AN ABSTRACT OF THE THESIS OF

María Mónica Giusti Hundskopf for the degree of Doctor of Philosophy in Food Science and Technology presented August 4, 1998. Title: Structure and Conformation of Red Radish (*Raphanus sativus* L.) Anthocyanins and Their Effect on Color and Pigment Stability.

Abstract approved :

Ronald E. Wrolstad

Red radish (*Raphanus sativus* L.) anthocyanins were extracted from liquid nitrogen powdered epidermal tissue using acetone and chloroform and isolated using C-18 resin. Chemical structure and conformation of major pigments were elucidated by 1-D and 2-D NMR, Mass Spectroscopy, HPLC, and spectral analyses. Two novel di-acylated anthocyanins, pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*- *trans*-*p*-coumaroyl-β-D-glucopyranoside)]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) and pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-feruloyl-β-D-glucopyranoside)]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) represented 70% of total pigment. Two mono-acylated anthocyanins (20%) were pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucopyranoside)]-5-*O*-(β-D-glucopyranoside) and pelargonidin 3-*O*-[2-*O* -(β-D-glucopyranosyl)-(6-*O*-*trans*-feruloyl-β-D-glucopyranoside)]-5-*O*-(β-D-glucopyranoside). NOESY revealed folding of the molecule.

Electrospray (ES-MS) and tandem mass spectroscopy (MS-MS) were tested as tools for anthocyanin characterization. Anthocyanins were semi-purified using C-18

resin, washed with acidified water and ethyl acetate, and recovered with acidified methanol. Samples were injected into a mass spectrometer in aqueous or methanolic solutions. Charged character of anthocyanins favored the fast and effective detection of intact molecular ions requiring minimal sample preparation and with little interference. MS-MS provided clear and characteristic fragmentation patterns.

Qualitative and quantitative anthocyanin pigment content of radish cultivars grown at 2 locations (Corvallis and Hermiston, OR) and harvested at 2 maturity stages, were evaluated. Pigment content depended on cultivar, root weight and location, with higher amounts obtained at Hermiston. Spring cultivars (n=22) had pigmentation in the skin, ranging from 39.3 to 185 mg anthocyanin/100g skin. Red-fleshed Winter cultivars (n=5) possessed from 12.2 to 53 mg anthocyanin/100g root. The major pigments were pelargonidin-3-sophoroside-5-glucoside, mono- or di-acylated with cinnamic and malonic acids; individual proportions varied among cultivars. Estimated pigment yields ranged from 1.3 to 15.8 kg/ha.

Acylated and non-acylated pelargonidin were isolated using semi-preparative HPLC to evaluate the effect of glycosylation and acylation in spectral characteristics, molar absorptivity and color. Molar absorptivity ranged from 15,600 to 39,590 for pelargonidin-3-glucoside and pg-3-rutinoside-5-glucoside acylated with p-coumaric acid, respectively. An hypsochromic shift on λmax was observed in presence of glycosylation. Pelargonidin-3,5-diglucoside and 3,5-triglucoside showed yellow-orange hue angle (>40°) in pH 1.0 buffer, higher than other pg-derivatives. Cinnamic acid acylation caused a bathochromic shift. Malonic acid acylation had little effect on color and no effect on λmax. The solvent system affected molar absorptivity and

visual color characteristic of the pigments. Acylation increased pigment resistance to acid hydrolysis.

Structure and Conformation of Red Radish (*Raphanus sativus* L.) Anthocyanins and Their Effect on Color and Pigment Stability

by

María Mónica Giusti Hundskopf

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Chair of Department of Food Science and Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Maria Monica Giusti Hundskopf, Author

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CONTRIBUTION OF AUTHORS

Ronald E. Wrolstad was involved on the experimental design, data analyses and writing of each chapter. Hamid Ghanadan participated in the experimental design of chapter 4 and assisted in NMR data collection and interpretation. Donald Griffin assisted on the selection of MS techniques and data collection of MS for chapter 5. Radish cultivars for chapter 6 were selected and planted by James R. Baggett, and Gary L. Reed, who were also involved in the experimental design of chapter 6. Robert W. Durst participated in cultivar selection, data gathering and interpretation for chapter 6. Luis E. Rodriguez-Saona was involved in experimental design and data interpretation of chapters 5, 6 and 7.

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A Danella,

who filled my life with new colors.

STRUCTURE AND CONFORMATION OF RED RADISH (*Raphanus sativus* L.) ANTHOCYANINS AND THEIR IMPACT ON COLOR AND PIGMENT STABILITY

CHAPTER 1. INTRODUCTION

Colorants are used widely in the food industry to ensure uniformity of color, to enhance or restore the natural appearance, giving attractive appearance and increasing acceptability, and to help protect flavor and light sensitive compounds during shelf-storage by a sun screen effect (Newsome, 1986). Color is also used by consumers as an indicator of quality.

There are seven synthetic colorants currently approved by the FDA for use in foods and in orally ingested drugs and cosmetics (Hallagan, 1991; FDA, 1991), among which, FD&C Red No. 40 is by far the one with the highest demand. The National Academy of Sciences 1977 survey of the amount of FD&C certified colorants consumed revealed that an average daily intake of 100 mg for FD&C No. 40 was much larger than that for other synthetic colorants. FD&C Yellow No. 5 at 43 mg/day was second.

Finding a natural red colorant to effectively replace FD&C Red No. 40 has been difficult, because few natural materials have its bright red color unmixed with other tones (LaBell, 1993). Natural colorants such as cochineal, concord grape extract, tart cherries extract, and carotenoids have been evaluated in various food systems (McLellan and Cash, 1979; LaBell, 1993; Sapers, 1994). Relatively good stability has been found; however, there have been limitations in reproducing the desired hue.

Radish (*Raphanus sativus* L.) is an easy vegetable to grow and it occurs in a variety of shapes: global, oval, long, flattened and pear-shaped roots; and colors: white, red, purple, yellow and black (George and Evans, 1981). The anthocyanin pigments responsible for red and purple radishes were characterized in the 60's by different researchers (Ishikura and Hayashi, 1962 and 1965; Harborne 1963; Fuleki, 1969), and the presence of pelargonidin- and cyanidin-derivatives was reported in red and purple radishes, respectively. A review on radish cultivars, composition, production, and anthocyanins has been published (Giusti, 1995).

Radish anthocyanin extract has proven to be an excellent potential alternative to the use of FD&C Red No. 40. Good pigment stability was obtained in a maraschino cherry system (Giusti and Wrolstad, 1996) and model juice systems (Rodriguez-Saona et al., 1998) and the color characteristic matched those obtained with FD&C Red No. 40.

In the present study, we conducted extensive analyses for elucidation of the chemical structure and conformation of radish anthocyanins and also evaluated the effect of sugar substitutions and acylations on spectral and color characteristics and pigment stability to acid hydrolysis. Electrospray mass spectroscopy and tandem mass spectroscopy techniques, which were found to be very useful in the characterization of radish anthocyanins, were applied to other anthocyanin extracts to evaluate those methodologies for anthocyanin characterization and detecting their presence in colored mixtures. Different radish cultivars were evaluated to determine the one that could be more suitable for potential colorant production. We analyzed pigment content and examined the qualitative pigment composition to make inferences regarding hue and

chroma. The effect of planting location and maturity on pigment yield were also evaluated by comparing results from 2 locations, and by harvesting at 2 maturity stages. Based on radish yield obtained and published data, potential yields of pigment/ha and pigment/ha-day were calculated, to determine the viability of pigment production on a commercial scale.

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CHAPTER 2. LITERATURE REVIEW
ANALYTICAL METHODS FOR THE ANALYSIS OF ANTHOCYANIN EXTRACTS

INTRODUCTION

Anthocyanins are part of a very large and widespread group of plant constituents known as flavonoids. They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzoprylium or flavylium salts (Mazza and Miniati, 1993).

Anthocyanins play an important role in plant taxonomy and biochemical systematics as chemical markers in plants and plant products (Harborne, 1963). They are of increasing interest for geneticists and plant breeders in the field of molecular biology (Strack and Wray, 1994). There is also a growing interest in anthocyanin producing cell cultures, as vehicles of secondary metabolites. In addition, anthocyanins have been shown to possess beneficial therapeutical properties, with strong antioxidant capacity (Wang et al., 1997; Tamura and Yagami, 1994; Brouillard, 1982).

Anthocyanin composition of fruits and vegetables has also been used to detect adulteration of anthocyanin based products (Wrolstad et al., 1981; Hale et al., 1986) and as indicators of product quality (Spayd and Morris, 1981; Jackman et al., 1987).

Anthocyanins have found considerable potential in the food industry as safe and effective food colorants (Strack and Wray, 1994). Interest in anthocyanins as potential natural food colors has increased in recent years. In 1980 the annual world production had been estimated to reach 10,000 tons from grapes alone (Timberlake, 1980).

Quantitative and qualitative anthocyanin composition are important factors in the determining the feasibility of the use of new plant materials as anthocyanin-based colorant sources.

The anthocyanin pigments consist of two or three portions (Fig. 2.1): the aglycon base (anthocyanidin), sugars and, often, acylating groups. There are 18 different aglycon groups known to naturally occur in nature, although from those only 6 occur frequently (Strack and Wray, 1994; Jackman and Smith, 1996). Since each aglycon may be glycosylated and acylated by different sugars, cinnamic and aliphatic acids, the number of anthocyanins is 15-20 times higher than the number of anthocyanidins, and there are about 300 known naturally occurring structures.

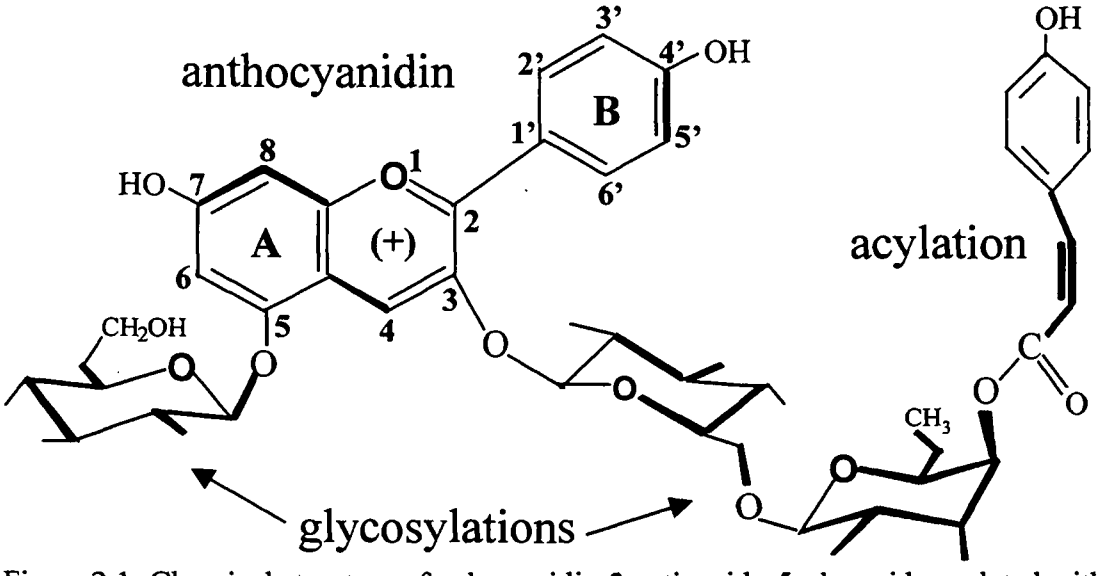

Figure 2.1. Chemical structure of pelargonidin-3-rutinoside-5-glucoside acylated with p-coumaric acid. Source: Rodriguez-Saona, et al. (1998).

Anthocyanins occur in solutions as a mixture of different secondary structures, quinonoidal base, carbinol pseudobase and the chalcone pseudobase (Brouillard, 1982; Strack and Wray, 1994). In addition, there are different mechanisms for the stabilization of anthocyanins which lead to the formation of tertiary structures such as self-association, inter- and intra-molecular copigmentation (Strack and Wray, 1994).

There are many analytical techniques that have been used for most of the century, which are still of importance. However, there have been considerable advances in new methodologies, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques, which have been particularly successful.

In this review, we summarize analytical procedures for quantitative and qualitative analyses of anthocyanin, including classical and modern techniques.

PIGMENT EXTRACTION AND CONCENTRATION

The extraction of anthocyanins is the first step in determination of total as well as individual anthocyanins in any type of plant tissue (Fuleki and Francis, 1968a). The choice of an extraction method is of great importance in the analysis of anthocyanins, and largely depends on the purpose of the extraction, the nature of the anthocyanins and the source material. A good extraction procedure should maximize anthocyanin recovery with minimum amount of adjuncts and minimal degradation or alteration of the natural (*in vivo*) state. Of course, one can never be sure that the extracted pigment is exactly the one occurring *in vivo* (Brouillard and Dangles, 1994). A knowledge of the factors that influence anthocyanin structure and stability is vital and those factors have been reviewed by Markakis (1982), Francis (1989), and Jackman and Smith

(1996). It is also desirable that the extraction procedure not be too complex, hazardous, time consuming or costly.

In most fruits and vegetables the anthocyanin pigments are located in cells near the surface (Jackman et al., 1987). Extraction procedures have generally involved the use of acidic solvents which cause rupture of cell membranes and simultaneously dissolve pigments. Maceration of crushed or ground material in methanol containing small amounts of HCl (< 1%) is commonly used at refrigerated temperatures, for times that may range from a few hours to overnight. The extracted material is usually too dilute for further analyses and the extraction procedure is usually followed by evaporation of the methanol using vacuum and mild temperatures (30-40°C). Ethanol would be preferred for food use to avoid the toxicity of methanolic solutions. However, recoveries are not as high as those obtained with methanol and concentration is more difficult because of its higher boiling point.

The solvent systems generally used for extraction purposes are by no means specific for anthocyanins (Markakis, 1974; Jackman et al., 1987). The acid tends to stabilize anthocyanins but it may also change the native form of the pigment in the tissue by breaking associations with metals, copigments, etc. Concentration procedures may also cause acid hydrolysis of labile acyl and sugar residues. Extraction with solvents containing HCl may result in pigment degradation during concentration, and is one of the reasons why acylations with aliphatic acids had been overlooked in the past (Strack and Wray, 1989). To minimize the decomposition of pigments, the use of milder pigment extraction procedures has been proposed using weaker organic acids, such as formic, acetic, citric or tartaric acids, or small amounts (0.5-3%) of stronger

more volatile acids such as trifluoroacetic acid, which could be then removed during pigment concentration (Jackman and Smith, 1996; Strack and Wray, 1994). Low HCl concentrations, in the order of 0.01-0.05% and procedures in the absence of acid have also been proposed (Jackman et al., 1987; Strack and Wray, 1994). These procedures need to be performed with care to avoid acid-dependent pigment degradation.

To obtain anthocyanins closer to their natural state a number of researchers have used neutral solvents for initial extraction such as 60% methanol, *n*-butanol, cold acetone, acetone/methanol/water mixtures, or simply water (Jackman et al., 1987). Metivier et al. (1980), compared the efficiency of extraction with 3 different solvents: methanol, ethanol and water, and using different acids, and found that methanol extraction was 20% more effective than ethanol and 73% more effective than water when used for anthocyanin recovery from grape pomace. They also reported that HCl was most effective when used in combination with ethanol, while of the organic acids, citric was more effective with methanol and acetic acid with water.

Depending on the means of extraction, decreasing the ratio of extraction solvent to plant material could avoid the need for a concentration step (Jackman and Smith, 1996).

An alternative procedure uses acetone as extracting solvent followed by partition with chloroform (Timberlake and Bridle, 1971; Wrolstad and Heatherbell, 1974; Abers and Wrolstad 1979; Wrolstad et al., 1990). Timberlake and Bridle (1971) compared this extraction procedure with the usual methanol-HCl extraction and concluded that the use of acetone with separation of the aqueous phase by addition of chloroform gave much cleaner and better defined bands and allowed for a better

assessment of anthocyanin composition. Recently, Wrolstad and Durst (1998) compared the acetone/chloroform method of extraction to acidified methanol and, depending on the material used, the anthocyanin recoveries were comparable or up to 30% higher with the use of acetone. An advantage of this procedure is that the chloroform-acetone mixture will partition lipids, chlorophyll and other water-insoluble materials from anthocyanins, yielding high recoveries of anthocyanins requiring few concentration and purification steps.

Liquid nitrogen has been used to freeze the sample and facilitate its conversion into a fine powder, minimizing anthocyanin degradation during extraction and maximizing recoveries (Wrolstad and Heatherbell, 1974; Price and Wrolstad, 1995). Advantages of the use of nitrogen powder are the inhibition of enzymatic reaction by lowering the temperature, the exclusion of oxygen by a nitrogen environment, and maximization of the surface area with recovery of a very fine powder.

QUANTITATIVE ANALYSIS OF ANTHOCYANINS

Frequently, the quantitative determination of anthocyanins is complicated by the presence of other compounds, which may interfere with the measurements. It is desirable to express anthocyanin determinations in terms that could be compared with the results from different workers (Fuleki and Francis, 1968a). The best way to express the results is in terms of the absolute quantities of anthocyanins present (Fuleki and Francis, 1968a). For this it is necessary to establish the identity of the pigments as well as their molar absorptivity coefficients.

The methods of quantitation of anthocyanins can be divided into three major groups: methods for samples with little or no interfering compounds, methods for samples with interfering compounds that absorb in the 480-550 nm range, and methods for quantitation of individual anthocyanin components.

Samples with no interfering materials: single pH method

Total anthocyanin content can be determined in crude extracts containing other phenolic materials by measuring absorptivity of the solution at a single wavelength. This is possible because anthocyanins have a typical absorption band in the 490-550 nm region of the visible spectra. This band is far from the absorption bands of other phenolics with spectral maximum in the UV-range (Fuleki and Francis, 1968a).

