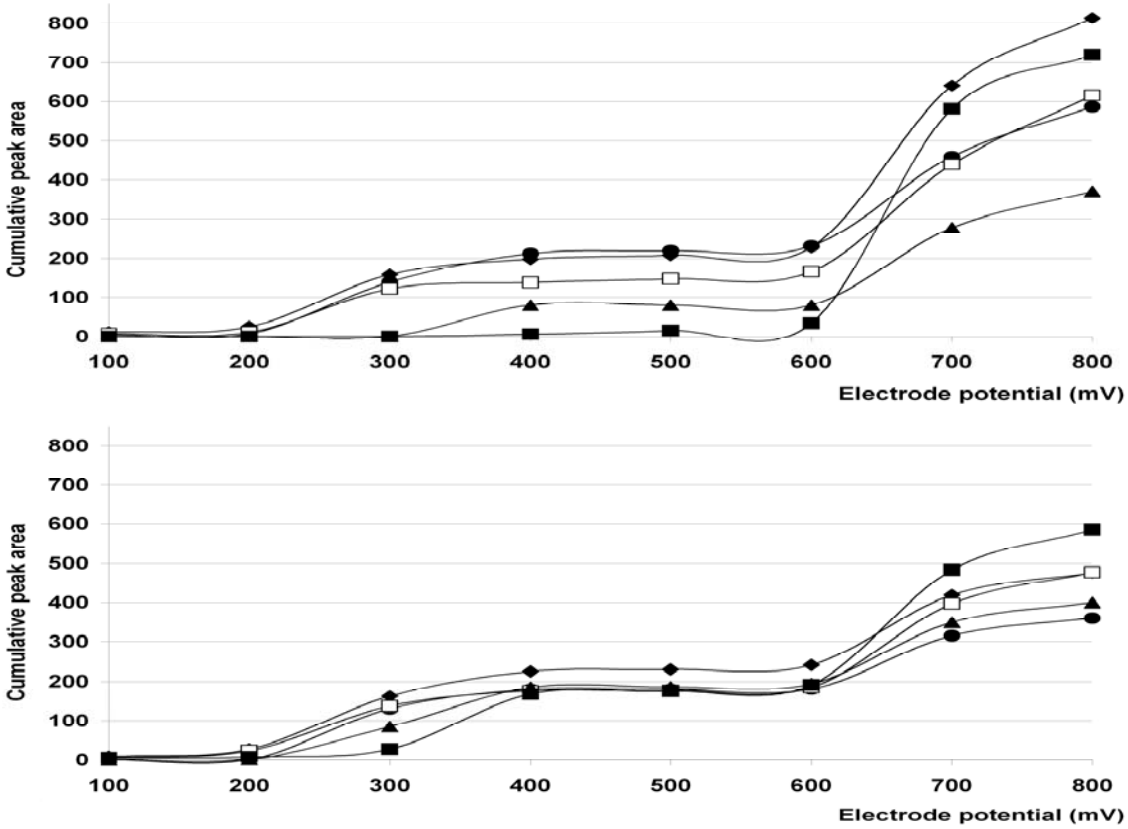


Figure 33. Top: Hydrodynamic voltammograms showing cumulative peak areas (μC/nmol anthocyanin) of 5-carboxypyranopelargonidin 3-glc (**28**) (■), 5-carboxypyranocyanidin 3-glc (**30**) (♦), 5-carboxypyranopeonidin 3-glc (**31**) (▲), 5-carboxypyranodelphinidin 3-glc (**32**) (●) and 5-carboxypyranopetunidin 3-glc (**33**) (□). **Bottom**: HDVs showing cumulative peak areas (μC/nmol anthocyanin) of pelargonidin 3-glc (**1**) (■), cyanidin 3-glc (**5**) (♦), peonidin 3-glc (**24**) (▲), delphinidin 3-glc (**22**) (●) and petunidin 3-glc (**26**) (□). glc = glucoside.

The reducing capacity of the 3-glucosides of pelargonidin (**1**), cyanidin (**5**), peonidin (**24**), delphinidin (**22**), petunidin (**26**), 5-carboxypyranopelargonidin (**28**), 5-carboxypyranocyanidin (**30**), 5-carboxypyranopeonidin (**31**), 5-carboxypyranodelphinidin (**32**), and 5-carboxypyranopetunidin (**33**) were also derived from coulometric analyses using HPLC coupled to a coulometric array detector set from 100 to 800 mV in increments of 100 mV. Hydrodynamic voltammograms (HDVs) for each of the 14 anthocyanins were achieved

by plotting the cumulative responses from 100 to 800 mV against the relative peak area in the chromatograms (Figure 33). Flavonoids present several waves of oxidation across the coulometric array, corresponding to several moieties capable of undergoing oxidation (Manach, 2003). According to Aaby et al. (2004) the cumulative responses at low to medium oxidation potentials (300-500 mV) were most relevant for addressing potential antioxidant capacity of various phenolics. Hence, in the present study the relative cumulative peak area at 400 mV was used as a measure for the reducing capacity of the individual anthocyanins.