The absorption maximum and absorptivity are very dependent on pH, temperature, solvent, and presence of other materials that could interact with anthocyanins. In addition, anthocyanin absorption follows a linear relationship with concentration only when present at low levels; therefore, considerable dilution is usually necessary (Fuleki and Francis, 1968a).

Samples with interfering materials: differential and subtractive methods

pH-DIFFERENTIAL METHOD

Frequently, a simple total anthocyanin determination cannot be applied because of interference from anthocyanins degradation products or melanoidins from browning reactions (Fuleki and Francis, 1968b). In those cases, the approach has been to measure the absorbance at 2 different pH values. The differential method relies on the

structural transformations of the anthocyanin chromophore as a function of pH (Jackman and Smith, 1996) This concept was first introduced by Sondheimer and Kertesz in 1948, who used pH values of 2 and 3.4 for analyses of strawberry jams (Francis, 1989). Since then, the use of other pH values has been proposed. Fuleki and Francis (1968b) used pH 1 and 4.5 buffers to measure anthocyanin content in cranberries, and modifications of this technique have been applied to a wide range of commodities (Wrolstad et al., 1982 and 1995). The pH-differential method has been described as fast and easy for quantitation of monomeric anthocyanins (Wrolstad et al., 1995).

SUBTRACTIVE METHODS

Subtractive methods are based on the use of bleaching agents that would decolor anthocyanins but would not affect interfering materials. A measurement of the absorbance at the visible maximum is obtained, followed by bleaching and remeasuring to give a blank reading (Jackman et al., 1987). The two most widely used bleaching agents are sodium sulfite (Somers and Evans, 1974; Wrolstad et al., 1982) and hydrogen peroxide (Swain and Hillis, 1959).

Taking advantage of the differential and subtractive methods, a few absorbance readings can be used to get an estimation of the amount of monomeric anthocyanin, polymeric color, color density, browning index (Wrolstad et al., 1982) and degradation index (Fuleki and Francis, 1968b). The absorbance of anthocyanins at two different pH levels (1 and 4.5) is measured at the visible λmax and at 700 nm, to correct for haze in the sample. The absorbance at 420 nm is used as an index for browning, and measured

in a sodium metabisulfite-bleached sample and a control. The anthocyanin present in the monomeric form (monomeric anthocyanin) is most susceptible to pH changes; polymerized material will not bleach with sodium bisulfite. Color density is defined as the sum of absorbance at the visible λmax and at 420nm, and the ratio between monomeric and total anthocyanin can be used to determine a degradation index.

Content of individual pigments

The situation where the amount of individual pigments is desired is usually a research situation (Francis, 1989). An analytical method for the quantitative determination of individual anthocyanins involves their separation from a mixture and a measurement of each individual pigment (Francis, 1982, Jackman et al., 1987). A chromatographic separation of individual pigments is usually the first step. Formerly, the individual anthocyanins were separated by paper chromatography followed by spectrophotometric estimation of the amount of individual pigment bound to the paper. Availability of an efficient method for separation of anthocyanins, such as HPLC, combined with a list of absorption coefficients, should simplify the quantitative estimation of individual anthocyanins (Francis, 1989). The use of HPLC coupled to a photodiode array detector allows for separation of anthocyanins from even complex mixtures and for the determination of the λmax of each individual anthocyanin (Andersen, 1985; Hong and Wrolstad, 1990).

Molar absorptivity

Regardless of the method used for anthocyanin quantitation, the determination of the amount present requires an absorptivity coefficient. Absorptivity coefficients

have been reported as the absorption of a 1% solution measured through a 1-cm path at the λmax, or as a molar absorption coefficient. Absorptivity coefficients of some known anthocyanins have been reported by different researchers (Figueiredo et al., 1996a; Dangles et al., 1993; Francis, 1982; Hrazdina et al., 1977; Swain, 1965;). Through the years there has been lack of uniformity on the values of absorptivity reported, mainly due to the difficulties of preparing crystalline anthocyanin, free from impurities, in sufficient quantities to allow reliable weighing under optimal conditions (Fuleki and Francis, 1968a; Francis, 1982). Another problem is that the anthocyanin mixtures may be very complicated and not all absorptivity coefficients may be known. Even when they are known, it is necessary to first evaluate if the objective is the estimation of total anthocyanin content or the determination of individual pigments, and then, to decide which absorption coefficient (s) to use. The absorptivity is dependent not only on the chemical structure of the pigment but also on the solvent used, and preferably the coefficient used should be one obtained in the same solvent system as the one used in the experiment. If the identity of the pigments is unknown, it has been suggested that it could be expressed as cyanidin-3-glucoside (Francis, 1989), since that is the most abundant anthocyanin in nature.

QUALITATIVE ANALYSIS OF ANTHOCYANINS

At present the most satisfactory method for mixture analysis is the multistep quantification, separation and isolation by HPLC and peak identification by FAB-MS and high field NMR (Mazza and Miniati, 1993). Well developed chromatographic and

spectroscopic techniques have been applied for rapid and accurate identification of anthocyanins (Jackman and Smith, 1996)

Purification of crude extracts

Fractionation of anthocyanin-containing extracts is often necessary. Since none of the solvents used for extraction is specific for anthocyanins, considerable amounts of other compounds may be also extracted and concentrated. The variety and concentration of other compounds will depend on the solvent and methodologies used. The presence of extraneous materials could influence the stability or analysis of anthocyanins. Therefore, the next step towards anthocyanin characterization is the pre-fractionation of those extracts.

ANTHOCYANIN PRECIPITATION

When appreciable amounts of lipids, chlorophyll or unwanted polyphenols are suspected to be present in anthocyanin-containing extracts; these materials may be removed by washing with petroleum ether, ethyl ether, diethyl ether, or ethyl acetate (Jackman and Smith, 1996). Centrifugation of the mixture can be used to favor precipitation of the anthocyanins, which may be then recovered in a suitable solvent, usually acidified methanol or acidified water.

OPEN COLUMN CHROMATOGRAPHY

Different resins have been used to clean up or pre-fractionate anthocyanins prior to isolation or characterization, including ion exchange resins, polyamide powders, and gel materials. Chromatography on Dowex or Amberlite ion exchange

resins, as well as polyamide powders (e.g. polyvinyl pyrrolidone, PVP) have been used to isolate polar non-phenolic compounds from crude anthocyanin extracts. Column chromatography on Sephadex LH-20 can be used for fractionation of crude extracts and is also particularly useful for purification of individual anthocyanins (Strack and Wray, 1989).

C-18 Sep Pak cartridges are becoming popular because of their ease of use and high efficiency for fractionating anthocyanins. In an aqueous phase, anthocyanins are bound to the solid phase, while polar compounds such as acids and sugars can be washed away with acidified water. The use of ethyl acetate has been suggested by Oszmianski and Lee (1990) to wash out phenolics other than anthocyanins. Finally, a relatively pure anthocyanin extract can be removed from the column with slightly acidified methanol.

A key factor in the use of these purification techniques is the stability of anthocyanins to the conditions used as well as the ease of anthocyanin recovery from the column (Strack and Wray, 1989).

Separation of individual anthocyanins

Chromatographic separation of anthocyanins plays a major role in analyses of the patterns of phenolic compounds in crude extracts (Strack and Wray, 1989).

THIN LAYER AND PAPER CHROMATOGRAPHY

Paper chromatography (PC) and thin layer chromatography (TLC) have been used since the 1940's. Preparative PC, on Whatman #3 paper, analytical PC on Whatman #1 paper, or analytical TLC on microcrystalline cellulose, silica gel or

polyamide have been applied with a variety of solvents and the behavior of anthocyanins has been similar in all media. Two-dimensional TLC allowed for the separation of several compounds, and has been used to clarify the anthocyanin composition of different commodities (Francis, 1982). Some of the solvent systems frequently used include *n*-butanol-acetic acid-water (4:1:5, BAW), n-pentanol-acetic acid-water (2:1:1, PAW), acetic acid-concentrated HCl-water (25:3:72, AHW), 1% aqueous HCl (water-concentrated HCl, 97:3). In general, anthocyanins with more glycosidic substitutions will have lower Rf values in solvents like BAW and PAW, but higher Rf in AHW and 1% HCl, while the addition of acyl groups usually has the opposite effect (Strack and Wray, 1989).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC has proved to be fast and sensitive for the analyses of phenolic plant constituents, and is especially useful for the analysis of anthocyanins (Strack and Wray, 1989). The first application of HPLC to anthocyanin analyses was in 1975, by Manley and Shubiak, and has now become the method of choice for the separation of mixtures of anthocyanins and anthocyanidins (Strack and Wray, 1994). HPLC is now used for analytical, quantitative and preparative work of anthocyanins, offering improved resolution compared to chromatographic procedures previously employed. It also allows for simultaneous rapid monitoring of the eluting anthocyanins.

The most popular system is the use of a reversed phase column (C-18), on a silica base column. However, the use of C-18 on a polymer-based column has been reported as providing better resolution, especially for the separation of complex

anthocyanin mixture containing acylated pigments (Hale et al., 1986, Wrolstad et al., 1995). Polymer-based columns also show better stability at low pH operating conditions.

The overall polarity and stereochemistry of anthocyanins are the key factor for their separation. The order of elution will be dependent on the hydroxyl or methoxyl substitutions of the pyrylium ring, the number and nature of sugar substituents and the presence, number and nature of acylating groups. In general, more glycosylations or hydroxylations increase anthocyanin mobility, while *O*-methylation and presence of acylations increase the elution time. However, this is not a strict rule, since deviations of this behavior may be dependent on the nature and position of the substitutions (Strack and Wray, 1994; Wrolstad et al., 1995). For example, 3-rutinosides show longer retention times than the corresponding 3-glucosides because of the non-polarity imparted by the C6 methylation of rhamnose.

Typical mobile phases used are gradients of acetic, phosphoric or formic acid in water-methanol or water acetonitrile gradient solvents (Strack and Wray, 1989), that separate anthocyanins as their flavylium cations and can be easily detected by their absorbance at their visible λmax. Different detectors are available for monitoring eluting anthocyanins, including monochromatic or multiple wavelength spectrophotometers, photodiode array detector, and more recently, mass spectrometer. The photodiode array detector scans UV-Vis spectral data of the eluting sample every few fractions of a second and allows for spectroscopic interpretation of the data and detection of impurities coeluting with the compounds of interest (Andersen, 1985; Hong and Wrolstad, 1990; Strack and Wray, 1994).

Characterization and identification

HYDROLYSIS OF ANTHOCYANINS

Identification of the anthocyanins of interest usually requires the breakdown of the pigments into its components for structural elucidation, using acid, alkaline and/or hydrogen peroxide hydrolyses (Strack and Wray, 1989).

Hydrolysis of anthocyanins by boiling them with strong acid (2N HCl) causes release of sugars and with them any acylating group present; however, it does not affect the structure of the aglycon, which can be then separated by HPLC. There are 18 known aglycons in nature, but only 6 are widely distributed, as compared to hundreds of anthocyanins known. Acid hydrolysis prior to HPLC analyses simplifies the chromatogram and provides definite conclusions on the identity of the aglycons, facilitating interpretation of the data. Anthocyanidins are unstable and precautions must be taken to achieve satisfactory results (Hong and Wrolstad, 1986), avoiding oxygen and light exposure, and cooling down rapidly after hydrolysis. Anthocyanidin analyses should be performed preferably immediately following hydrolysis. Controlled acid hydrolysis has been found helpful for the rapid structural elucidation of an unknown anthocyanin. Samples are hydrolyzed for different time periods and the appearance and disappearance of intermediate glycosides formed from the original pigment is monitored, until finally the aglycon is left (Francis and Harborne, 1966). The pattern of intermediate glycosides will reflect number and site of attachments of the sugars: e.g. a 3-diglycoside will produce only one 3-glycoside as intermediate step,

while a 3,5-diglycoside will produce two different intermediates, the 3-glycoside and the 5-glycoside (Strack and Wray, 1989).

Saponification, or hydrolysis with a base (10% KOH for 8 min) will not affect glycosidic bonds; however, it will break down the ester linkage of acylating groups. This procedure can be used to determine with certainty which anthocyanins are acylated and to separate cinnamic or aliphatic acids from the anthocyanin for later separation and determination of their identity (Wrolstad et al., 1995).

Anthocyanin oxidation with hydrogen peroxide has been used to separate intact sugar substituents from anthocyanins. Hydrogen peroxide, under alkaline conditions, reacts with the anthocyanin breaking down the basic structure of the aglycon (C2 and C3 positions of the anthocyanin) liberating intact sugars. Anthocyanins are dissolved in methanol or water and treated with 30% H_2O_2, and allowed to stand until the pigment is bleached. The solution is then treated with NH_4OH, and finally evaporated to dryness (Strack and Wray, 1989). Intact sugars are liberated, which can later be analyzed after derivatization using gas chromatographic procedures.

SPECTRAL CHARACTERISTICS

Substantial information can be obtained from the spectral characteristics of anthocyanins. Two distinctive bands of absorption, one in the UV-region (260-280 nm) and another in the visible region (490-550 nm) are shown by all anthocyanins. The different aglycons have different visible λmax values, ranging from 520 nm for pelargonidin to 546 for delphinidin, and their monoglucosides exhibit their visible λmax at about 10-15 nm lower (Strack and Wray, 1989). The shape of the spectra may

give information regarding the number and position of glycosidic substitutions and number of cinnamic acid acylations. The ratio between the absorbance at 440 nm and the absorbance at the visible λmax is almost twice as much for anthocyanins with glycosidic substitutions in position 3 as compared to those with substitutions in positions 3 and 5 or position 5 only. The presence of glycosidic substitutions at other positions, e.g. 3,7-diglycosides, can be recognized because they exhibit a different spectral curve from those of anthocyanins with common substitution patterns. The presence of cinnamic acid acylation is revealed by the presence of a third absorption band in the 310-360 nm range. The ratio of absorbance at 310-360 nm to the absorbance at the visible λmax will give an estimation of the number of acylating groups (Harborne, 1967; Hong and Wrolstad, 1990). The solvent media used for spectral determination will affect the position of the absorption bands and therefore must be taken into consideration when comparing data available.

The availability of HPLC systems coupled to a photodiode array detector allows for the on-line spectral characterization of anthocyanins.

MASS SPECTROSCOPY

The use of mass spectroscopic analyses for characterization of anthocyanins has been increasing over the last decade, with most of those reports using HPLC coupled to a MS detector or isolating individual pigments prior to the mass spectroscopic analysis (Baublis et al., 1994; Bakker et al., 1997; Saito, et al., 1996; Shi et al., 1993). Accurate molecular weight determination is very important for structure elucidation of complex anthocyanins since small components with little UV-Vis

absorption that show weak or no NMR signals may be overlooked if the molecular weight is not known (Goto, 1987). The ionization technique more often reported has been fast atom bombardment (FAB), although the use of electrospray (ES) or ion spray (IS) MS has also been reported.

The introduction of fast atom bombardment mass spectroscopy has had an enormous influence on anthocyanin structural studies (Strack and Wray, 1989). FAB MS provides the molecular ion as well as various fragmentation ions that give direct information on the structure of the molecule. Only a small amount of sample is required (1-2 μL may be enough). After solvent evaporation, a liquid matrix is introduced (glycerol, thioglycerol or 3-nitrobenzyl alcohol). A neutral-atom beam of xenon is then used to bombard the droplet surface from which material is sputtered. The mass/charge ratio of the intact molecular ion is obtained, and the fragmentation of the molecule allows the side chains to be determined although their position is ambiguous (Strack and Wray, 1989 and 1994).

Electrospray ionization mass spectrometry (ES-MS) has emerged as a powerful technique for the characterization of biomolecules, and is the most versatile ionization technique in existence today (Covey, 1995, Snyder, 1995). This soft ionization technique can produce intact ions from large and complex species in solution, even from thermally labile, non-volatile, polar compounds (Black and Fox, 1995; Hutton and Major, 1995; Fenn et al., 1989). A chromatogram with only the base peak for every mass spectrum provides a more readily interpretable data because of fewer interference peaks (Sagesser and Deinzer, 1996).

Tandem mass spectroscopy (MS-MS) allows for the formation of low-energy collisionally induced dissociation fragments (Hutton and Major, 1995). The first region of the mass analyzer is used as a separation device before inducing fragmentation with a suitable gas (usually argon) in a collision cell followed by analysis of the fragment ion in the second (McLafferty and Turecek, 1993; Jennings, 1996; Lawson, et al., 1996). This process provides structural information on the components of a mixture, which leads to the unambiguous identification of fragmentation pathways (Lawson, et al., 1996). Tandem MS has been used in combination with FAB and ES-MS ionization techniques.

The coupling of reversed phase liquid chromatography to MS allows the molecules to be characterized by retention time, UV-visible response and mass spectral information for the individual components and fragments (Hutton and Major, 1995) and is becoming a technique gaining in popularity over recent years. The development of electrospray ionization as a sensitive technique, as well as a specific and versatile detector for liquid sample introduction has contributed enormously to the establishment of liquid chromatography LC-MS as an analytical technique for mixture analysis (Gaskell, 1996).

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

NMR spectroscopy is the most powerful method for structural elucidation in solution, and advances in NMR techniques have made a significant impact on anthocyanin studies (Strack and Wray, 1989 and 1994). Complete structural characterization of anthocyanins is possible with 1- and 2-dimensional NMR

techniques. However, relatively large quantities of purified material are required for resolution of proton signals associated with sugars and positions C6 and C8 of the flavylium nucleus (Jackman and Smith, 1996).

It was in the late 70's that the first successful ^{1}H-NMR was recorded on natural anthocyanins by Goto et al. (1978), which made it possible to determine the complete structure and stereochemistry of complex anthocyanins (Goto, 1987). ^{13}C-NMR has also been reported. 1-D and 2-D NMR information allow for determination of the *trans* configuration of cinnamic acid acylation, the ß-glucopyranoside conformation of the sugars present, and the exact position of attachment of the acylating group to the sugar moiety (Goto, 1987).

The nature of the aglycon, together with the number of glycosylations and acylating groups present in the molecule can normally be assessed from 1-D ^{1}H-NMR. When the 1-D spectrum is too complex, it is useful to do a 2-D *J*-resolved experiment, to simplify the spectra and clarify the magnitude of the coupling constants. Coupling constants gives information regarding *trans* vs. *cis* configurations and equatorial or axial protonations in the glycosidic moiety, as well as information regarding the configuration of the anomeric carbon in the sugars. Two-dimensional shift correlation and total correlation analyses (2D NMR COSY and TOCSY) assist in assignment of all individual proton signals from the individual sugars moieties. Acylation causes a low field shift of 0.5-1.0 ppm for the geminal proton relative to the non-acylated pigment (Strack and Wray, 1989).

HMQC and HMBC experiments enable us to trace connectivities between ^{1}H and ^{13}C atoms through indirect detection of the low natural abundance nuclei ^{13}C, via

^{1}H nuclei (Agrawal, 1992). The HMQC spectra provides correlation between directly bonded ^{1}H and their corresponding ^{13}C. HMBC is a long-range heteronuclear chemical shift correlation technique, and provides intra-residue multiple bond correlation; this information is valuable for confirming ^{13}C and/or ^{1}H assignments. At the same time, it also provides inter-residue multiple bond correlation between the anomeric carbon and the aglycon proton and thus serves to identify the inter-glycosidic linkages (Agrawal, 1992).

Two dimensional NOESY experiments gives valuable information in mapping specific through-space internuclear distances (Keepers and James, 1984) which could be sufficient to determine the molecular three dimensional structure. Cross-peaks are observed in NOESY spectra between proton pairs that are close in space, typically less than 5 Å, close enough to allow through-space interactions (Agrawal, 1992), and the greater the signal, the closer together those hydrogens are in space (Kemp, 1991). It has been proposed (Goto, 1987; Figueiredo et al., 1996a and 1996b) that stacking between the aromatic nuclei of the anthocyanin and the planar ring of the aromatic acid would be through the formation of π-π hydrophobic interactions.

COLOR MEASUREMENTS AS A MEANS FOR QUALITY ASSESMENT OF ANTHOCYANIN EXTRACTS

The evaluation of an extract as a potential food colorant involves two general approaches: overall appearance and analysis of pigment content. For the analysis of pigment content, different approaches have been presented. However, the main reason to use a food colorant is to improve visual color and this is measured by the use of a tristimulus colorimeter or a spectrophotometer (Francis, 1989).

Numerous methods have been devised in the past to enable numeric quantitation of color. In 1905 Munsell devised a method for expressing colors using a great number of color chips classified according to their hue, lightness and saturation. Other methods were developed by the Commission Internationale de l'Eclairage (CIE), that devised the Yxy color space in 1931. This method gave quantitative measurement of color but did not easily translate into visual color characteristics. Later a new system was devised, the CIELab, based on the tristimulus values XYZ defined by CIE but corrected so that numerical changes would better correlate with visual changes.

The CIELab established a system of numerical coordinates that was able to locate individual colors in a 'color solid' based on uniform visual spacing (Voss, 1992). The 'color solid' is a three-dimensional geometric representation of colors using three coordinates (Fig, 2.2): lightness (L*), redness or greenness (a*) and yellowness or blueness (b*). The color coordinates are directly obtained with a tristimulus colorimeter by reflection or transmittance. With a spectrophotometer, tristimulus data can be obtained from mathematical transformation of reflection or transmission curves in the visible range from 380 to 710 nm.

The a* and b* coordinates can be readily transformed into hue angle (arctan b*/a*) and chroma $(a^{*2} + b^{*2})^{1/2}$. The central axis of the solid of color (a* and b* = 0) is an achromatic scale, with white at the top (L* = 100) and black at the bottom (L* = 0), and shades of gray in between. The segments of the color solid represent the hues. The hue angle is an indicator of the color itself, and values of 0, 45, 90, 180 and 270° corresponds to red-purple, orange, yellow, bluish-green and blue colors, respectively.

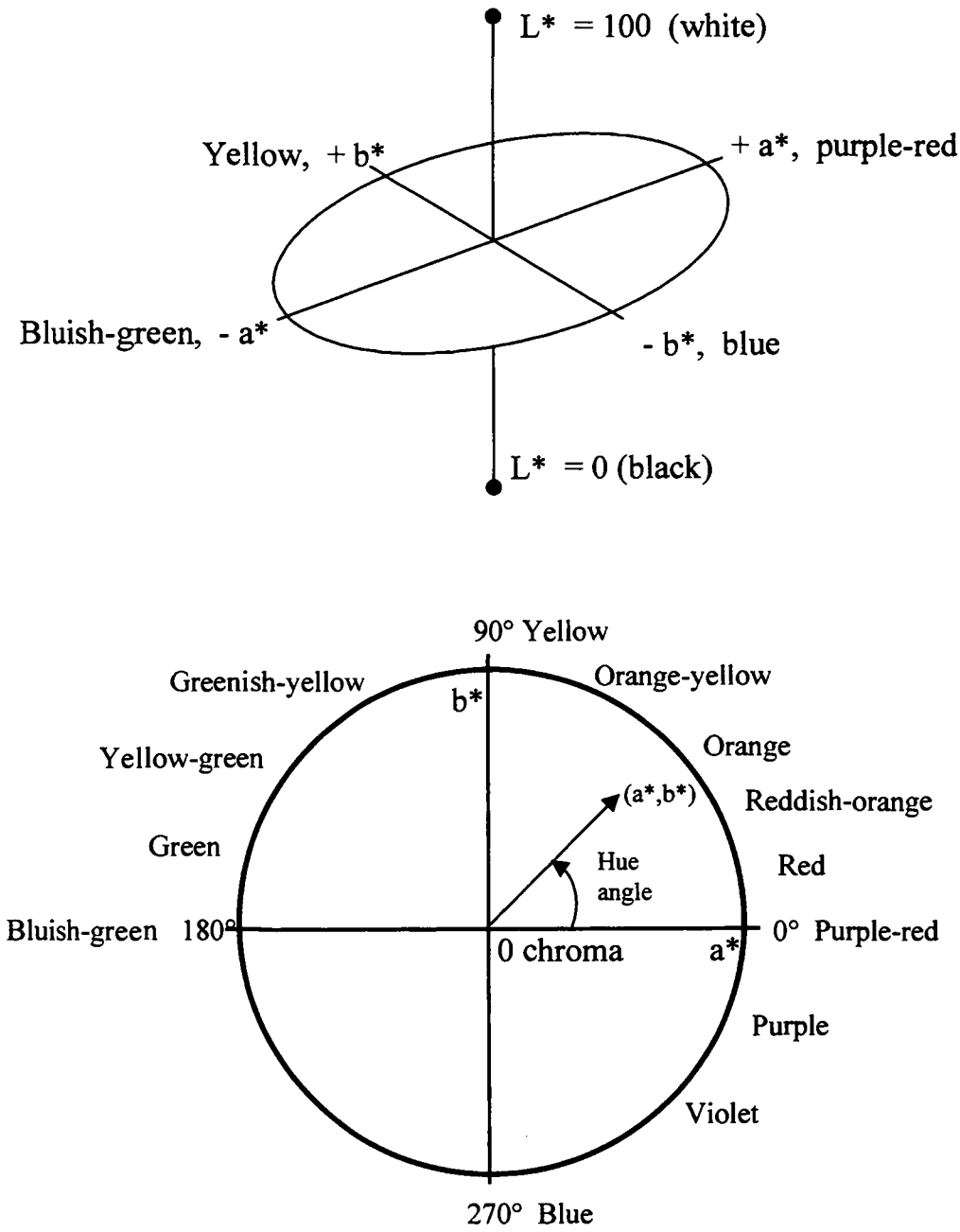

Figure 2.2. CIEL*a*b* three-dimensional representation of color.

Chroma is a measure of the saturation or intensity of the color. Near the central axis (chroma = 0), the colors are grayish; near the outer surface of the color solid, they are saturated or pure (Francis, 1989; McGuire, 1992; Minolta, 1993). The color solid is not symmetrical, with high-chroma yellows representing relatively light colors and conversely the high-chroma reds and purples are relatively dark colors (Voss, 1992).

The use of absorption data at one or two wavelengths has been used as a measure of tinctorial power of color extracts. The measurement is simple and most laboratories are equipped with spectrophotometers. Dilutions of the solutions should be avoided since they may not follow Lambert and Beer's law due to copigmentation effects (Francis, 1989), and it is preferably to use smaller path cells. The use of CIELab tristimulus data, however, is likely to be more accurate since it measures color based on absorption over the whole visible range as opposed to one or two wavelengths.

SUMMARY

Characterization of anthocyanins and anthocyanin extracts involves the quantitative and qualitative analysis of the pigments. Different approaches have been discussed, and a combination of techniques is usually the best approach. The objective of the analysis will determine the methodologies to choose. Routine analyses will mainly involve spectrophotometric measurements for quantification of pigments. HPLC can be used as a fast and simple procedure for monitoring qualitative composition. Characterization of new anthocyanins will require the use of a combination of several procedures including MS and NMR. The advances on

analytical techniques over the last years allow for a simpler, faster and more accurate structure and conformation determination than ever before.

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CHAPTER 3

CHARACTERIZATION OF RED RADISH ANTHOCYANINS

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ABSTRACT

Red radish (*Raphanus sativus* L.) anthocyanins were extracted from liquid nitrogen powdered epidermal tissue using acetone, partitioned with chloroform and isolated by using C-18 resin. Four major pigments were identified by electrospray mass spectroscopy, HPLC, and spectral analyses as pelargonidin-3-sophoroside-5-glucoside derivatives. The two major pigments were acylated with malonic acid and either ferulic or *p*-coumaric acid. The two other pigments were acylated with only ferulic or *p*-coumaric acid. Acylation of the pigments increased pigment resistance to acid hydrolysis.

INTRODUCTION

Interest in anthocyanins has increased because of their potential use as natural colorants and possible beneficial health effects (Francis, 1982; Mazza and Miniati, 1993). Anthocyanins have been consumed for many years without apparent adverse effects, have bright attractive colors, and are water soluble, facilitating their incorporation into aqueous food systems (Markakis, 1982). These qualities make them attractive alternatives to synthetic dyes. Anthocyanin-containing products, however, are susceptible to color deterioration during processing and storage, resulting in combined anthocyanin degradation and brown pigment formation (Abers and Wrolstad, 1979; Wrolstad et al., 1970; Markakis, 1982; Skrede et al., 1992). The chemical instability of anthocyanin extracts, coupled with high cost and low tinctorial strength, has limited commercial applications of anthocyanin pigments (Parkinson and

Brown, 1981). However, there is a continuing effort to produce anthocyanin-based colorants which have improved stability. Acylated anthocyanin pigments show greater stability during processing and storage (Bassa and Francis, 1987; Hong and Wrolstad, 1990; Murai and Wilkins, 1990; Rommel et al., 1992; Shi et al., 1992). These acylated pigments also respond differently to pH change than do non-acylated ones (Price and Wrolstad, 1995). The presence of acylating groups is believed to protect the oxonium ion from hydration, thereby preventing formation of the hemiketal (pseudobase) or chalcone forms (Brouillard, 1981; Francis, 1989).

The pigments of red radish (*Raphanus sativus* L.) have been identified by previous workers (Ishikura and Hayashi, 1962 and 1963; Harborne, 1963; Fuleki, 1969), the major ones being pelargonidin-3-sophoroside-5-glucoside (raphanusin) with *p*-coumaric, ferulic and caffeic acid esterified to the sugar substituents. Pelargonidin (Pg) has an orange-red hue, and acylation would be expected to shift the hue to a longer wavelength (red hue) and impart improved stability.

In a previous study (Giusti, 1995), we confirmed the presence of raphanusin as the basic structure in radish pigments and found 2 of them acylated with p-coumaric acid, while two others acylated with ferulic acids. The objectives of this study were to further identify the anthocyanin pigments in red radish epidermal tissue to determine the differences between those pigments, apparently having the same chemical structure but different retention times. Also, we compared the stability of radish anthocyanins to acid hydrolysis with other pg-based anthocyanins.

MATERIALS AND METHODS

Plant material

Red radishes (*Raphanus sativus* L.) cultivar Fuego, grown by SIRI Produce Inc. (Oregon City, OR.), were obtained from Cub Foods (Corvallis, OR). The leaves were cut, the roots were washed with cold water to eliminate extraneous matter and refrigerated at 1°C. Radishes were peeled manually and the epidermal tissue was frozen under liquid nitrogen and stored at –23°C.

Standards

Caffeic, ferulic, *p*-coumaric and malonic acids were obtained from Sigma Chemical Co. (St. Louis, MO). Pelargonidin-3-glucoside (Pg-3-glu) and pelargonidin-3-sophoroside (Pg-3-soph) were extracted from strawberry juice concentrate and from Nasturtium flowers, respectively.

Pigment extraction

The extraction was done as described by Wrolstad et al. (1990). Frozen red radish epidermal tissue was liquid nitrogen powdered using a stainless steel Waring blendor. Powdered samples were blended with ca. 2 L of acetone per kg skin and filtered on a Buchner funnel. The filter cake residue was re-extracted with aqueous acetone (30:70 v/v) until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with chloroform (1:2 acetone:chloroform v/v) and stored overnight at 1°C. The aqueous portion (top portion) was collected and placed on a

Büchi rotovapor at 40°C (for 5 to 10 min) until all residual acetone was evaporated. The aqueous extract was made up to a known volume with distilled water.

Anthocyanin purification

The aqueous extract was passed through a C-18 mini-column (high load C-18 tube), 20 mL capacity and 5 g sorbent weight (Alltech Assoc., Inc., IL), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the mini-column; sugars, acids and other water soluble compounds were eluted with 2 volumes of 0.01% aqueous HCl and anthocyanins were subsequently recovered with methanol containing 0.01% HCl (v/v). The methanolic extract was concentrated using a Büchi rotovapor at 35°C and pigments were dissolved in deionized water containing 0.01% HCl.

Alkaline and acid hydrolysis of anthocyanins

Purified pigment was hydrolyzed (saponified) with 10% aqueous KOH at room temperature in the dark, as described by Hong and Wrolstad (1990). Saponified pigment was then hydrolyzed for 45 min at 100°C, and the hydrolysate purified using a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), as previously described. Sugars and acids obtained from the saponification procedure (not retained by the C-18 Sep-Pak) were also collected.

Stability of Pg-derivatives to partial acid hydrolysis

The stability to partial acid hydrolysis of different Pg-derivatives was evaluated. Pigments used were Pg-3-glu (from strawberry juice), Pg-3-soph (from

Nasturtium flowers), Pg-3-soph-5-glu (from saponified radish anthocyanins) and Pg-3-soph-5-glu acylated with malonic and ferulic or *p*-coumaric acids (from radish). Pelargonidin-derivatives were isolated by manual collection from a preparatory HPLC column (System IV, described later). Pigment (c.a. 0.5 mg) was hydrolyzed with 10 mL of 2N HCl for different times (ranging from 5 to 70 min). All other steps and parameters of acid hydrolysis were as previously described. The partially hydrolyzed pigments were analyzed by HPLC, and the monomeric anthocyanin content was calculated as described by Giusti (1995).

High performance liquid chromatography (HPLC)

Apparatus: A Perkin-Elmer Series 400 liquid chromatograph, equipped with a Hewlett-Packard 1040A photodiode array detector and a Hewlett-Packard 9000 computer system, was used. Simultaneous detection was at 280, 320 and 520 nm. The spectra (detection wavelengths from 250 to 600 nm) were recorded for all peaks.

Columns and Mobile Phase. System I: Polymer Labs PLRP-S column (5 micron) 250 x 4.6 mm id (Polymer Labs, Amherst, MA), fitted with a Polymer Labs, 1.5 cm x 4.6 mm id guard column. Solvent A: 100% HPLC grade acetonitrile; B: 4% phosphoric acid (aqueous). **System II**: Supelcosil LC-18 column (5 micron), 250 x 5 mm id (Supelco, Inc., PN), fitted with a ODS-10, 4 cm x 4.6 mm id, Micro-Guard column (Bio-Rad Laboratories). Solvent A: 100% HPLC grade acetonitrile, B: 1% phosphoric acid, 10% acetic acid, 5% acetonitrile and water. Flow rate: 1 mL/min. **System III**: Coupled Spherisorb ODS-2 and Spherisorb ODS columns (5 micron), 250 x 4.6 mm id (Alltech Associates, Inc., IL) . Solvent: phosphate buffer pH 2.4 (27.2 g

KH_2PO_4 made up to 1 L with deionized distilled water, pH adjusted with concentrated phosphoric acid). Flow rate 0.7 mL/min. Injection volume (Systems I, II and III): 50 µL. **System IV**: Supelcosil PLC-18 column (18 micron), 250 x 21.2 mm id (Supelco, Inc., PN), fitted with a ODS-10, 4 cm x 4.6 mm id, Micro-Guard column (Bio-Rad Laboratories). Solvents: same as System II. Flow rate: 10 mL/min. Injection volume: 2 mL. Solvents and samples were filtered through a 0.45 µm Millipore filter type HA (Millipore Corp., Bedford, MA). The spectra (from 250 to 600 nm) were recorded.

Anthocyanin and anthocyanidin analysis conditions

Radish anthocyanins were separated by using System I. The program followed a linear gradient from 15 to 20% A in 40 min. Saponified anthocyanins, anthocyanidins, and partially hydrolyzed pigments were separated with System II. The program used was a linear gradient from 0 to 30% A in 30 min for saponified anthocyanins and anthocyanidins; for partially hydrolyzed Pg-derivatives, a gradient from 0 to 12% A in 13 min and to 20% A in 15 min was used. Simultaneous detection was done at 280, 320 and 520 nm.