When examining the reducing capacity of the individual anthocyanins, the most pronounced effect was observed for 5-carboxypyranopelargonidin 3-glucoside (**28**). This pigment remained without any significant cumulative responses even at electrode potentials as high as 600 mV. In full agreement with FRAP measurements the reducing capacity of this compound with a D-ring and only one hydroxyl group on the B-ring, was very low compared to the other examined anthocyanins. In fact the relative order of the reducing capacity of the 5-carboxypyranoanthocyanidin 3-glucosides were alike whether determined by coulometric array detection or FRAP. With exemption of a slightly decreased value for delphinidin 3-glucoside (**22**) measured by coulometric array detection, there was similarly agreement between the relative reducing capacity of the examined anthocyanidin 3-glucosides.

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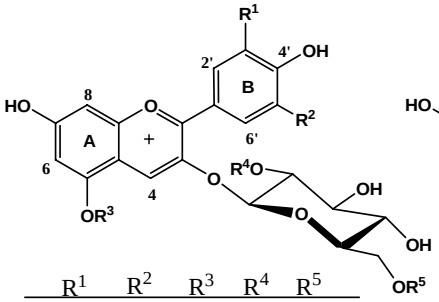
APPENDIX A-D

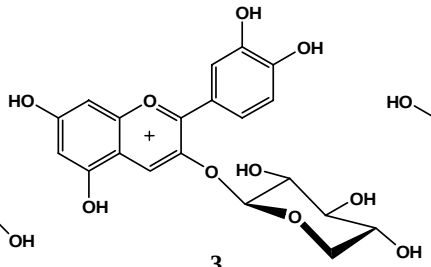
APPENDIX A. Presentation of pigments involved in the thesis

Pigment	**No**	Struc. (Fig.)	^{1}H NMR	^{13}C NMR	Paper ref.
Pelargonidin 3-glc	**1**	B-1			III(**1**); VIII(**1**)
Pelargonidin 3-[6-(rha)glc] (Pg rutinoside)	**2**	B-1	Tab. C-1	Tab. D-1	IV(**2**)
Cyanidin 3-xyl	**3**	B-1			II(**7**)
Cyanidin 3-gal	**4**	B-2	Tab. C-2	Tab. D-2	III(**5**); IV(**4**); VIII(**13**)
Cyanidin 3-glc	**5**	B-1			II(**3**); III(**2**);VIII(**2**)
Cyanidin 3-[6-(ara)glc] (Cy vicianoside)	**6**	B-1	Tab. C-1	Tab. D-1	III(**7**)
Cyanidin 3-[2-(xyl)gal]	**7**	B-1			III(**11**)
Cyanidin 3-[2-(xyl)glc] (Cy sambubioside)	**8**	B-1			III(**12**)
Cyanidin 3-[6-(rha)glc]	**9**	B-1	Tab. C-1	Tab. D-1	II(**4**); III(**9**); IV(**1**)
Cyanidin 3-[6-(glc)glc] (Cy gentiobioside)	**10**	B-1			III(**8**)
Cyanidin 3-xyl-5-glc	**11**	B-3	Tab. C-1	Tab. D-1	I(**1**)
Cyanidin 3,5-di-glc	**12**	B-1			III(**13**)
Cyanidin 3-[2-(xyl)-6-(rha)glc]	**13**	B-1			III(**10**)
Cyanidin 3-[2-(xyl)glc]-5-glc	**14**	B-1			III(**14**)
Cyanidin 3-[6-*E*-(caf)glc]	**15**	B-1	Tab. C-2	Tab. D-2	II(**8**)
Cyanidin 3-[6-*Z*-*p*-(cum)glc]	**16**				II(**9**)
Cyanidin 3-[6-*E*-*p*-(cum)glc]	**17**	B-1	Tab. C-2	Tab. D-2	II(**10**)
Cyanidin 3-xyl-5-[6(mal)glc]	**18**	B-3	Tab. C-1	Tab. D-1	I(**2**)
Cyanidin 3-xyl-5-[6(Me-mal)glc]	**19**	B-3	Tab. C-1	Tab. D-1	I(**3**)
Cyanidin 3-[2-(xyl)-6-*Z*-*p*-(cum)glc]-5-glc	**20**	B-1			III(**15**)
Cyanidin 3-[2-(xyl)-6-*E*-*p*-(cum)glc]-5-glc	**21**	B-1			III(**16**)
Delphinidin 3-glc	**22**	B-2	Tab. C-2	Tab. D-2	II(**1**); III(**3**); V(**1**); VI(**3**); VII(**1**); VIII(**4**)
Delphinidin 3-[6-(rha)glc]	**23**	B-1			II(**2**)
Peonidin 3-glc	**24**	B-1			II(**5**); III(**4**); VIII(**3**)
Peonidin 3-[6-(rha)glc]	**25**	B-1			II(**6**); III(**6**)
Petunidin 3-glc	**26**	B-2	Tab. C-2	Tab. D-2	V(**3**); VI(**2**); VII.(**2**); VIII(**5**)
Malvidin 3-glc	**27**	B-2	Tab. C-2	Tab. D-2	V(**5**); VI(**1**);VII(**3**); VIII(**6**)
5-CPpelargonidin 3-glc	**28**	B-2			VIII(**7**)
5-CPcyanidin 3-gal	**29**	B-2			VIII(**14**)
5-CPcyanidin 3-glc	**30**	B-2			VIII(**8**)
5-CPpeonidin 3-glc	**31**	B-2			VIII(9)
5-CPdelphinidin 3-glc	**32**	B-2	Tab. C-4	Tab. D-4	V(**2**); VII(**4**); VIII(**10**)
5-CPpetunidin 3-glc	**33**	B-2	Tab. C-4	Tab. D-4	V(**4**); VII(**5**); VIII(**11**)
5-CPmalvidin 3-glc	**34**	B-2	Tab. C-4	Tab. D-4	V(**6**); VII(**6**); VIII(**12**)
Quercetin 3-[6-(rha)glc]	**35**	B-2			VII(**7**)
Cyanidin 3-gal (hemiketal) major (a)	**4a**	B-2	Tab. C-3	Tab. D-3	
Cyanidin 3-gal (hemiketal) minor (b)	**4b**	B-2	Tab. C-3	Tab. D-3	
Delphinidin 3-glc (hemiketal) major (a)	**22a**	B-2	Tab. C-3	Tab. D-3	
Delphinidin 3-glc (hemiketal) minor (b)	**22b**	B-2	Tab. C-3	Tab. D-3	
Petunidin 3-glc (hemiketal) minor (a)	**26a**	B-2	Tab. C-3	Tab. D-3	
Petunidin 3-glc (hemiketal) minor (b)	**26b**	B-2	Tab. C-3	Tab. D-3	
Malvidin 3-glc (hemiketal) major (a)	**27a**	B-2	Tab. C-3	Tab. D-3	
Malvidin 3-glc (hemiketal) minor (b)	**27b**	B-2	Tab. C-3	Tab. D-3	

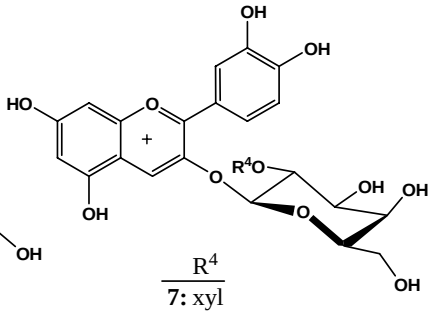
APPENDIX B. Structures of pigments involved in the thesis



	R^1	R^2	R^3	R^4	R^5
1:	H	H	H	H	H
2:	H	H	H	H	rha
5:	OH	H	H	H	H
6:	OH	H	H	H	ara
8:	OH	H	H	xyl	H
9:	OH	H	H	H	rha
10:	OH	H	H	H	glc
12:	OH	H	glc	H	H
13:	OH	H	H	xyl	rha
14:	OH	H	glc	xyl	H
15:	OH	H	H	H	*E*-caf
16:	OH	H	H	H	*Z*-cum
17:	OH	H	H	H	*E*-cum
20:	OH	H	glc	xyl	*Z*-cum
21:	OH	H	glc	xyl	*E*-cum
23:	OH	OH	H	H	rha
24:	OMe	H	H	H	H
25:	OMe	H	H	H	rha



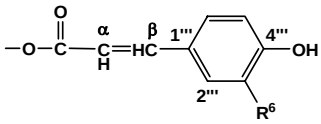
3



R^4

7: xyl

	R^6
15:	OH
16:	H
17:	H
20:	H
21:	H



R5

Figure B-1. Structures of **1** (pg 3-glc), **2** (pg 3-[6-(rha)glc]), **3** (cy 3-xyl), **5** (cy 3-xyl), **6** (cy 3-[6-(ara)glc]), **7** (cy 3-[2-(xyl)gal], **8** (cy 3-[2-(xyl)glc], **9** (cy3-[6-(rha)glc]), **10** (cy 3-[6-(glc)glc]), **12** (cy 3,5-di-glc), **13** (cy 3-[2-(xyl) 6-(rha)glc]), **14** (cy 3-[2-(xyl)glc]-5-glc), **15**, (cy 3-[6-*E*-*p*-(cum)glc]), **16** (cy 3-[6-*Z*-*p*-(cum)glc]), **17** (cy 3-[6-*E*-*p*-(cum)glc]), **20** (cy 3-[2-(xyl)-6-*Z*-*p*-(cum)glc]-5-glc), **21** (cy 3-[2-(xyl)-6-*E*-*p*-(cum)glc]-5-glc), **23** (dp 3-[6-(rha)glc]), **24** (pn 3-glc), **25** (pn 3-[6-(rha)glc]).