Isolation of purified Pg-derivatives

Pg-derivatives were isolated using System IV. The conditions used varied for the different pigments. **Pg-3-glu** was isolated by using a linear gradient from 0 to 15% A in 20 min. **Pg-3-soph** was isolated by using a linear gradient from 0 to 7% A in 5 min, and isocratic conditions at 7% A for 7 min. **Pg-3-soph-5-glu** was isolated by using a linear gradient from 0 to 8% A in 15 min. **Acylated Pg-3-soph-5-glu** was

isolated by using a linear gradient from 4 to 10% A in 5 min and from 10 to 12% A in 15 min.

HPLC conditions for phenolic acid analysis

Phenolic acids, obtained from radish anthocyanin saponification, were separated using the 2 systems described. With System I, the program used was a linear gradient from 10 to 15% A in 15 min, a linear gradient from 15 to 25% A in 15 min and isocratic at 25% A for 5 min. System II was run isocratic at 5% A, with simultaneous detection at 280 and 320 nm. The identity of the acyl groups was confirmed by the 2 HPLC systems. Retention times and spectra were compared to pure standards.

HPLC conditions for organic acid analysis

Organic acids, obtained from the saponification of radish pigments, were separated using System III with the program running under isocratic conditions. Elution of peaks was monitored with simultaneous detection at 214 and 224 nm. Spectra and retention times of the acids from saponified radish were compared to pure standards.

Mass spectroscopy (MS) of radish anthocyanins

Low-resolution MS was done using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ Mass Spectrometer, equipped with an Ion Spray source (ISV=4700, orifice voltage of 80) and loop injection. Partially purified radish anthocyanin extract was injected directly into the system.

RESULTS AND DISCUSSION

HPLC separation of radish anthocyanins

Anthocyanins from radish were separated by HPLC (Fig. 3.1). Two anthocyanins (peaks 5 and 6) represented ca. 70% of the total area, and two others (peaks 3 and 4) ca. 20%. Four other minor peaks were also detected (Peaks 1, 2, 7 and 8), each accounting for 1 to 3% of the total area. The spectra of all peaks showed a maximum absorbance in the 310 nm region, corresponding to the presence of acylating groups. The ratios of A_{max} acyl/A_{max} visible found for radish anthocyanins were 62.5, 58.3, 58.1, and 52.4% for peaks 3, 4, 5 and 6, respectively. These ratios suggest that all major anthocyanins found in radish epidermal tissue (peaks 3, 4, 5 and 6) were acylated in a 1/1 molar ratio of the cinnamic acid to the anthocyanin.

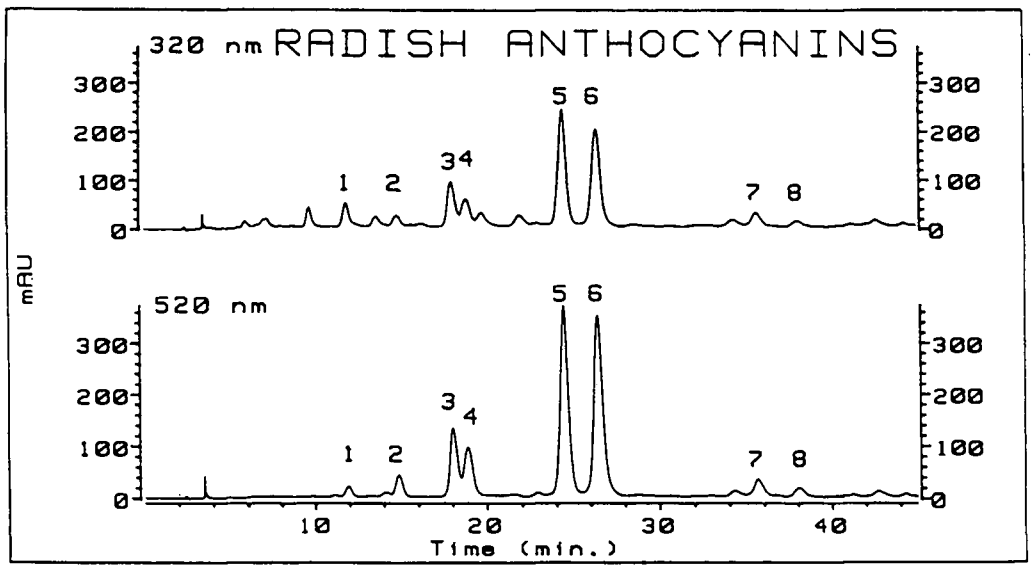

Figure 3.1. HPLC separation of red radish anthocyanins. Polymer Labs PLRP-S, 250 x 4.6 mm i.d. column. Solvent A: 100% acetonitrile, B: 4% phosphoric acid. Linear gradient from 15 to 20% A in 40 min. Flow rate: 1 mL/min. Injection volume: 50 µL.

Radish anthocyanins saponification and acid hydrolysis

Saponification of radish anthocyanins revealed the presence of two major acylating groups accounting for 49 and 41% of the total area at 320 nm detection. Comparison of retention times and spectra with pure standards permitted the identification of acylating acids as *p*-coumaric and ferulic acids. Saponification of radish anthocyanins produced only one peak detected at 520 nm. The retention time of the saponified anthocyanin was shorter than that of Pg-3-glu from strawberry, suggesting the presence of additional glycosidic substitutions. These results were in agreement with the findings of Ishikura and Hayashi (1962), who determined that the pigment in red radish epidermal tissue was a mixture of different acylated anthocyanins with a common basic component, Pg-3-soph-5-glu, raphanusin.

Acid hydrolysis of the saponified radish anthocyanins resulted in only one anthocyanidin. The retention time and spectrum of the hydrolyzed pigment matched that of pelargonidin from strawberry, confirming previous identification of radish anthocyanins as Pg derivatives (Ishikura and Hayashi, 1962; Harborne, 1963; Fuleki, 1969, Giusti, 1995).

Mass spectroscopy

Results from MS (Fig. 3.2) showed molecular weights of 902 and 932, corresponding to Pg-3-soph-5-glu acylated with *p*-coumaric and ferulic acids, respectively, in confirmation of previous identifications of radish anthocyanins by Ishikura and Hayashi (1962), Harborne (1963), Fuleki (1969) and Giusti (1995). However, two molecules were found with molecular weights 86 units higher than each

of the others, revealing the presence of another acylating molecule attached to the anthocyanins. The molecular weight, and the fact that it was not detected by phenolic acid analysis, suggested the presence of an organic acid.

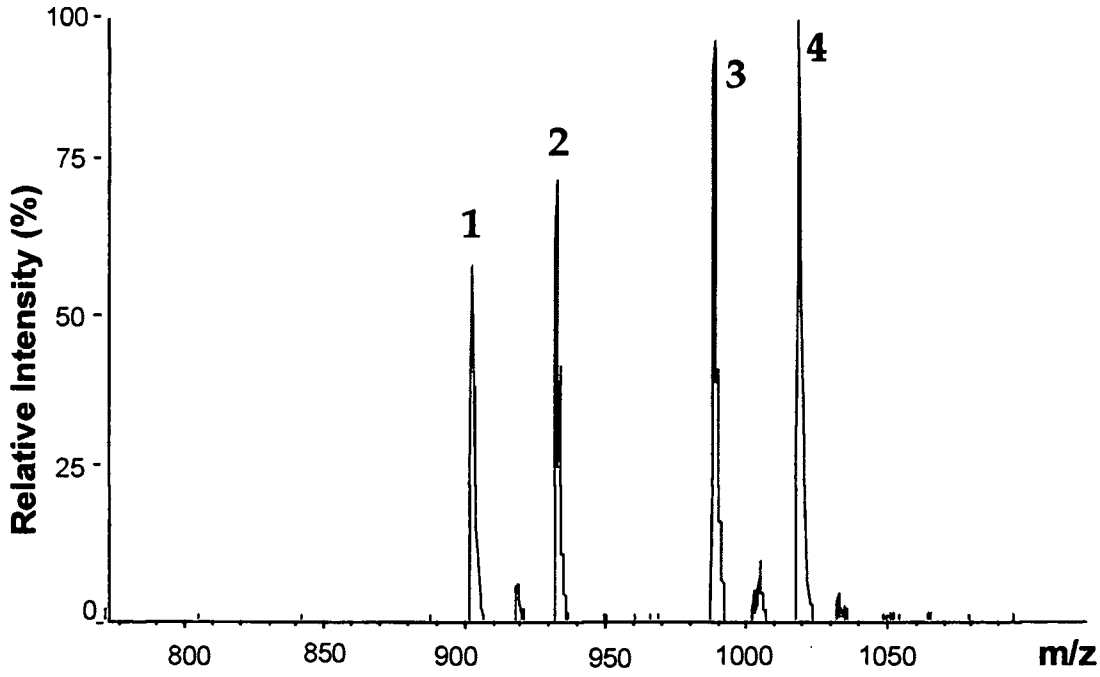

Figure 3.2. MS of radish anthocyanins. The molecular weights obtained correspond to Pg-soph-5-glu acylated with *p*-coumaric acid (903.6), ferulic acid (933.6), *p*-coumaric and malonic acids (989.6) and ferulic and malonic acids (1019.6).

Organic acids analysis

The HPLC analysis of organic acids from saponified radish anthocyanins revealed the presence of malonic acid. This acid has been reported as a frequently found acylating group in anthocyanins (Macheix, et al., 1990); however, its presence has not been reported in radish. According to Teusch and Formann (1987), acylation with dicarboxylic aliphatic acids such as malic, succinic and malonic acids, has often

been overlooked in earlier studies due to their hydrolysis under typical extraction procedures with methanolic HCl.

Peak identification

Combined information from HPLC data of radish anthocyanins, the saponified pigments and acid-hydrolyzed pigments showed that all the anthocyanins present were Pg-3-soph-5-glu with one or more acylating groups. The two major pigments were identified as Pg-3-soph-5-glu, acylated with malonic acid and, one with *p*-coumaric acid and the other with ferulic acid in a 1/1 molar ratio. Since the retention time of *p*-coumaric acid is shorter than that of ferulic acid under these HPLC conditions, we hypothesized that peak 5 corresponded to Pg-3-soph-5-glu acylated with *p*-coumaric and malonic acids, while peak 6 corresponded to Pg-3-soph-5-glu acylated with ferulic and malonic acids. Peaks 3 and 4 (Figure 3.1) showed the same basic composition, but with no malonic acid attached to the molecule (Pg-3-soph-5-glu acylated with *p*-coumaric and ferulic acid, respectively).

Stability of Pg-derivatives to partial acid hydrolysis

The percent purity obtained for the Pg-derivatives (as percent peak area by HPLC) were 94.1, 100, 99.2, and 90.8 % for Pg-3-glu, Pg-3-soph, Pg-3-soph-5-glu, and acylated Pg-3-soph-5-glu (mixture of Pg-3-soph-5-glu acylated with malonic acid and with either ferulic or *p*-coumaric acids), respectively. The contaminant present in Pg-3-glu corresponded to Pg-glu acylated with organic acid, and in the acylated Pg-3-glu-5-glu, the impurity was the same basic anthocyanin structure without malonic acid.

Pg-3-glu and Pg-3-soph showed similar stability to acid hydrolysis. Nearly all of these pigments (86 and 93%, respectively) were hydrolyzed after 30 min. Diglycosides are usually more stable than corresponding monoglycosides (Mazza and Miniati, 1993). However, diglycosides hydrate more rapidly and to a larger extent than the corresponding monoglycoside (Dangles et al., 1993). We found that Pg-3-glu hydrolysis resulted in the formation of only the aglycon (Pg) while Pg-3-soph hydrolysis resulted in formation of both, Pg-3-glu and Pg. However, this intermediate step did not seem to increase pigment resistance to acid hydrolysis. After 40 min, 97% of both pigments had been destroyed.

Acid hydrolysis of Pg-3-soph-5-glu resulted in the formation of 5 additional pigments (Fig. 3.3): Pg, Pg-5-glu, Pg-3-glu, Pg-3-soph, and Pg-3-glu-5-glu.

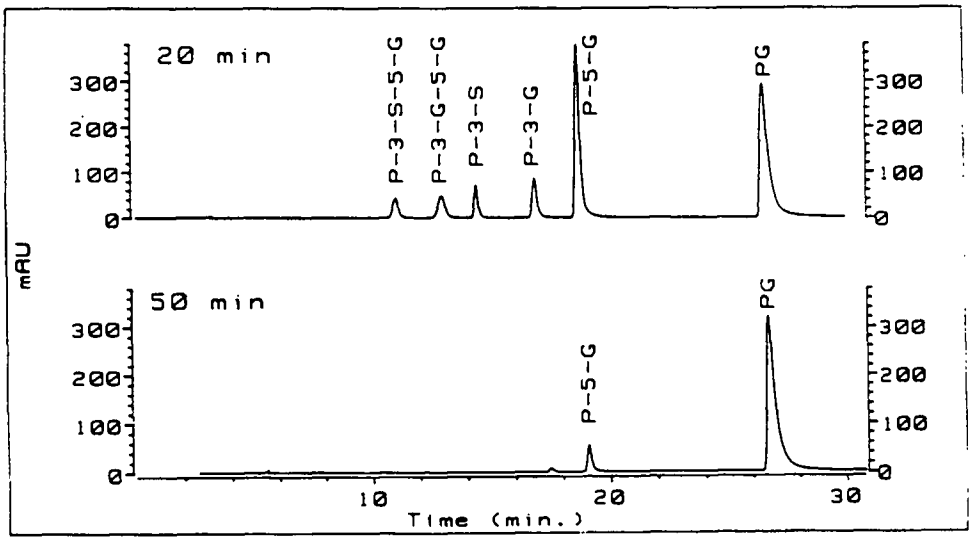

Figure 3.3. HPLC separation (detection at 520 nm) of partially hydrolyzed Pg-3-soph-5-glu, after 20 and 50 min of acid hydrolysis. Code: P-: Pelargonidin, G: glucose, S:sophorose, PG: Pelargonidin (aglycon). Supelcosil LC-18, 250 x 5 mm i.d. column. Solvent A: 100% acetonitrile; B: 1% phosphoric acid, 10% acetic acid, 5% acetonitrile, and water, run isocratically at 5% A. Flow rate: 1 mL/min. Injection volume: 50 μL.

The identity of the different Pg-derivatives formed was determined from retention times of purified Pg-3-glu, Pg-3-soph, and from their spectra. Our results confirmed those reported by Harborne (1967), that the ratio of absorbance at 440 nm to the absorbance at visible maximum wavelength of 3-glucosides was about twice as much as for the 3,5-diglucosides (Fig. 3.4). Our results also showed that this ratio was the same for 5-glucosides as for 3,5-di or 3,5-triglucosides. Similarly, the ratio was the same for 3-mono and 3-diglucosides. The shoulder for the aglycon was intermediate in height compared to the two previously described. Pg-5-glu showed a tendency to accumulate with time while all others seemed to degrade at a similar rate (Fig. 3.3). Pg-5-glu is not reported to occur in nature, and no information regarding its stability as compared to sugar substitution in other positions was found. Our finding suggested that substitution in position 5 may contribute to improved pigment stability. In the case of Pg-3-soph-5-glu, 50 min destroyed most of the pigment (91%), with Pg-5-glu the only remaining anthocyanin (9%).

The chromatogram from partial hydrolysis of the acylated anthocyanin was very complex (Fig. 3.5). Hydrolysis for 10 min was sufficient to hydrolyze 60% of the malonic acid, resulting in formation of other acylated Pg-derivatives, the major ones being Pg-3-soph-5-glu acylated with *p*-coumaric or ferulic acids. In addition, different non acylated Pg-derivatives were formed. Acylated radish anthocyanins showed higher stability to acid hydrolysis than non-acylated forms. After 30 min 47% of the pigment was in the aglycon form and after 70 min, most of the pigment (87%) was present as

Pg. Prolonged hydrolysis of acylated Pg-3-soph-5-glu seemed to promote polymerization, as shown by the presence of broad, late-eluting peaks (Fig. 3.5).

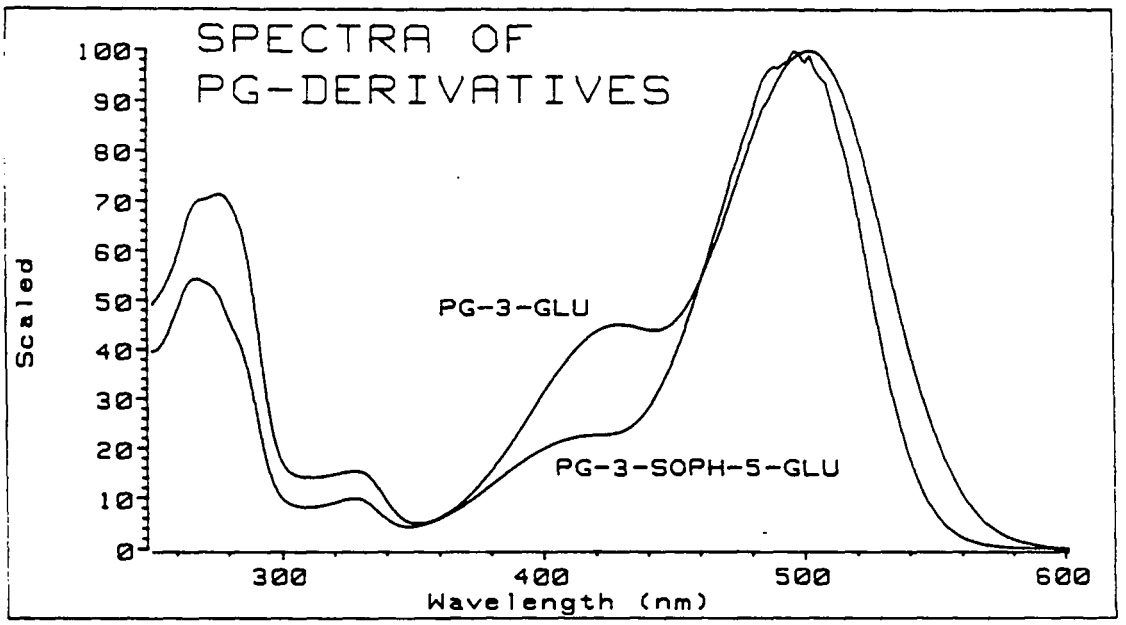

Figure 3.4. Comparative spectra of saponified radish anthocyanin and pelargonidin glucoside (from strawberry), same HPLC conditions as for Figure 3.3.