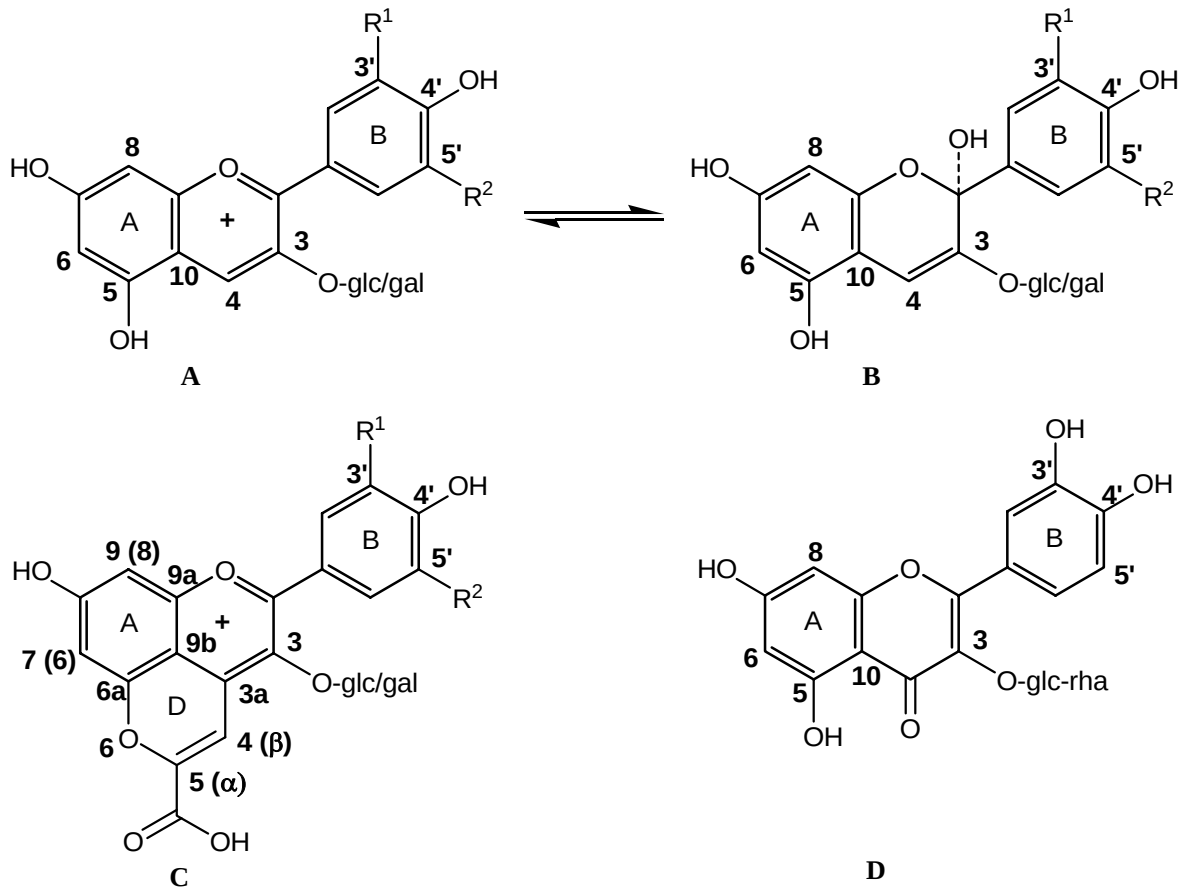


Figure B-2. **A**: Flavylium cation form of anthocyanins (**4**, **22**, **26**, **27**); **B**: Hemiketal forms of anthocyanins (**4a,b**, **22a,b**, **26a,b**, **27a,b**); **C**: Carboxypyranoanthocyanins (**28-34**); **D**: Rutin (**35**). **4 =** cy 3-gal (R^1 = OH, R^2= H); **22** = dp 3-glc (R^1, R^2 = OH); **26** = pt 3-glc (R^1 = OCH_3, R^2 = OH); **27** = mv 3-glc (R^1, R^2 = OCH_3); **28** = 5-CPpg 3-glc(R^1, R^2 = H); **29** = 5-CPcy 3-gal (R^1 = OH, R^2 = H); **30** = 5-CPcy 3-glc (R^1 = OH, R^2 = H); **31** = 5-CPpn 3-glc (R^1 = OMe R^2 = H); **32** = 5-CPdp 3-glc (R^1, R^2 = OH); **33** = 5-CPpt 3-glc (R^1 = OCH_3, R^2 = OH); **34** = 5-CPmv 3-glc (R^1, R^2 = OCH_3); **35** = quercetin 3-[6-(rha)glc]. The numbers in brackets in **C** shows the normal nomenclature for positions in anthocyanins.

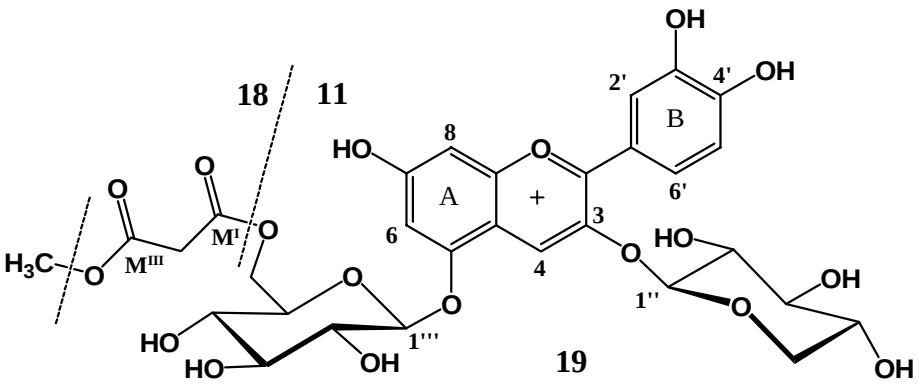


Figure B-3. Structure of pigments **11** (cy 3-xyl-5glc), **18** (cy 3-xyl-5-[6(mal)glc]) and **19** (cy 3-xyl-5-[6(Me-mal)glc]).

APPENDIX C. ^{1}H NMR data reported in the thesis (chemical shifts are given in ppm and coupling constants are given in Hz)

Table C-1 ^{1}H NMR spectral data for pigment **2**, **6**, **9**, **11**, **18** and **19** dissolved in CD_3OD–CF_3COOD (95:5, v/v) recorded at 25°C.

	2	**6**	**9**	**11**	**18**	**19**
4	9.10 s	9.06 s	9.09 s	9.04 s	9.06 s	9.05 s
6	7.20 d, 2.9	6.74 d, 1.6	6.78 s	7.13 s	7.10 s	7.08 d, 1.8
8	6.77s	6.97 d, 1.4	7.02	7.17 s	7.18 s	7.17 d, 1.6
2'	8.69 d, 9.2	8.11 d, 2.3	8.17 d, 2.1	8.15 d, 2.3	8.15 d, 2.0	8.15 d, 2.2
3'	7.14 d, 9.2					
5'	7.14 d, 9.2	7.09 d, 8.7	7.14 d, 9.2	7.12 d, 8.8	7.11 d, 8.9	7.11 d, 8.8
6'	8.69 d, 9.2	8.33 dd, 2.3, 8.9	8.70 dd, 9.2, 2.1	8.42 dd, 2.3, 8.8	8.44 dd, 2.0, 8.9	8.42 dd, 2.3, 8.7
	3-glucoside	*3-glucoside*	*3-glucoside*	*3-xyloside*	*3-xyloside*	*3-xyloside*
1"	5.37 d, 7.8	5.33 d, 7.7	5.36 d, 7.7	5.49 d, 7.0	5.48 d, 6.9	5.49 d, 6.9
2"	3.75 dd, 9.0, 7.8	3.77	3.77 dd, 9.1, 7.7	3.81 dd, 7.0, 9.4	3.80 dd, 7.0, 9.3	3.80 dd 9.3, 7.0
3"	3.61 m	3.63	3.62 m	3.66 m	3.65 t 9.4	3.65 t, 9.3
4"	3.50 m	3.56	3.50 t, 9.8	3.75 ddd, 12.3, 9.4, 4.5	3.74 m	3.75 m
5(A)"	3.81 m	3.80	3.81 m	4.11 dd, 4.9, 11.6	4.10 dd 11.3, 5.0	4.11 dd, 4.9, 11.5
5B"				3.60 dd, 9.4, 11.5	3.59 dd, 9.3, 11.5	3.60 dd, 9.3, 11.5
6A"	4.16 dd, 11.1, 1.5	4.23	4.15 dd, 11.2, 1.7			
6B"	3.68 m	3.86	3.68 m			
	6''-rhamnosyl	*6''-arabinosyl*	*6''-rhamnosyl*	*5-glucoside*	*5-glucoside*	*5-glucoside*
1'''	4.74 d, 1.6	4.26 d, 7.1	4.74 d, 1.6	5.28 d, 7.9	5.29 d, 7.7	5.29 d, 7.7
2'''	3.90 dd, 3.5, 1.6	3.63	3.90 dd, 3.5, 1.6	3.74 m	3.73 m*	3.74 m
3'''	3.71 m	3.50	3.72 m	3.69 m	3.65 t, 9.3	3.66 t, 9.0
4'''	3.41 m	3.85	3.42 m	3.55 t, 9.4	3.54 t, 9.3	3.56 m
5(A)'''	3.65 m	3.90	3.65 m	3.65 m*	3.89 ddd, 9.2, 6.9, 1.9	3.88 ddd, 9.5, 6.8, 1.9
5B'''		3.50				
6(A)'''	1.27 d, 6.2		1.26 d, 6.2	4.04 dd, 2.0, 12.1	4.62 dd, 2.0, 12.0	4.63 dd, 1.9, 11.9
6B'''				3.84 dd, 12.1, 5.7	4.42 dd, 12.0, 6.9	4.42 dd, 11.9, 6.8
					6'''-O-*Malonyl*	*6'''*-O-*Malonyl*
M^{II}					#	3.44
M^{IV}					#	3.76

*Overlap; s, singlet; d, doublet, t, triplet; m, multiplets;
#, not detected adequately. (For structures see Fig. B-1 and B-3)

Table C-2 ^{1}H NMR spectral data for pigment **15** and **17** dissolved in CD_3OD–CF_3COOD (95:5, v/v), and pigment **22**, **26**, **27** and **4** dissolved in CD_3OD. All spectra are recorded at 25^{o}C.