The monomeric anthocyanin content was measured in all hydrolyzed samples at the following time intervals: after 5, 10, 20, 30 and 40 min of hydrolysis for Pg-3-glu; after 10, 20, 30, 40, 50, and 60 min for Pg-3-soph, Pg-3-soph-5-glu and acylated Pg-3-soph-5-glu, and an additional hydrolysis time, 70 min, for acylated Pg-3-soph-5-glu (Fig. 3.6). A limitation of the methodology was that the formed anthocyanidin contributed substantially to the absorbance at 520 nm when measurements were made soon after hydrolysis. The aglycons are very reactive, and after 24 hr, the solutions showed very low monomeric anthocyanin content, probably because of reaction of

anthocyanidins with anthocyanins. Consequently, all monomeric anthocyanin measurements were taken 6 hr after hydrolysis. From monomeric anthocyanin measurements, only acylation appeared to improve the stability of Pg-derivatives to acid hydrolysis (Fig. 3.6).

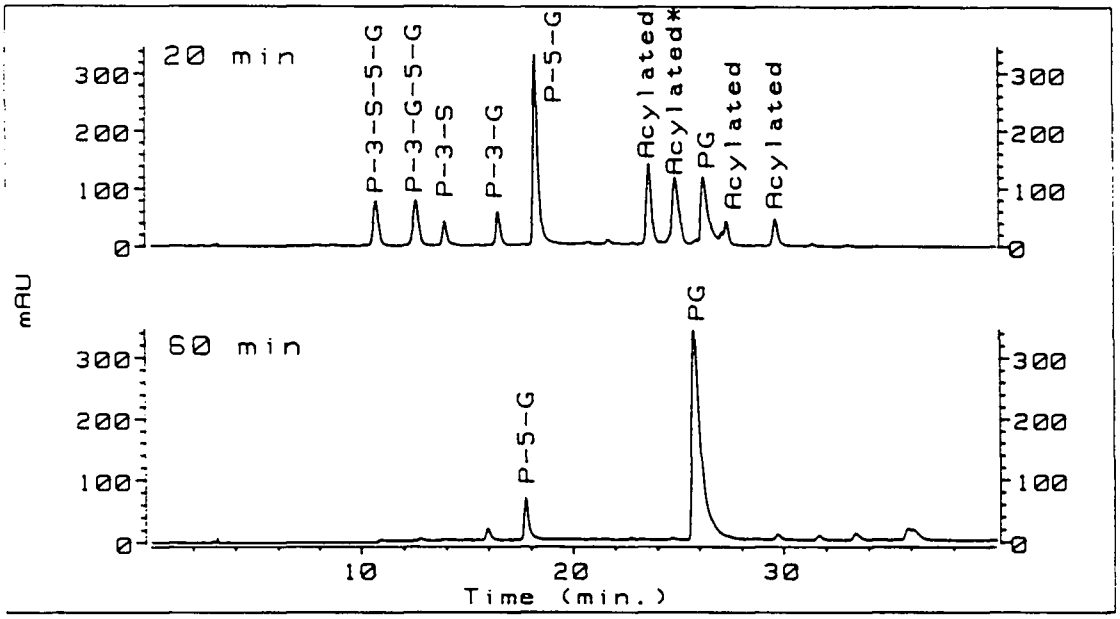

Figure 3.5. HPLC separation (detection at 520 nm) of partially hydrolyzed Acylated Pg-3-soph-5-glu, after 20 and 60 min of acid hydrolysis. P-: Pelargonidin, G: glucose, S:sophorose, PG: Pelargonidin aglycon; Acylated: Pg-3-soph-5-glu, acylated with either ferulic or *p*-coumaric acids; Acylated*: same, plus malonic acid. Same HPLC conditions as for Figure 3.3.

Some observations were made regarding the visual appearance of these pigments in pH 1.0 buffer. Purified Pg-3-glu and Pg-3-soph showed an attractive orange-red color while Pg-3-soph-5-glu showed a pale yellow-orange color. The wavelength of maximum absorbance in the buffer media was the same for all non acylated Pg-derivatives (between 496-498 nm). The main spectral difference between

Pg-3-soph-5-glu and the other two pigments, was in the ratio of absorbance at 440 to the absorbance at 500 nm (0.23 for Pg-triglucoside and 0.5 for the Pg-mono and diglucosides). No differences were found among them in other regions of the spectra (Fig. 3.4). The greater absorbance in the region of 440 nm has an influence on the color characteristics of the pigment. In addition, the presence of acylating groups caused a shift of ca. 10 nm in the wavelength of maximum absorbance and a more intense red hue.

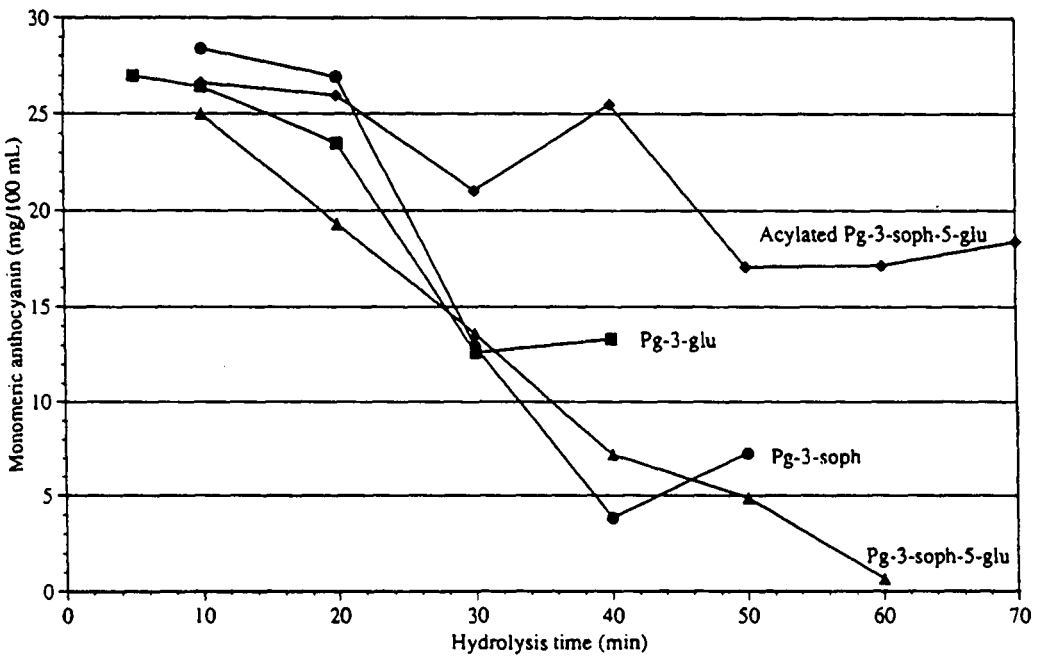

Figure 3.6. Changes in monomeric anthocyanin content of Pg-derivatives after different times of acid hydrolysis.

CONCLUSIONS

HPLC, MS and UV-visible spectral analyses of radish anthocyanins confirmed previous identifications of the pigments as Pg-3-soph-5-glu acylated with *p*-coumaric and/or ferulic acids. Malonic acid was an acyl substituent of the two major anthocyanins; this has not been previously reported for radish. Eight anthocyanins were separated, four of them representing ca. 90% of the total area. They were identified as Pg-3-soph-5-glu acylated with *p*-coumaric or ferulic acid and malonic acid, and as Pg-3-soph-5-glu acylated with *p*-coumaric and ferulic acids.

The study of the stability of different Pg-pigments to acid hydrolysis by HPLC and spectral measurements showed that the presence of acylating groups greatly increased the resistance of these Pg-derivatives to acid hydrolysis, the cinnamic acids being more resistant than malonic acid. The sugar substituent at the C-5 position was also resistant to hydrolysis. The attractive red hue of radish anthocyanins and their stability to heat and acid hydrolysis suggest that radishes may be a desirable colorant source for various food applications.

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CHAPTER 4

ELUCIDATION OF THE STRUCTURE AND CONFORMATION OF RED RADISH (*Raphanus sativus*) ANTHOCYANINS, USING ONE AND TWO DIMENSIONAL NUCLEAR MAGNETIC RESONANCE TECHNIQUES.

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ABSTRACT

Different 1- and 2-dimensional NMR techniques were used to elucidate the structural conformation of purified anthocyanins obtained from red radish (*Raphanus sativus*). Two novel diacylated anthocyanins, pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucopyranoside)]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) and pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-feruloyl-β-D-glucopyranoside)]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) were characterized. Two other mono-acylated anthocyanins were determined to be pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucopyranoside)]-5-*O*-(β-D-glucopyranoside) and pelargonidin 3-*O*-[2-*O* -(β-D-glucopyranosyl)-(6-*O*-*trans*-feruloyl-β-D-glucopyranoside)]-5-*O*-(β-D-glucopyranoside). Three dimensional conformation of the molecule was investigated using NOESY techniques, which showed proximity between hydrogens from the cinnamic acid acylating group and the C-4 of the pelargonidin, revealing folding of the molecule.

Key words: Anthocyanins, red radish (*Raphanus sativus* L.), 1D and 2D NMR, pelargonidin derivatives.

INTRODUCTION

Interest in anthocyanins has increased in recent years because of their potential use as natural alternatives to artificial colorants. New subfamilies of anthocyanins have emerged over the last decades including the zwitterionic and the polyacylated

anthocyanins (Dangles et al., 1993). The zwitterionic anthocyanins are characterized by the presence of the positively charged aglycon and the negative charge from the presence of an aliphatic dicarboxylic acid as acylating group. At the same time, many polyacylated anthocyanins have been found over the last years, most of them having 2 or more cinnamic acids as acylating groups and also exhibiting increased stability (Rommel et al., 1992; Shi et al., 1992; Baublis et al., 1994; Baublis and Berber-Jiménez, 1995; Giusti and Wrolstad, 1996a, b). In addition, anthocyanins with both types of acyl groups: aliphatic and cinnamic acids, have been found.

Radish anthocyanin pigments were first characterized in the 60's by different researchers (Ishikura and Hayashi, 1962 and 1965; Harborne 1963; Fuleki, 1969), who determined the presence of pelargonidin-3-sophoroside-5-glucoside (Pg-3-soph-5-glu) derivatives, with cinnamic acids attached to the glycosidic moieties. Giusti and Wrolstad (1996a) confirmed the presence of this basic structure in red radishes and found the presence of malonic acid as an additional acylating group. Those findings would place the main anthocyanins present in radishes into both categories mentioned, first with a zwitterionic characteristic given by the charges from the aglycon and the dicarboxylic acid acylation (malonic acid), second with more than one acylating group, malonic acid and a cinnamic acid group, ferulic or *p*-coumaric acids. Giusti and Wrolstad (1996b) also investigated the potential of red radish anthocyanin extract as a natural colorant for maraschino cherries and determined that radish extract could provide color characteristics very close to those of FD&C Red No. 40 and keep good color and pigment stability for at least 6 mo at room temperature. The high stability was attributed to the presence of the acylating groups.

There is a wide variety of chemical structures that can be encountered among anthocyanins: different glycosylating patterns, different acylating groups, many different hydroxyl groups available for esterification of the acylating groups and finally presence of cinnamic acids in different stereoisomeric forms (Dangles, et al., 1993). In the case of radish anthocyanins, the components of the molecule have been determined but no information was available regarding the position of the acylating groups, the type of cinnamic acid stereoisomer present, or the self protecting effect of potential interactions of intramolecular copigmentation. The objectives of this study were to determine the positions of the acylating groups, the three dimensional configuration of radish anthocyanins, and the intramolecular copigmentation of the acylated pigments by 1- and 2-dimensional nuclear magnetic resonance (NMR) techniques. These NMR techniques were also used to confirm previous identification of the diglucoside as a sophorose unit, to determine the anomeric configuration of the sugars, the types of chemical bonds and the stereoisomeric conformation of the acylating cinnamic acids.

MATERIALS AND METHODS

Pigment extraction

Red radishes cultivar Fuego were grown at the Oregon State University Lewis-Brown Horticultural Farm, Corvallis, Oregon. Pigment extraction was done following the procedure described by Giusti and Wrolstad (1996b). Radishes were manually peeled and the frozen epidermal tissue was liquid nitrogen powdered by using a stainless steel Waring Blendor. Powdered samples were blended with 1 volume of

acetone and filtered on a Buchner funnel using Whatman #1 paper. The filter cake residue was re-extracted with aqueous acetone (30:70 v/v) until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with 2 volumes of chloroform (1:2 acetone: chloroform v/v) and stored overnight at 1°C. The aqueous portion (top layer) was collected and placed on a Büchi rotovapor at 40°C until all residual acetone was evaporated (5 to 10 min). The aqueous extract was made up to a known volume with distilled water.

Anthocyanin purification

The aqueous extract was passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the mini-column; sugars, acids and other water soluble compounds were removed with 2 volumes of 0.01% aqueous HCl and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotovapor at 35°C and pigments were dissolved in distilled deionized water containing 0.01% HCl.

Pigment isolation

Semi-purified pigments were isolated using semi-preparative HPLC. Individual anthocyanins were collected and further purified by passing them through a C-18 Sep-Pak cartridge. Pigments were recovered from the cartridge with 90% methanol and 10% acidified methanol (0.01% HCl methanol). The methanol was evaporated in a Büchi rotovapor at 35°C and pure methanol added and evaporated again to favor the

removal of HCl or water remaining in the sample. This procedure was repeated twice, and a third time using d-methanol (CD_3OD). The flask containing dried pure anthocyanins was cooled in a desiccator, and the weight recorded. Pure samples were dissolved in d-methanol containing 10% d-TFA (CF_3COOD).

Alkaline hydrolysis of anthocyanins

Approximately 30 mg of purified pigment was hydrolyzed (saponified) in a screw-cap test tube with 10 mL of 10% aqueous KOH for 8 min at room temperature in the dark, as described by Hong and Wrolstad (1990). The solution was neutralized by using 2N HCl, and the hydrolysate was purified by using semi-preparative HPLC and C-18 Sep-Pak cartridge, as previously described.

High performance liquid chromatograph (HPLC)

Apparatus : A semi-preparative Dynamax Rainin Model SD-300 High Performance Liquid Chromatograph was used, equipped with a Hewlett-Packard 1040A photodiode array detector and a Gateway 2000 P5-90 computer with a Hewlett-Packard HPLC2D ChemStation software. A 1 mL injection loop was used.

Columns and mobile phase: A Microsorb™ C-18 reverse phase column (5μ), 250 x 21.4 mm i.d. fitted with a 50 x 21.4 mm i.d. guard module (both from Rainin Instrument Co., Inc., Emeryville, CA) was used. The solvents used were A: 100% HPLC grade acetonitrile and B: 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v:v:v) in water. Flow rate: 20 mL/min. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA).

HPLC conditions for anthocyanin separation and isolation

Radish anthocyanins and saponified radish anthocyanins were separated using isocratic conditions at 10% A. The identity of anthocyanins was verified by collecting data at 280, 310, and 520 nm and collecting peak spectra (from 260 to 600 nm) of all peaks at 520 nm. A total of 15, 25, 50, 60, and 55 mg of Pigments 1 through 5, respectively, were collected.

Electrospray mass spectroscopy

Low-resolution MS was done by using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ Mass Spectrometer, equipped with an Ion Spray source (ISV=4700, orifice voltage of 80) and loop injection. Purified anthocyanins were injected directly into the system.

Nuclear magnetic resonance (NMR)

Apparatus: 1-dimensional (1D) and 2-dimensional (2D) NMR spectra were collected on a Bruker 600 operating system at 14.1T with an inverse probe.

Conditions for analyses: Samples analyzed were dissolved in d-MeOH (CD_3OD) containing 10% d-TFA (CF_3COOD) and all analyses were carried out at 25°C.

Hydrogen NMR: 1-D ^{1}H-NMR was recorded by using a spectral width of 9 ppm, with center at 5 ppm. Two-dimensional correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) were collected using the average of 32 transients for each of the 256 increments in t1, with 4096 complex points in t2. A spectral width of 10 ppm with center at 5 ppm were used. Spin coupling information was obtained

by using homonuclear J-resolved spectroscopy, with a spectral width of 10 ppm and center at 5 ppm.

Carbon NMR: The proton detected ^{13}C heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra were obtained by using spectral widths of 9 ppm with center at 5 ppm in the proton dimension and 200 ppm with the center at 100 ppm in the carbon dimensions. A total of 32 transients were averaged for each of the 512 increments in t1, with 2048 complex points in t2. The (1/2J) delays in the pulse sequence were optimized for proton-carbon coupling constants of approximately 160 Hz.

Nuclear Overhauser effect spectroscopy (NOESY) spectra were collected by using the average of 64 transients for each of the 256 increments in t1, with 4096 complex points in t2. The spectral width used was 10 ppm with the center at 5 ppm.

RESULTS AND DISCUSSION

HPLC analysis and mass spectroscopy of radish anthocyanins

All acylated and saponified radish anthocyanins were isolated by using semi-preparative HPLC, and the purity of the isolated pigments ranged from 92 to 99%, as indicated by their percent area at 280 nm. Previous chemical characterization of radish anthocyanins (Giusti and Wrolstad, 1996a) revealed the presence of 4 major anthocyanins, all sharing a basic structure: pelargonidin aglycon with a diglucoside on position 3, identified as sophorose based on reported identification of the pigments (Ishikura and Hayashi, 1963; Fuleki, 1969; Harborne 1963), and a glucose unit on position 5 of the anthocyanidin structure. Two of those pigments (ca. 20% of total

pigment content) were mono-acylated with *p*-coumaric (pigment 2) or ferulic (pigment 3) acids, and 2 others (ca. 70% of total pigment content) were di-acylated anthocyanins, containing the same cinnamic acid acylations plus malonic acid (pigments 4 and 5, respectively). The remaining anthocyanin content (ca. 10%) was composed of 4 other minor peaks with the same basic structure but with either no acylation, mono-acylation with caffeic acid, or di-acylation with cinnamic acids.