	15	**17**	**22**	**26**	**27**	**4**
4	8.99	8.94	9.056 d 0.8	9.090 d 0.8	9.132 d 0.8	9.113 d 1.0
6	6.63 d, 1.9	6.60 d 1.9	6.732 d 2.0	6.739 d 2.0	6.756 d 2.1	6.738 d 2.0
7						
8	6.89 d, 1.9	6.82 d, 1.9	6.949 dd 0.9, 2.0	6.994 dd, 0.7, 2.0	7.060 dd, 0.7, 2.1	6.982 dd, 1.0, 2.0
2'	8.09 d, 2.3	8.07 d, 2.3	7.861 s	8.075 d, 2.2	8.091 s	8.158 d, 2.3
5'	7.09 d, 8.7	7.07 d, 8.7				7.105 d, 8.8
6'	8.32 dd, 2.3, 8.7	8.27 dd, 2.3, 8.7	7.861 s	7.867 dd, 0.6, 2.2	8.091 s	8.358 dd, 8.8. 2.3
OMe				4.088 s	4.100 s	
3-gly	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*galactoside*
1"	5.41 d, 7.7	5.39[b]	5.41 d, 7.8	5.43 d, 7.8	5.44 d, 7.8	5.35 d, 7.7
2"	3.79	3.82 dd, 2.1, 12.1	3.79 dd, 7.8, 9.1	3.76 dd, 7.8, 9.0	3.73 dd, 7.7, 9.2	4.08 dd, 7.7, 9.6
3"	3.66	3.69	3.65 t 9.1	3.64 t, 9.0	3.63 t, 9.2	3.76 dd, 9.6, 3.4
4"	3.56	3.58 dd, 9.8, 9.2	3.54 dd, 9.1, 9.8	3.51 dd, 9.0, 9.9	3.49 dd, 9.2, 9.9	4.04 dd, 0.5, 3.4
5"	3.92	3.92	3.65 m	3.66 m	3.66 m	3.89 m
6A"	4.61	4.61 dd, 2.1, 12.1	4.00 dd, 2.3, 12.2	4.01 dd, 2.3, 12.1	4.01 dd, 2.2, 12.2	3.89 m
6B"	4.45	4.45 dd, 7.7, 12.1	3.82 dd, 6.3, 12.2	3.79 dd, 6.3, 12.1	3.78 dd, 6.3, 12.2	3.86 m
	6″-O-E-*caffeoyl*	*6″*-O-E-p-*coumaroyl*				
1‴						
2‴	6.99 d, 1.8	7.36 d, 8.6				
3‴		6.86 d, 8.6				
4‴						
5‴	6.84 m	6.86 d, 8.6				
6‴	6.86 m	7.36 d, 8.6				
α	6.26 d, 15.9	6.31 d, 15.9				
β	7.45 d, 15.9	7.51 d, 15.9				

*Overlap; s, singlet; d, doublet, t, triplet; m, multiplets; gly, glycoside; [b]chemical shift value from the COSY spectrum, anomeric signal was overlapped by the water signal. (For structures see Fig. B-1 and B-2)

Table C-3 ^{1}H NMR spectral data for the hemiketal forms **a** (major) and **b** (minor) of pigment **4**, **22**, **26** and **27** dissolved in CD_3OD. All spectra are recorded at 25^{o}C.

	4a	**4b**	**22a**	**22b**	**26a**	**26b**	**27a**	**27b**
4	6.610 d, 0.7	6.658 d, 0.7	9.056 d, 0.8	6.636 d, 0.6	6.579 d, 0.7	6.646 d, 0.7	6.582 d, 0.8	6.651 d, 0.8
6	6.044 d, 2.2	6.049 d, 2.2	6.732 d, 2.0	6.047 d, 2.2	6.051 d, 2.0	6.055 d, 2.0	6.065 d, 2.2	6.059 d, 2.2
7								
8	5.999 dd, 0.7, 2.2	6.017 dd, 0.7, 2.2	6.949 dd, 0.9, 2.0	6.016 dd, 0.7, 2.2	6.022 dd, 0.6, 2.2	6.028 dd, 0.6, 2.3	6.046 dd, 0.7, 2.2	6.043 dd, 0.7, 2.2
2'	7.105 d, 2.2	7.106 d, 2.2	7.861 s	6.678 s	6.857 d, 1.9	6.814 d, 1.9	6.968 s	6.944 s
5'	6.792 d, 8.3	6.826 d, 8.3						
6'	7.011 dd, 8.3, 2.2	6.991 dd, 8.3, 2.2	7.861 s	6.678 s	6.795 dd, 0.6, 2.0	6.792 dd, 0.6, 2.0	6.968 s	6.944 s
OMe					3.909 s	3.967 s	3.908 s	3.911 s
3-gly	*galactoside*	*galactoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*
1"	4.90 d, 7.7	4.72 d, 7.7	4.93 d, 7.8	4.758 d, 7.8	4.95 d, 7.8	4.79 d, 7.8	4.97 d, 7.8	4.83 d, 7.8
2"	3.69 dd, 7.7, 9.8	3.66 dd, 7.7, 9.8	3.38 dd, 7.8, 9.1	3.34 dd, 7.8, 9.4	3.37 dd, 7.8, 9.1	3.34 dd, 7.8, 9.3	3.36 dd, 7.7, 9.1	3.34 dd, 7.8, 9.2
3"	3.64 dd, 9.8, 3.4	3.56 dd, 9.8, 3.4	3.52 m	3.45 t 9.4	3.52 t 9.1	3.45 t 9.3	3.52 t 9.1	3.46 t 9.2
4"	3.97 dd, 1.1, 3.4	3.95 dd, 1.1, 3.4	3.47 m	*	3.45 m	3.49 m	3.44 dd, 9.1, 9.9	*
5"	3.78 m	3.74 m	3.54 m	3.48 m	3.52 m	3.48 m	3.52 m	3.58 m
6A"	3.84 m	3.89 m	3.96 m	3.82 m	3.95 m	3.99 m	3.78 m	3.82 m
6B"	3.80 m	3.86 m	3.81 m	3.99 m	3.79 m	3.83 m	3.94 m	3.99 m

*Overlap; s, singlet; d, doublet, t, triplet; m, multiplets; gly, glycoside. (For structures see Fig. B-2)

Table C-4 ^{1}H NMR spectral data for pigment **32**, **33** and **34** in dissolved in CD_3OD–CF_3COOD (95:5, v/v) recorded at 25^{o}C.

	32	**33**	**34**
4	8.12 s	8.08 s	8.07 s
7 (6)	7.29[b] d, 1.9	7.25[b] d, 1.9	7.27[b] d, 1.9
8 (7)			
9 (8)	7.24[b] d, 1.9	7.34[b] d, 1.9	7.41[b] d, 1.9
2'	7.65 s	7.82 d, 2.2	7.81 s
6'	7.65 s	7.62 d, 2.2	7.81 s
OMe		3.99 s	
3-glucoside			
1"	4.81 d, 7.7	4.81 d, 7.8	4.79 d, 7.8
2"	3.74 dd, 7.7, 9.3	3.71 dd, 7.8, 9.2	3.70 dd, 7.8, 9.2
3"	*	3.46	*
4"	3.35 dd, 9.0, 8.8	3.32 dd, 9.0, 8.7	3.32 dd, 9.1, 8.9
5"	3.24 ddd, 9.0, 6.8, 1.9	3.24 ddd, 9.0, 6.8, 1.9	3.24 ddd, 9.1, 6.8, 1.9
6A"	3.82 dd, 11.7, 1.9	3.82 dd, 11.7, 1.9	3.81 dd, 11.6, 1.9
6B"	3.48 dd, 11.7, 6.8	3.48 dd, 11.7, 6.8	3.48 dd, 11.6, 6.8

*Overlap; s, singlet; d, doublet, t, triplet; m, multiplets; coupling constant (Hz), [b]assignments may be reversed. (For structures see Fig. B-2)

APPENDIX D. ^{13}C NMR data reported in the thesis (chemical shifts are given in ppm)

Table D-1 ^{13}C NMR spectral data for pigment **2**, **6**, **9**, **11**, **18** and **19** dissolved in CD_3OD–CF_3COOD (95:5, v/v) recorded at 25°C.

	2	**6**	**9**	**11**	**18**	**19**
2	165.02	164.47	163.65	164.8	165.1	165.1
3	146.25	145.59	145.33	145.3	146.2	146.0
4	136.52	137.65	136.25	135.0	135.0	134.6
5	159.55	159.24	158.68	157.0	156.4	156.0
6	103.21	103.52	104.01	105.6	105.8	105.6
7	172.12	170.71	170.46	169.1	169.3	168.9
8	96.50	95.35	99.56	97.5	97.3	97.3
9	157.58	157.86	157.40	155.5	157.1	157.2
10	112.26	113.46	113.30	113.3	113.2	113.1
1'	121.14	121.24	121.34	120.5	121.0	120.7
2'	135.05	118.51	118.02	118.9	118.5	118.4
3'	118.50	147.41	146.67	147.3	147.6	147.3
4'	166.54	155.79	149.90	157.0	156.7	156.4
5'	118.50	117.46	117.48	117.9	117.6	117.6
6'	135.05	128.35	128.52	129.4	129.1	129.1
	3-glucoside	*3-glucoside*	*3-glucoside*	*3-xyloside*	*3-xyloside*	*3-xyloside*
1"	103.75	104.28	102.92	104.1	103.9	103.9
2"	78.71	74.81	74.51	74.4	74.1	74.1
3"	77.89	77.93	77.78	78.9	77.4	77.0
4"	70.85	71.13	70.89	71.0	70.7	70.7
5"	77.04	77.73	77.22	67.4	67.0	66.9
6A"	67.69	69.51	67.50			
6B"	67.69	69.51	67.50			
	6"-rhamnosyl	*6"-arabinosyl*	*6"-rhamnosyl*	*5-glucoside*	*5-glucoside*	*5-glucoside*
1"'	102.03	105.28	102.05	102.8	102.4	102.3
2"'	71.65	72.38	71.25	74.9	74.6	74.6
3"'	72.38	74.14	72.33	78.1	77.4	77.7
4"'	73.72	69.58	73.70	71.5	71.2	71.3
5"'	69.66	66.90	69.55	77.7	75.9	75.8
6"'	17.69		17.58	62.5	65.3	65.2
					6"'-O-*Malonyl*	*6"'*-O-*Malonyl*
M^I					168.7	167.9
M^{II}					#	#
M^{III}					#	168.6
OCH_3 (M^{IV})						52.9

[#] Not detected adequately. (For structures see Fig. B-1 and B-3)

Table D-2 ^{13}C NMR spectral data for pigment **15** and **17** dissolved in CD_3OD–CF_3COOD (95:5, v/v), and pigment **22**, **26**, **27** and **4** dissolved in CD_3OD. All spectra are recorded at 25°C.