Electrospray mass spectroscopy confirmed previously reported m/z of the molecular ions as 903, 933, 989, and 1019 for pigments 2, 3, 4, and 5 respectively (Giusti and Wrolstad, 1996a). Saponification of the pigments yielded only one pigment (pigment 1), pelargonidin tri-glucoside, with a m/z ratio of 721.

Purified non-acylated raphanusin did not bind to the C-18 resin as easily and tightly as other anthocyanins did, due to its increased polarity and high hydrophilicity caused by the presence of three sugar substituents. This increased hydrophilicity also accounted for a low affinity and very short retention time on the C-18 HPLC column. Several semi-preparative HPLC runs were needed to collect amounts large enough to perform the NMR analyses on the non-acylated pigment. Even then, the concentration was relatively low and only a limited number of NMR analyses were performed on this sample, and no carbon data were obtained for it.

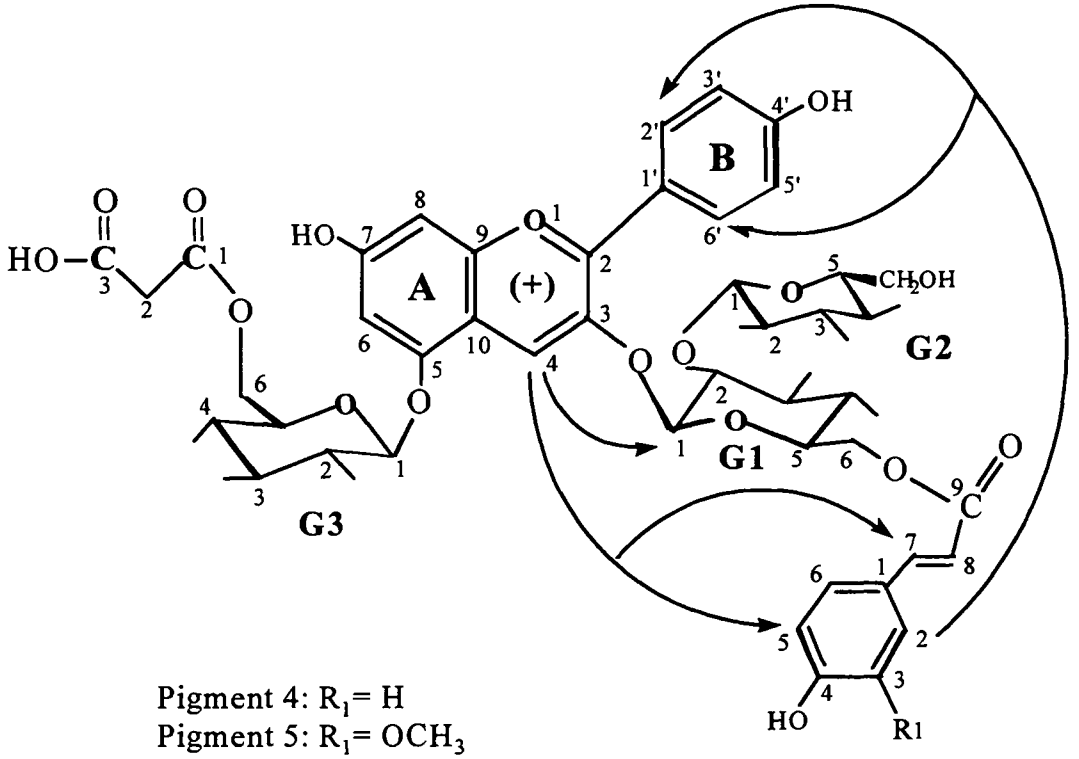
NMR of saponified radish anthocyanins

Saponification of radish anthocyanin yielded only one pigment (pigment 1) previously identified (Giusti and Wrolstad, 1996a) by HPLC and electrospray mass spectroscopy as pelargonidin-3-sophoroside-5-glucoside. The chemical shifts (δ)

obtained from the ^{1}H-NMR analysis of saponified radish anthocyanins (Table 4.1) confirmed the identity of the pigment.

Hydrogens from the aglycon gave chemical shifts that ranged from 7.1 to 9.19 ppm, in agreement with the aromaticity of the A and B rings (Fig. 4.1) of the aglycon molecule and with previously reported data for similar compounds (Hosokawa et al., 1995; Fossen et al., 1996).

Figure 4.1. Chemical structure of pelargonidin derivatives encountered in red radish. Arrows indicate hydrogens close in space.



Pigment 4: R_1= H
Pigment 5: R_1= OCH_3

Table 4.1. 1H-NMR Spectra of Radish Anthocyanins (in 10% d-TFA d-MEOH). Chemical shifts (ppm). 1 = pg-3-soph-5-glu, 2 = pg-3-soph-5-glu acylated with *p*-coumaric acid, 3 = pg-3-soph-5-glu acylated with ferulic acid, 4 = pg-3-soph-5-glu acylated with *p*-coumaric acid and malonic acid, 5 = pg-3-soph-5-glu acylated with ferulic and malonic acids.

	Pigment			
	1	2	4	5
Aglycon				
C4	9.19	9.03	8.97	8.98
C6	7.15	6.97	6.98	6.96
C8	7.15	7.02	6.97	6.94
C2'	8.66	8.60	8.58	8.57
C3'	7.10	7.10	7.10	7.08
C5'	7.10	7.09	7.10	7.08
C6'	8.66	8.61	8.58	8.57
Sophorose glycoside (G1)				
C1	5.49	5.61	5.61	5.61
C2	4.12	4.12	4.13	4.13
C3	3.81	3.84	3.86	3.88
C4	3.63	3.61	3.63	3.64
C5	3.50	3.86	3.96	3.97
C6	3.78	4.46	4.45	4.38
	3.95	4.53	4.54	4.59
Sophorose glycosyl (G2)				
C1	4.85	4.79	4.81	4.82
C2	3.21	3.25	3.27	3.28
C3	3.30	3.33	3.36	3.35
C4	3.26	3.27	3.26	3.28
C5	3.00	3.08	3.05	3.07
C6	3.50	3.49	3.52	3.52
	3.60	3.60	3.62	3.60
Glucoside (G3)				
C1	5.20	5.19	5.22	5.20
C2	3.73	3.76	3.80	3.78
C3	3.60	3.60	3.60	3.57
C4	3.48	3.49	3.48	3.42
C5	3.69	3.61	3.82	3.80
C6	3.77	3.77	4.28	4.21
	3.96	3.99	4.56	4.54
Acyl group				
C2		7.27	7.19	6.85
C3		6.78	6.72	---
C5		6.78	6.72	6.72
C6		7.27	7.19	6.82
C7		7.35	7.35	7.34
C8		6.16	6.18	6.18
Malonyl				
C2			3.45	3.49

Three signals corresponding to the chemical shifts of acetal groups formed by the anomeric carbons from sugar residues were detected and assigned to the different sugars present in the molecule using previously reported data for similar compounds (Terahara et al., 1996; Hosokawa et al., 1995) as reference. The chemical shift of 5.49 ppm was assigned to the proton at the anomeric carbon of the glycoside residue from sophorose (G1, Fig. 4.1), 4.85 ppm assigned to glycosyl residue from sophorose (G2), and 5.20 ppm assigned to the anomeric proton of glucoside residue (G3). Important information can be obtained from the H-H coupling constants of neighboring hydrogens. Neighboring diaxial hydrogens can be clearly identified by large coupling constants (7-9 Hz), which contrast with smaller coupling constants (2-4 Hz) of protons in di-equatorial or axial-equatorial configurations (Agrawal, 1992; Breitmaier, 1993). This difference allows for elucidation of the configuration of sugars. Clear duplets were observed for the anomeric protons of the saponified radish anthocyanin, and the J-couplings for these protons were determined from the ^{1}H-NMR spectra. The large coupling constants obtained for hydrogens at the anomeric carbon of the sugar residues (7.1, 7.77 and 7.76 Hz for G1, G2 and G3, respectively) showed that the hydrogens at the anomeric carbon and at C2 of the glucose units were all in axial configuration, revealing that all glucose units were in the C1-β-D-glucopyranoside conformation.

The vast majority of proton resonance from non-anomeric sugar methine and methylene groups appear in a small spectral width of 3.0-4.2 ppm, with a subsequent overlap problem (Agrawal, 1992). Significant overlap makes it difficult to locate the resonance of individual nuclei and to assign these resonances to a specific monosaccharide residue. Two-dimensional shift correlation and total correlation

analyses (2D NMR COSY and TOCSY) assisted in assigning all individual chemical shifts of the hydrogens from the different sugars (Table 4.1), using as a reference the already assigned resonances of the anomeric carbons and correlating spins in a step-wise manner around the spin system of the ring. The higher chemical shift obtained for the proton at position 2 in G1 (4.12 ppm as compared to 3.21 and 3.73 ppm for H-2 in G2 and G3, respectively) showed the presence of a substitution on that position, in agreement with the bond type 1-2 of sophorose.

NMR of acylated radish anthocyanins

The NMR spectra obtained for all radish anthocyanins had similar chemical shifts for the aglycon (pelargonidin) as well as for most protons from the sugar substitutions (Table 4.1). Differences were found in the shifts of protons at position 6 of G1 (on pigments 2, 4 and 5) and G3 (on pigments 4 and 5), higher than those obtained for H-6 of the sugars in the saponified pigment. This suggested that the acylating groups were attached to the hydroxyl groups of these positions.

Additional signals were detected in all acylated pigments analyzed in the region between 6.1 and 7.4 ppm, corresponding to protons from the cinnamic acid acylating groups. Another additional signal detected on the ^{1}H-NMR spectra of pigments 4 and 5 in the 3.4 ppm region, was identified as coming from the proton at position 2 of the malonic acid residue. This signal was not evident at first due to its proximity to signals from protons located on the sugar residues.

Two-dimensional HMQC and HMBC spectra were collected (Table 4.2) for pigments 2, 4 & 5 to confirm the position of the ester linkage. HMQC and HMBC

Table 4.2. ^{1}H and ^{13}C NMR spectra of pelargonidin-3-sophoroside-5-glucoside acylated with malonic and p-coumaric (Pigment 4) or ferulic (Pigment 5) acids.

	Pigment 4					Pigment 5				
	H-NMR		(J, Hz)	HMQC	HMBC	1H-NMR		(J, Hz)	HMQC	HMBC
Aglycon										
C2					164					164.2
C3					144.5					144.5
C4	8.97	d	(2.4)	134.7		8.98	d	(3.0)	134.8	
C5					156.5					155.8
C6	6.98	d	(1.56)	105.0		6.96	br s		104.6	
C7					168					169.0
C8	6.97	d	(1.56)	96.3		6.94	br s		96.5	
C9					156.5					155.8
C10					112.5					112.3
C1'					119.5					119.7
C2'	8.58	d	(9.05)	135.5		8.57	d	(8.9)	135.5	
C3'	7.10	d	(9.04)	116.9		7.08	d	(8.89)	117.4	
C4'					166.5					166.7
C5'	7.10	d	(9.04)	116.9		7.08	d	(8.89)	117.4	
C6'	8.58	d	(9.05)	135.5		8.57	d	(8.9)	135.5	
Sophorose glycoside (G1)										
C1	5.61	d	(7.14)	100.5		5.61	d	(6.98)	110.5	
C2	4.13	dd	(7, 8.5)	80.8		4.13	dd	(6.3, 9.3)	80.5	
C3	3.86	dd	(8.25, 9.25)	76.8		3.88	dd	(7.2, 9.0)	76.6	
C4	3.63	dd	(8.5, 10)	70.8		3.64	dd	(8.4, 10.2)	70.5	
C5	3.96	m		74.5		3.97	m		74.2	
C6	4.45	dd	(7.65,	63.5		4.38	dd	(7.5, 11.7)	63.0	
	4.54	br d	(11.85)			4.59	dd	(2.7, 11.7)		
Sophorose glycosyl (G2)										
C1	4.81	d	(7.77)	103.6		4.82	d	(7.61)	103.0	
C2	3.27	dd	(7.5, 9.5)	74.7		3.28	dd	(8.1, 9.9)	74.8	
C3	3.36	dd	(8.75, 9.75)	76.8		3.35	dd	(7.5, 10.5)	76.5	
C4	3.26	dd	(8.5, 10)	70.7		3.28	dd	(8.1, 9.9)	70.3	
C5	3.05	m		76.9		3.07	dd	(6.0, 9.6)	77.0	
C6	3.52	br s		61.6		3.52	dd	(6.3, 12.3)	61.0	
	3.62	dd	(8.5, 10)			3.60	dd	(8.4, 10.2)		
Glucose (G3)										
C1	5.22	d	(7.76)	101.5		5.20	d	(7.69)	101.5	
C2	3.80	dd	(7.75, 9.75)	74.0		3.78	dd	(7.5, 9.3)	73.8	
C3	3.60	dd	(8.5, 9.5)	76.5		3.57	dd	(8.7, 9.9)	76.6	
C4	3.48	dd	(8.5, 10)	70.0		3.42	dd	(7.5, 10.5)	69.9	
C5	3.82	m		74.7		3.80	dd	(6.6, 9.6)	74.5	
C6	4.28	dd	(6.28,	64.2		4.20	dd	(6.6, 11.4)	64.1	
	4.56	br d	(11.85)			4.54	dd	(2.7, 12.3)		
Cinnamic acid										
C1					126					126.5
C2	7.19	d	(8.53)	130.5		6.85	br s		111.0	
C3	6.72	d	(8.55)	115.5						148.0
C4					160.5					149.7
C5	6.72	d	(8.55)	115.5		6.72	d	(8.16)	115.5	
C6	7.19	d	(8.53)	130.5		6.82	d	(9.65)	123.0	
C7	7.35	d	(15.87)	145.8		7.34	d	(15.84)	147.0	
C8	6.18	d	(15.89)	113.5		6.18	d	(15.85)	114.0	
C9					168					168.0
Malonyl										
C1					167					167.5
C2	3.45	s		40.5		3.49	s		40.5	
C3					167					168.0

experiments enable us to trace connectivities between ^{1}H and ^{13}C atoms through indirect detection of the low natural abundance nuclei ^{13}C, via ^{1}H nuclei (Agrawal, 1992). The HMQC spectra provide correlation between directly bonded ^{1}H and their corresponding ^{13}C. The ^{13}C spectra for these pigments were obtained by using this procedure and the results are presented in Table 4.2. HMBC is a long-range heteronuclear chemical shift correlation technique, and provides intra-residue multiple bond correlation; this information is valuable for confirming ^{13}C and/or ^{1}H assignments. At the same time, it also provides inter-residue multiple bond correlation between the anomeric carbon and the aglycon proton and thus serves to identify the inter-glycosidic linkages (Agrawal, 1992). HMQC techniques were useful for confirmation of the signal at 3.4 ppm as coming from malonic acid, since this ^{1}H chemical shift was the only one that correlated with ^{13}C signals of 40.5 ppm, typical for such structure and in contrast with the all other ^{13}C chemical shifts of these molecules, higher than 60 ppm.

Figure 4.2 shows the HMBC plot for Pigment 4. The HMBC spectra of pigments 2 and 4 showed correlation between the H-6 of G1 (δ 4.45 and 4.54 ppm) and a carbonyl carbon from the *p*-coumaric acid (δ 167.8 ppm), confirming that the *p*-coumaric acid was esterified to sugar G1 through the hydroxyl group at C-6. The HMBC spectra of Pigment 4 (Fig. 4.2) also showed correlation between H-6 of G3 (δ 4.28 and 4.56 ppm) and the carbon from the carbonyl group of malonic acid (δ of 167.1 ppm). These results showed that *p*-coumaric acid was attached to the position 6 of G1 through an ester bond, while malonic acid was attached through an ester bond to position 6 of G3. Similar results were obtained for Pigment 5, where the positions of

the ester linkage between the ferulic acid and sugars was determined to be at C-6 of G1; and malonic acid was attached to C-6 of G3. The position of attachment of the acyl groups were confirmed by the higher chemical shifts obtained for H-6 of G1 and G3 and by the correlation found between these hydrogens and the carboxylic groups of the adjacent acylating acids.