	15	**17**	**22**	**26**	**27**	**4**
2	164.54	164.03	164.22	164.09	163.99	164.42
3	145.08	144.71	145.87	145.81	145.81	145.72
4	137.11	136.71	136.12	136.63	137.08	136.98
5	158.60	158.34	159.16	159.22	159.35	159.21
6	103.64	103.41	103.19	103.25	103.87	103.29
7	170.62	170.41	170.24	170.44	170.69	170.42
8	95.18	95.04	94.96	95.13	95.37	95.09
9	157.73	157.61	157.63	157.74	157.96	157.69
10	112.81	112.99	113.20	113.42	113.06	113.39
1'	121.22	120.99	120.01	119.96	119.87	121.27
2'	118.36	118.27	112.55	109.32	110.63	118.46
3'	147.48	147.09	144.78	149.77	149.78	147.41
4'	155.82	155.54	147.55	145.21	146.25	155.78
5'	117.39	115.34	144.78	147.51	149.78	117.41
6'	128.31	128.09	112.55	113.69	110.63	128.23
OMe				57.15	57.17	
3-gly	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*galactoside*
1"	103.18	102.91	103.63	103.73	103.76	104.45
2"	74.70	74.61	74.76	74.94	75.04	72.07
3"	77.88	77.72	78.05	78.18	78.25	74.92
4"	71.70	71.55	71.04	71.15	71.22	70.10
5"	76.06	75.84	78.81	78.89	78.97	77.79
6A"	64.42	64.53	62.32	62.38	62.42	62.33
6B"	64.42	64.53	62.32	62.38	62.42	62.33
	6″-O-E-*caffeoyl*	*6″*-O-E-p-*coumaroyl*				
1‴	127.57	126.60				
2‴	115.39					
3‴	146.75					
4‴	149.62	131.00				
5‴	116.46	116.64				
6‴	122.83	161.06				
α	114.63	114.20				
β	147.19	146.85				
C=O	168.96	168.8				

gly, glycoside. (For structures see Fig. B-1 and B-2)

Table D-3 ^{13}C NMR spectral data for the hemiketal forms **a** (major) and **b** (minor) of pigment **4**, **22**, **26** and **27** dissolved in CD_3OD. All spectra are recorded at 25^{o}C.

	4a	**4b**	**22a**	**22b**	**26a**	**26b**	**27a**	**27b**
2	103.10	102.96	103.19	103.11	103.82	102.75	103.11	103.60
3	145.48	145.53	145.55	145.39	145.47	145.47	145.39	145.27
4	98.63	99.59	98.78	98.57	98.64	99.72	98.57	99.79
5	154.59	154.44	154.31	154.57	153.26	154.40	154.57	153.17
6	97.05	97.10	96.98	97.19	97.07	97.30	97.19	97.13
7*	158.4	158.4	158.4	158.4	158.4	158.4	158.4	158.4
8	95.09	95.25	95.14	95.29	95.13	95.29	95.10	95.25
9	153.19	153.19	153.17	152.95	153.17	152.52	152.95	154.11
10	101.77	101.92	101.76	101.75	101.69	101.78	101.75	101.89
1'	133.27	133.32	132.41	132.15	132.56	132.32	132.15	132.56
2'	115.59	115.48	107.37	105.90	103.81	107.24	105.90	103.60
3'	146.64	145.64	146.12	148.52	148.98	146.28	148.52	148.77
4'	146.56	146.66	134.47	136.81	135.22	134.56	136.81	135.48
5'	115.36	115.45	146.12	148.52	145.69	146.28	148.52	145.62
6'	119.77	119.67	107.37	105.90	109.81	107.24	105.90	103.60
OMe					56.67	56.69	56.69	56.81
3-gly	*galactoside*	*galactoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*	*glucoside*
1"	102.73	103.35	102.73	102.11	102.98	103.55	102.11	102.63
2"	71.89	71.89	71.89	74.78	74.57	74.66	74.78	74.64
3"	74.84	74.46	74.84	77.88	78.02	77.81	77.88	77.67
4"	70.05	69.99	70.05	71.25	71.09	70.95	71.25	70.93
5"	76.84	77.00	76.84	78.29	78.04	78.18	78.29	78.24
6A"	62.16	62.26	62.16	62.45	62.25	62.44	62.45	62.45
6B"	62.16	62.26	62.16	62.45	62.25	62.44	62.45	62.45

*^{13}C NMR data obtained from the HMBC spectra. gly, glycoside.
(For structures see Fig. B-2)

Table D-4 ^{13}C NMR spectral data for pigment **32**, **33** and **34** in dissolved in CD_3OD–CF_3COOD (95:5, v/v) recorded at 25^{o}C.

	32	**33**	**34**
2	166.45	166.15	165.68
3	136.25	136.18	136.08
3a (4)	149.41	149.70	e
4	107.40	107.44	e
5	155.68[c]	155.66[c]	156.43[c]
COOH	161.33	161.11	161.48
6a (5)	154.39	154.55	154.52
7 (6)	101.34[b]	101.92[b]	101.98[b]
8 (7)	169.39	169.68	169.68
9 (8)	101.75[b]	101.58[b]	101.82[b]
9a (9)	154.34[c]	154.50[c]	154.56[c]
9b (10)	110.71	110.94	111.01
1'	120.21	120.23	120.23
2'	112.12	108.66	110.08
3'	147.24	149.93	149.56
4'	143.38	143.93	144.95
5'	147.24	147.31	149.56
6'	112.12	113.45	110.08
OMe		57.24	57.26
3-glucoside			
1"	105.73	105.47	105.36
2"	75.43	75.64	75.71
3"	77.65	77.75	77.79
4"	71.39	71.62	71.61
5"	78.93	79.11	79.12
6A"	62.77	62.85	62.83
6B"	62.77	62.85	62.83

e, signal is missing; [b,c]assignments may be reversed; numbers in brackets represent anthocyanin positions. (For structures see Fig. B-2)