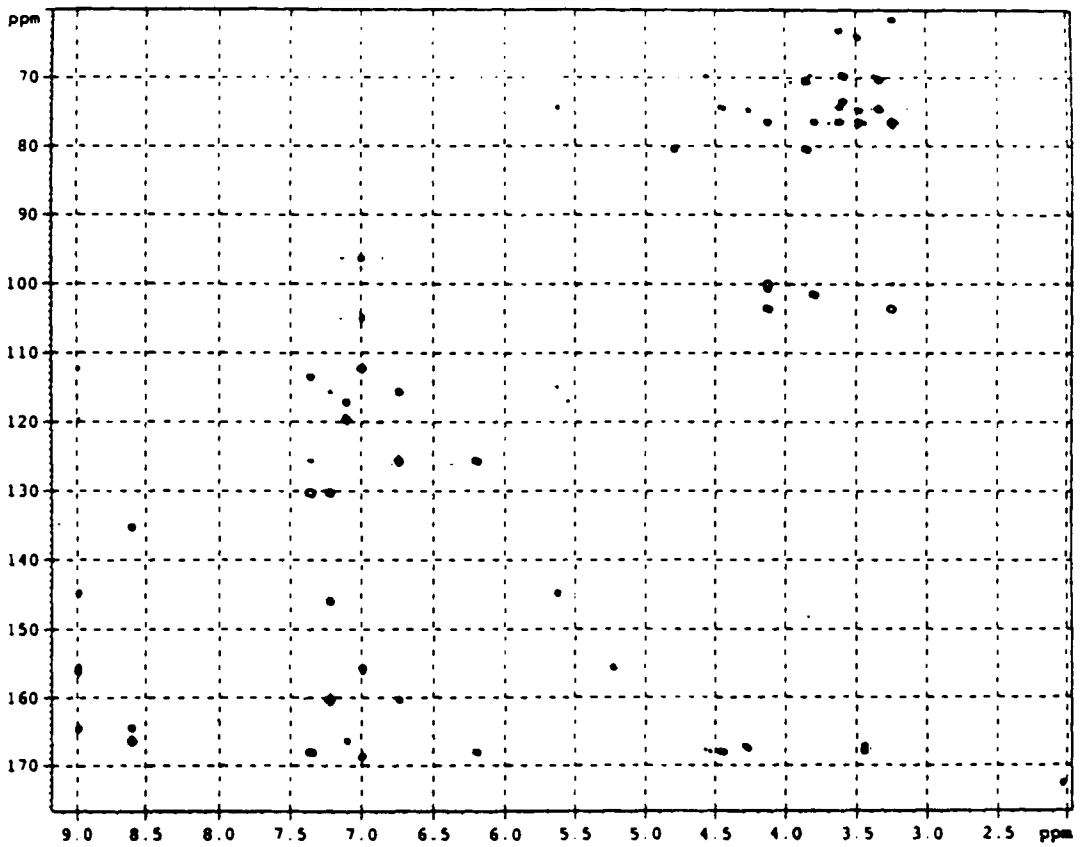


Figure 4.2. Heteronuclear multiple bond correlation (HMBC) of pigment 4.

The chemical shift and large J-coupling (15.89 Hz) of H-7 and H-8 (Table 4.2) for *p*-coumaric acid (in pigment 2 & 4) and ferulic acids (pigment 5) showed that both cinnamic acids were in the *trans* configuration. Thus, Pigment 2 was identified as pelargonidin-3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucopyranoside)]-5-*O*-(β-D-glucopyranoside); Pigment 4 was pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-*p*-coumaroyl β-D-glucopyranoside)]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside), and Pigment 5 was pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-(6-*O*-*trans*-feruloyl-β-D-glucopyranoside)]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside), where the latter two are novel anthocyanins.

Pigment 3 was not analyzed by NMR. However, using the information obtained for pigments 2, 4 and 5, combined with the fact that the degradation of pigments 4 and 5 results in the formation of pigments 2 and 3, respectively with release of malonic acid (Giusti and Wrolstad, 1996b), suggest the chemical structure of Pigment 3 as pelargonidin 3-O-[2-O-(β-D-glucopyranosyl)-(6-O-trans-feruloyl-β-D-glucopyranoside)]-5-O-(β-D-glucopyranoside).

Intramolecular copigmentation of acylated radish anthocyanins

Two-dimensional NOESY experiments provide valuable information in mapping specific through-space internuclear distances (Keepers and James, 1984) which could be sufficient to determine the molecular three dimensional structure. Crosspeaks are observed in NOESY spectra between proton pairs that are close in space, typically less than 5 Å, close enough to allow though-space interactions (Agrawal, 1992), and the greater the signal, the closer together those hydrogens are in

space (Kemp, 1991). NOESY experiments performed on anthocyanin pigments 4 and 5 showed high correlation between H-4 of the pelargonidin moiety and the hydrogens in positions H-3 & H-5 of *p*-coumaric acid, as well as a small correlation with H-8. These findings demonstrated that the molecule was folded in space. It has been proposed (Goto, 1987; Figueiredo et al., 1996a and 1996b) that the stacking between the aromatic nuclei of the anthocyanin and the planar ring of the aromatic acid would be through the formation of π-π hydrophobic interactions.

Folding of malonic acid over the pyrylium ring of the anthocyanin could not be determined due to the weak signal obtained for hydrogens from malonic as well as the interference of the signals of hydrogens from the sugar moieties, very close to those of malonic acid. However, the zwitterionic character of pigments 4 and 5 would make likely an ionic attraction between the positively charged portion of the oxonium form, and the negatively charged free carboxylic group from malonic acid. The positions of attachment of the acylating groups to the sugars, at position C6 in both cases, would allow for free rotation and the subsequent folding of the acyl groups over the pyrylium ring in a sandwich type conformation, where cinnamic and malonic acids would be on top and bottom of the pelargonidin portion of the molecule.

Several studies (Brouillard, 1981; Goto, 1987; Goto and Kondo, 1991; Figueiredo et al., 1996a and 1996b) have suggested that the intramolecular copigmentation or interaction within anthocyanins may play an important role in the increased stability of acylated anthocyanins. The flexible saccharide chains can act as linkers allowing the folding of the acyl aromatic rings over the planar pyrylium ring preventing the addition of nucleophiles, especially water, to the C2 and C4 positions of

the anthocyanin, protecting the chromophore against hydration and diminishing the formation of the colorless pseudobase (Figueiredo et al., 1996a). Copigmentation has also been reported to have an impact on the anthocyanin spectral characteristics (Mazza and Brouillard, 1990) causing a hyperchromic and bathochromic effect, increasing the color intensity as well as the wavelength of maximum absorbance of the pigment. The folding observed in acylated radish anthocyanins may account for the high color and pigment stability reported (Giusti and Wrolstad, 1996b) for these pigments in food systems.

CONCLUSIONS

One and two dimensional NMR techniques were employed to chemically characterize red radish anthocyanins. Two novel zwitterionic di-acylated anthocyanins and two mono-acylated anthocyanins were found and their molecular structure and configuration elucidated. Folding of the molecule was evidenced by correlation between the cinnamic acid acylating group and the anthocyanidin moiety revealed by NOESY techniques. This intramolecular copigmentation may play an important role in the high pigment stability previously reported for these pigments.

LIST OF ABBREVIATIONS

ACN : anthocyanins

COSY: correlation spectroscopy

HMQC: heteronuclear multiple quantum coherence

HMBC: heteronuclear multiple bond correlation

HPLC: high performance liquid chromatography

MS: mass spectroscopy

NOESY: nuclear Overhouser effect spectroscopy

TOCSY: total correlation spectroscopy

1D and 2D NMR: one dimensional and two dimensional nuclear magnetic resonance.

Pg-3-soph-5-glu : pelargonidin-3-sophoroside-5-glucoside, also called raphanusin

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CHAPTER 5

ELECTROSPRAY AND TANDEM MASS SPECTROMETRY AS A TOOL FOR ANTHOCYANIN CHARACTERIZATION

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ABSTRACT

The usefulness of electrospray and tandem mass spectroscopy (ES-MS and MS-MS) in anthocyanin characterization was tested using different anthocyanin extracts. Anthocyanins were semi-purified using a C-18 resin, washed with acidified water followed by ethyl acetate, and recovered with acidified methanol. Samples were directly injected into a mass spectrometer in either aqueous or methanolic solutions. Positive change of anthocyanins favored fast and effective ES-MS detection of intact molecular ions. Little interference from other compounds was observed when the ethyl acetate cleaning procedure was used. Tandem mass spectroscopy provided clear and characteristic fragmentation patterns. Voltage used only affected the proportions at which these fragments were present. ES-MS may be used as a fast procedure for identification of anthocyanins, requiring minimal sample preparation. In combination with HPLC, ES-MS and MS-MS could be a very powerful tool for anthocyanin characterization and monitoring authenticity of anthocyanin-containing fruit juices and vegetable extracts.

Key words: Anthocyanins, ES-MS, Tandem spectroscopy, MS-MS,

INTRODUCTION

Anthocyanin composition of fruits and vegetables can be used as a fingerprint to monitor authenticity of juices. Analytical techniques for analysis of anthocyanins usually involve the use of spectrophotometric and chromatographic techniques. These techniques have proven to be very useful for routine analyses and HPLC coupled to a diode array detector has become the method of choice for monitoring anthocyanin

profiles in juices (Strack et al., 1980; Hong and Wrolstad, 1990; Wrolstad et al., 1995; Gao and Mazza, 1994). However, there are hundreds of anthocyanins in nature and the use of other detection methods are often required since these methodologies are sometimes not enough to positively discriminate between compounds that possess very similar spectral characteristics.

Most mass spectroscopic as well as gas chromatographic techniques, require volatility of the sample, and water soluble polar compounds would require derivatization before analysis. Ionspray mass spectrometry has been described as an ideal method for analysis of water soluble flavonol glycosides in hops (Sagesser and Deinzer, 1996). Furthermore, electron impact ionization MS has been successfully used for determining structures of all types of flavonoids (Aramendia, et al., 1995).

Electrospray ionization mass spectrometry (ES-MS) has emerged as a powerful technique for the characterization of biomolecules, and is the most versatile ionization technique in existence today (Covey, 1995, Snyder, 1995). The use of ES-MS has grown in exponential proportion over the last decade (Snyder, 1995) because this soft ionization technique can produce intact ions from large and complex species in solution, even from thermally labile, non-volatile, polar compounds (Black and Fox, 1995; Hutton and Major, 1995; Fenn et al., 1989). The only absolute pre-requisite for ES ionization is that the analytes of interest be soluble in some solvent. The ability of ES to work with liquid sample introduction techniques has launched it into prominence as one of the most important detectors for high pressure liquid chromatography and capillary zone electrophoresis (Covey, 1995).

Tandem mass spectroscopy (MS-MS) allows for the formation of low-energy collisionally induced dissociation fragments (Hutton and Major, 1995). Individual molecules are selected by the first quadrupole mass analyzer, fragmented in the collision cell using a suitable gas, usually argon, and their fragments detected by the second quadrupole mass analyzer (Hutton and Major, 1995).

In this study we evaluate the use of ES-MS and MS-MS as tools for anthocyanin characterization and potentially for monitoring their presence and detecting the presence of adulterants in colored mixtures.

MATERIALS AND METHODS

Pigment material

Anthocyanin pigments from radish (*Raphanus sativus*) epidermal tissue and from red-fleshed potatoes (*Solanum tuberosum*)were extracted following the procedures described by Giusti and Wrolstad (1998). Frozen radish epidermal tissue was liquid nitrogen powdered using a stainless steel Waring Blendor. Powdered samples were blended with 1 volume of acetone and filtered on a Buchner funnel using Whatman #1 paper. For red fleshed potatoes, frozen slices were directly blended with 1 volume of acetone and filtered as described. The filter cake residue was re-extracted with aqueous acetone (30:70 v/v) until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with 2 volumes of chloroform and stored overnight at 1°C. The aqueous portion was collected and placed on a Büchi rotavapor at 40°C until all residual acetone was evaporated (5 to 10 min) and brought to a known volume with distilled water.

Other juice concentrates and anthocyanin extracts were obtained from their manufacturers: Red cabbage (*Brassica oleracea*) extract from Warner Jenkinson (St. Louis, Missouri); chokeberry (*Aronia melanocarpa*) dried concentrate from Artemis International (Fort Wayne, Indiana); and Concord grape (*Vitis labrusca*) juice concentrate, from Welch's (Westfield, NY). Roselle (*Hibiscus sabdariffa* L.) dried powder extract was supplied by the Food Technology Center, Malasian Agricultural Research and Development Institute (Kuala Lumpur, Malaysia);

Dried powders were dissolved in distilled water, and juice concentrates were diluted to single strength juice.

Anthocyanin purification

Anthocyanin containing solutions were passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the mini-column; sugars, acids and other water soluble compounds were removed with 2 volumes of 0.01% aqueous HCl. Anthocyanins were separated from other phenolics using a modification of the procedure described by Oszmianski and Lee (1990). The non-anthocyanin phenolics were washed away from the column using 2 volumes of ethyl acetate, and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotovapor at 35°C and pigments were dissolved in distilled deionized water containing 0.01% HCl.

High performance liquid chromatography (HPLC)

Apparatus: A high performance liquid chromatograph Perkin-Elmer Series 400, equipped with a Hewlett-Packard 1040A photodiode array detector, Gateway 2000 P5-90 computer with a Hewlett-Packard HPLC^{2D} ChemStation software and a Beckman 501 autosampler with a 50 μL loop was used, with simultaneous detection at 280, 310 and 520 nm. The spectra (detection wavelengths from 250 to 600 nm) were recorded for all peaks.

Columns and mobile phase: a Supelcosil LC-18 column (5 micron), 250 x 5 mm i.d. (Supelco, Inc., PN), fitted with an ODS-10, 4 cm x 4.6 mm i.d., Micro-Guard column (Bio-Rad Laboratories) was used. The solvents used were A: 100% HPLC grade acetonitrile and B: 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v:v:v) in water. Flow rate: 1 mL/min. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA).

HPLC conditions for anthocyanin separation and isolation

Anthocyanins were separated using a gradient from 0 to 30% A in 30 min. Peak assignments were made based on published literature and spectral data.

Electrospray mass spectroscopy

Low-resolution MS was done using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ Mass Spectrometer, equipped with an Ion Spray interface (ISV=4700, orifice voltage of 80). The mass spectrometer was operated in the positive ion mode. Samples were introduced into the ES-MS by loop injection (5 μL injection loop) dissolved in distilled water or HPLC grade methanol containing 0.01%

HCl into a flow stream of 1:1 acetonitrile:water with 0.1% TFA. Purified anthocyanins were injected directly into the system.

Tandem mass spectrometry (MS-MS)

Collision induced dissociation (CID) experiments were carried out using Argon as target gas. The mass of the parent ion of interest was scanned in the first quadrupole (Q1), m/z selected and collisionally activated in Q2, and the daughter ions were analyzed in the third quadrupole (Q3). MS-MS was performed using different voltages ranging from 15-30 eV to compare the fragmentation pattern of the targeted anthocyanins at different energy levels.

RESULTS AND DISCUSSION

HPLC anthocyanin profile

HPLC analysis of the anthocyanin pigments of fruits and vegetables is very useful in characterization and authenticity testing since pigment profiles are quite distinctive for different commodities (Fig. 5.1 - 5.5).

Different sources of anthocyanins were used for this study, including approved natural colorants and anthocyanin extracts that are being proposed as natural colorants. Peak assignments were made based on available literature on these pigments and data obtained from HPLC, ES-MS and MS-MS. The anthocyanin profiles ranged from the simple, in the case of Roselle, with only 2 non-acylated major peaks (Pouget et al., 1990), to the more complex picture of Concord grape, with the presence of 5 different anthocyanidins, and with both, acylated and non-acylated pigments (Wrolstad et al.,

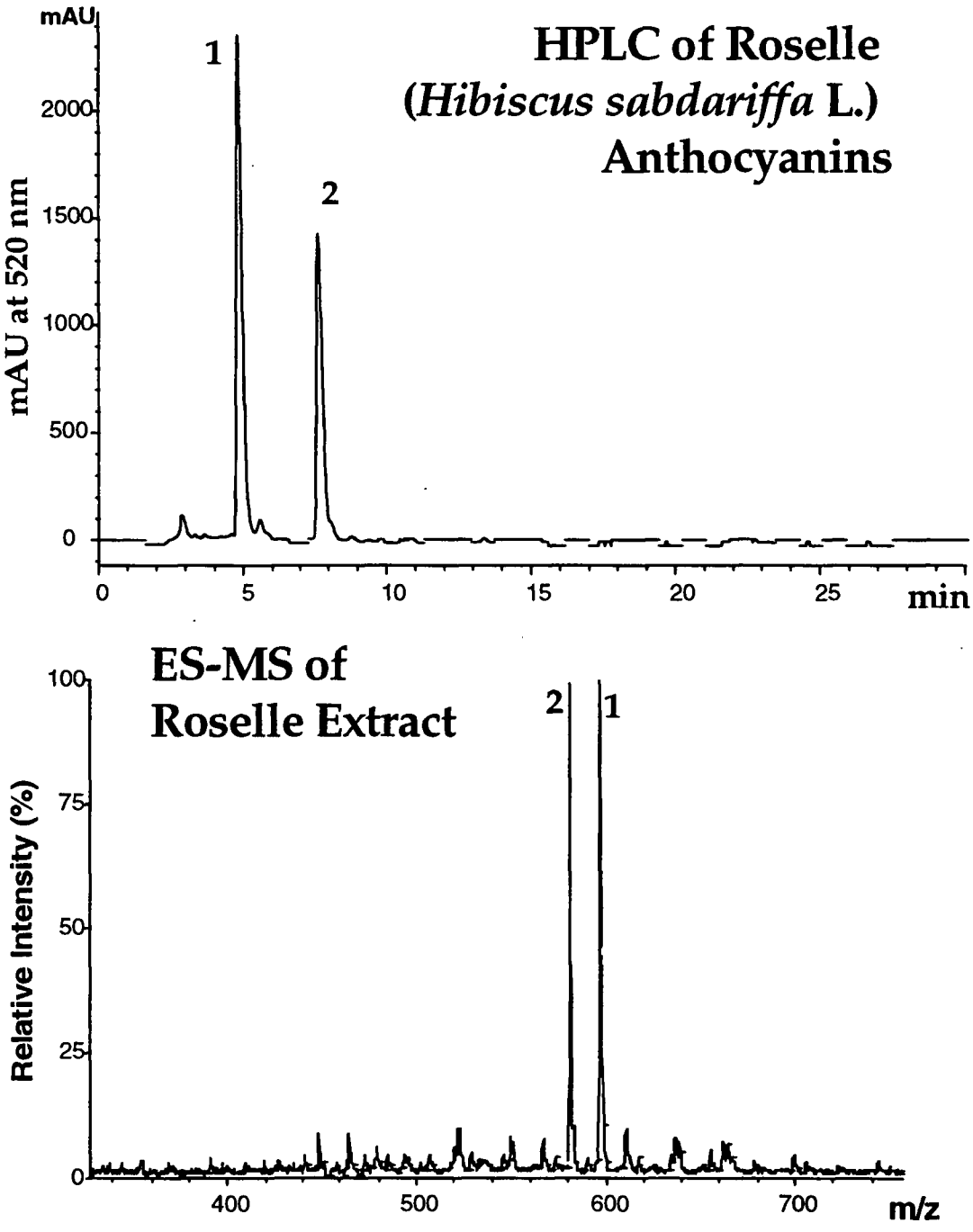


Figure 5.1. HPLC and ES-MS separation of Roselle (*Hibiscus sabdariffa* L.) dried powder extract anthocyanins. Tentative peak assignments, 1: dpd-3-xyl-glu, 2: cyd-3-xyl-glu.

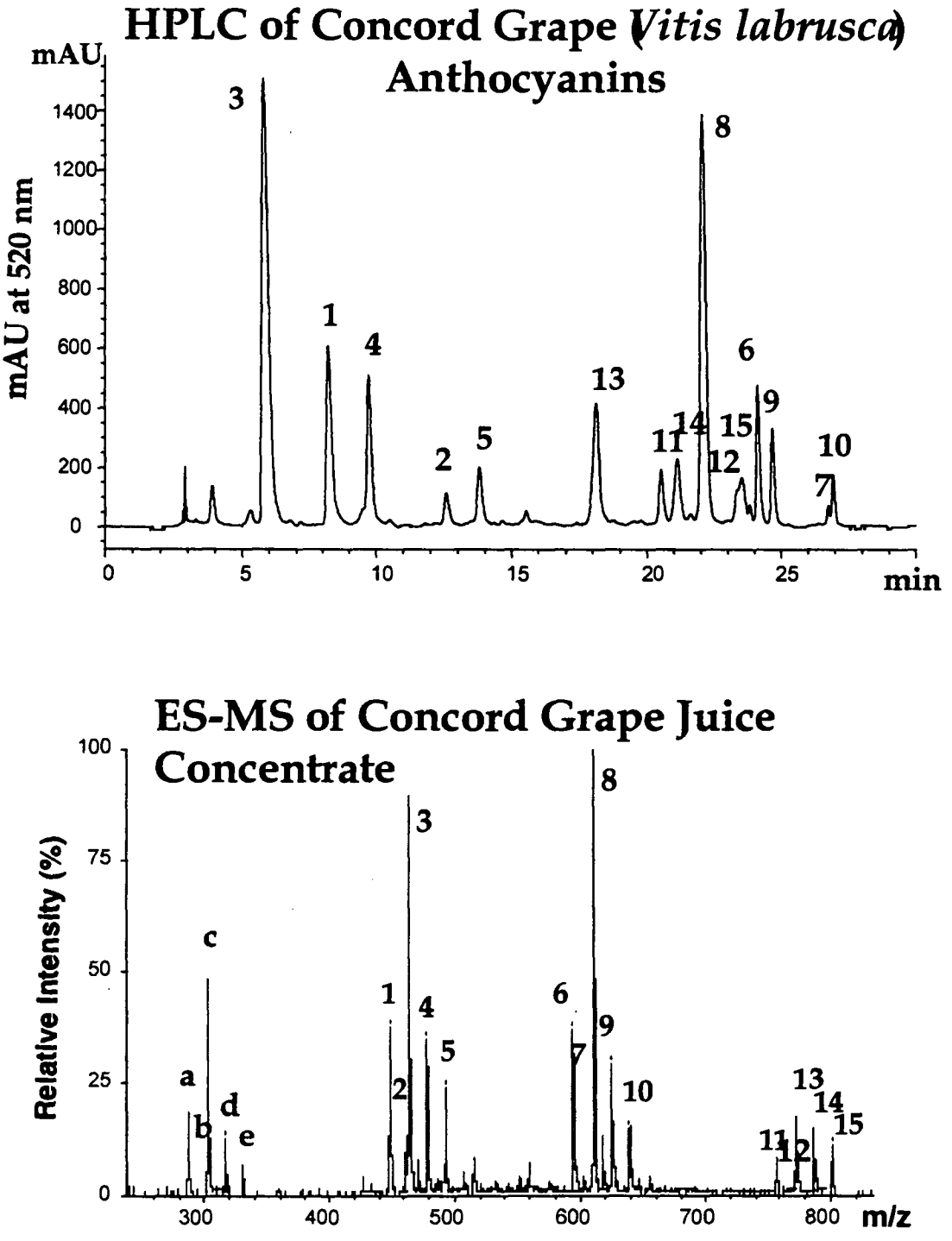


Figure 5.2. HPLC and ES-MS separation of Concord grape (*Vitis labrusca*) juice concentrate anthocyanins. Tentative peak assignments, a: cyd, b: pnd, c: dpd, d: ptd, e: mvd, 1 - 5: their respective 3-glucosides, 6-10: their respective -3-glucosides acylated with *p*-coumaric acid, 11-15: their respective di-glucosides acylated with *p*-coumaric acid.

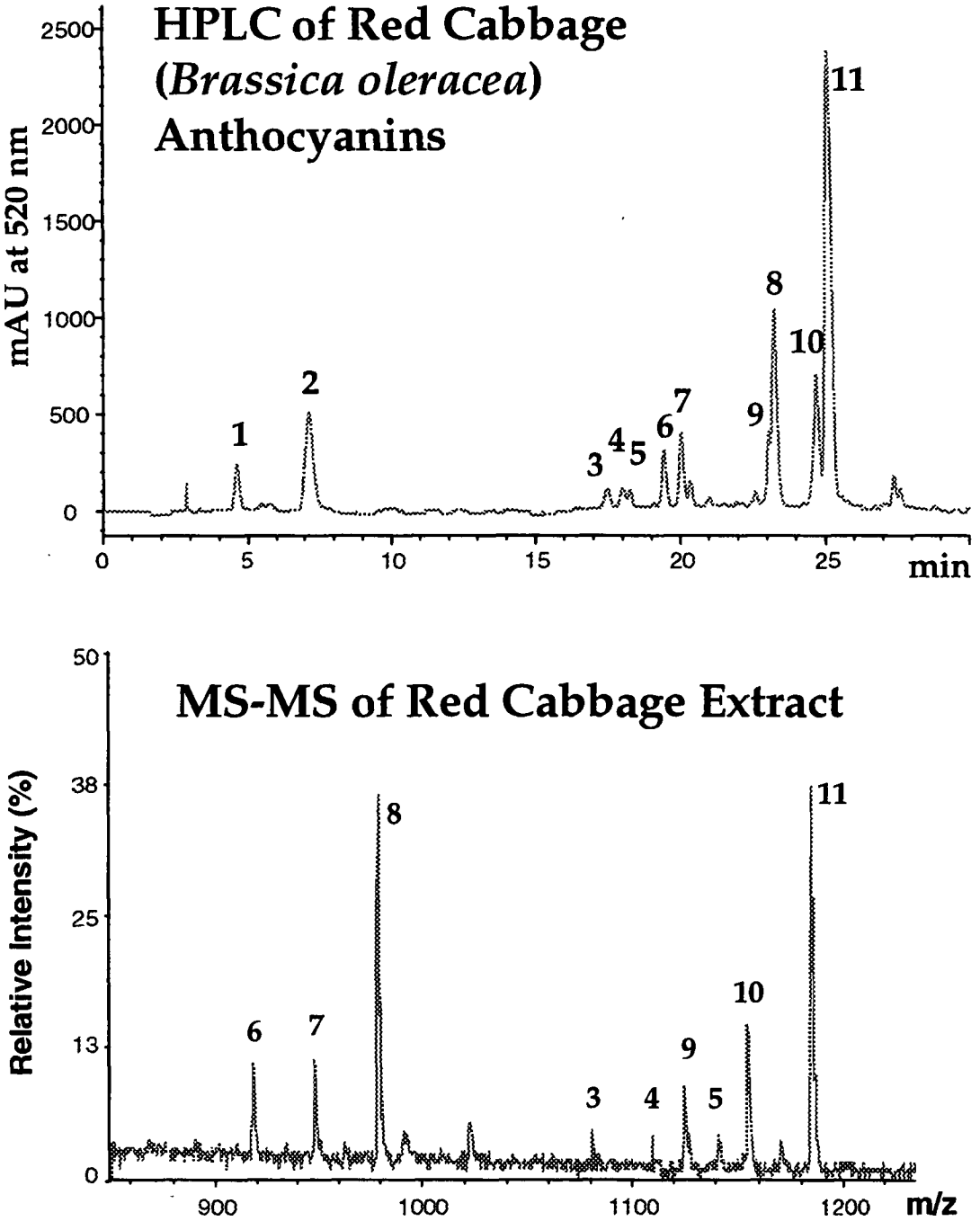


Figure 5.3. HPLC and ES-MS separation of red cabbage (*Brassica oleracea*) extract anthocyanins. Tentative peak assignments, 1: cyd-3-diglu-5-glu, 2: cyd-3-glu-5-glu, 3: cyd-3-triglu-5-glu acylated with p-coumaric acid, 4: cyd-3-triglu-5-glu acylated with ferulic acid, 5: cyd-3-triglu-5-glu acylated with sinapic acid, 6: cyd-3-diglu-5-glu acylated with p-coumaric acid, 7: cyd-3-diglu acylated with ferulic acid, 8: cyd-3-diglu-5-glu acylated with sinapic acid, 9: cyd-3-diglu-5-glu acylated with p-coumaric and sinapic acids, 10: cyd-3-diglu-5-glu acylated with ferulic and sinapic acids, 11: cyd-3-diglu-5-glu acylated with 2 sinapic acids.

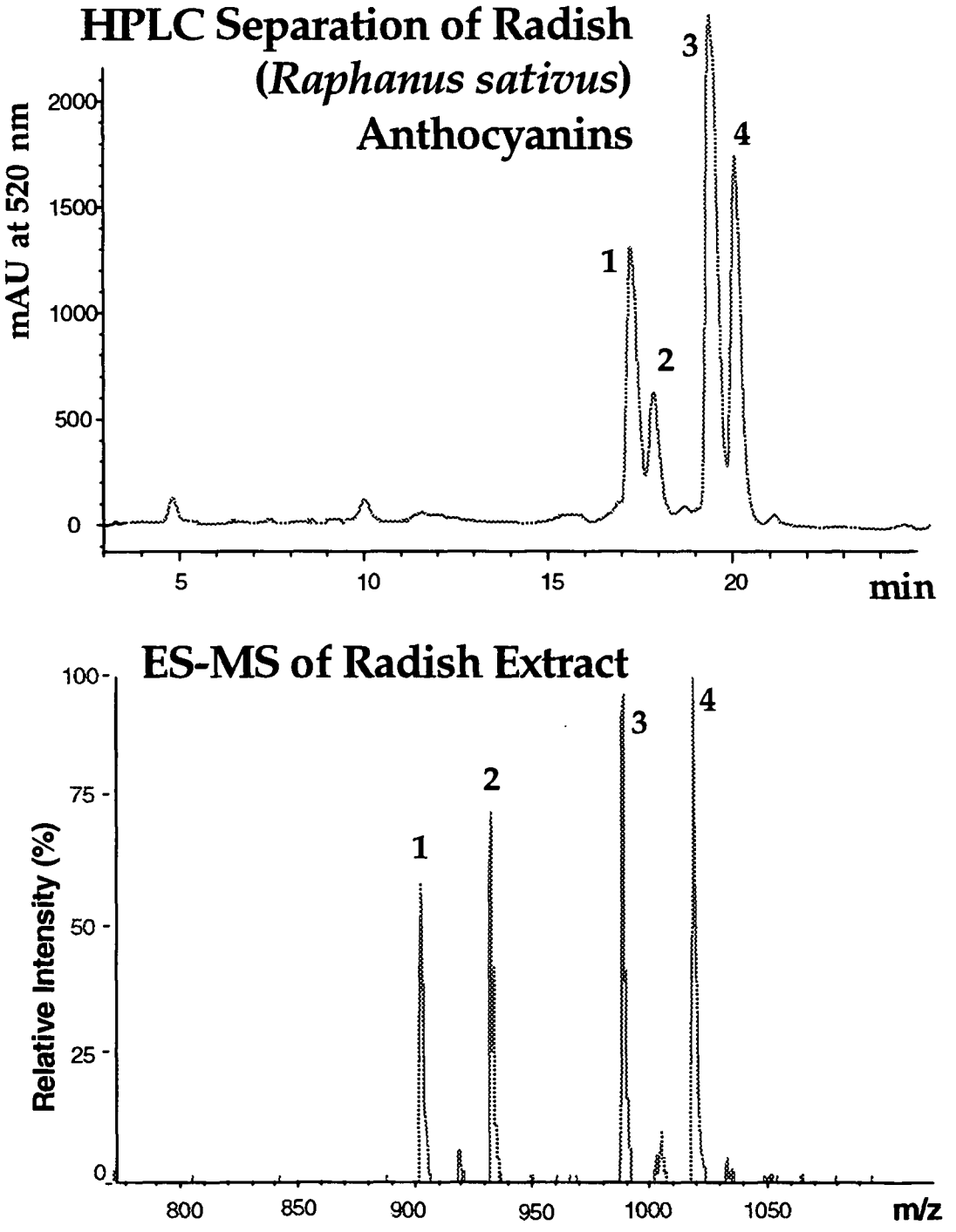


Figure 5.4. HPLC and ES-MS separation of radish (*Raphanus sativus*, L.) anthocyanins. 1: pgd-3-soph-5-glu acylated with p-coumaric acid, 2: pgd-3-soph-5-glu acylated with ferulic acids, 3: pgd-3-soph-5-glu acylated with p-coumaric and malonic acid, 4: pgd-3-soph-5-glu acylated with ferulic and malonic acids.

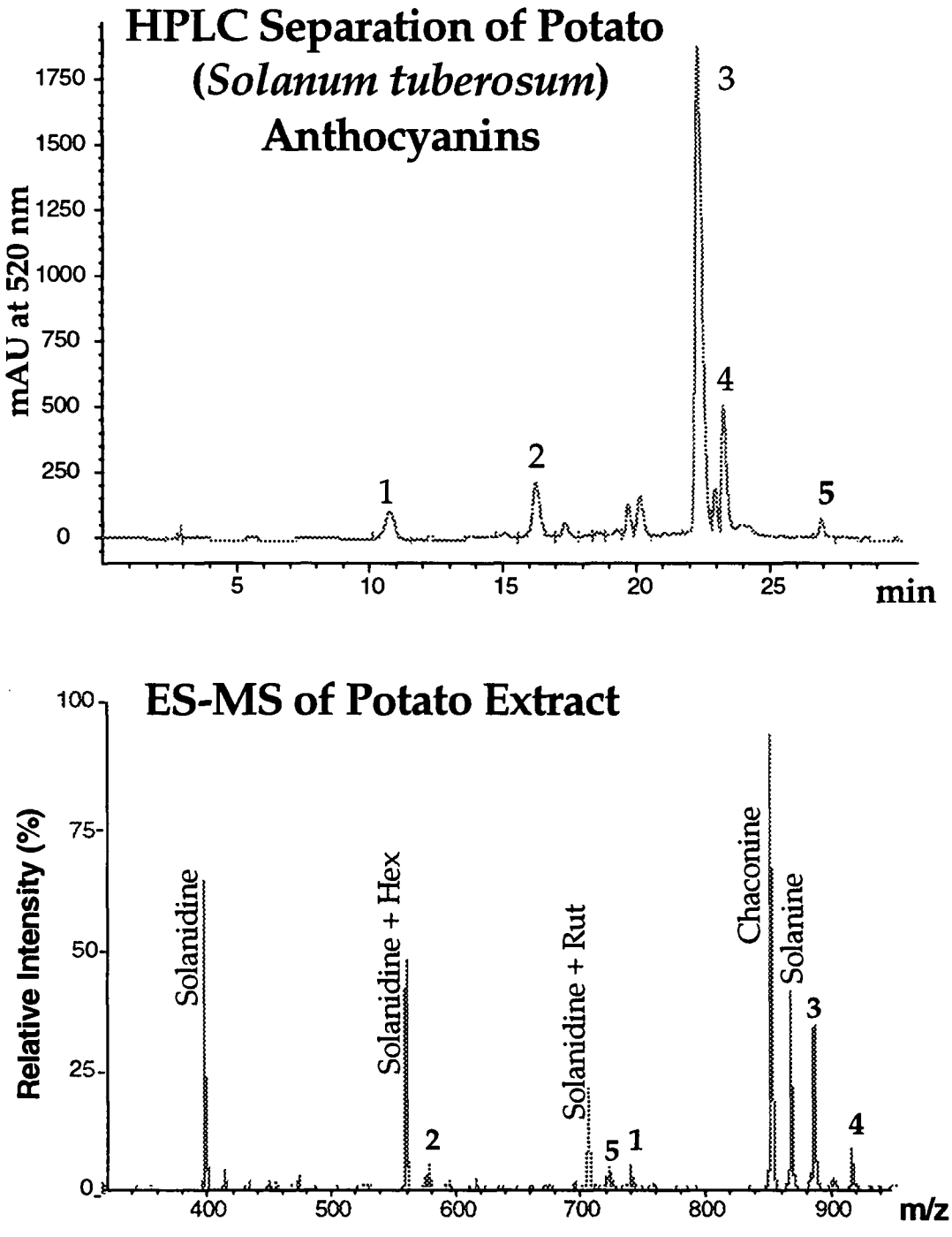


Figure 5.5. HPLC and ES-MS separation of red-fleshed potato (*Solanum tuberosum*) anthocyanins. 1: pgd-3-rut-5-glu, 2: pgd-3-rut, 3: pgd-3-rut-5-glu, 4: pgd-3-rut-5-glu acylated with ferulic acid, 5: pgd-3-rut-5-glu acylated with p-coumaric acid.

1995; Baublis et al., 1994). We also evaluated red cabbage, with a complex anthocyanin profile that shows different acylating patterns with only one anthocyanidin group (Idaka, 1987, Baublis et al., 1994, Ichi et al., 1996). Radish and red-fleshed potatoes have been extensively studied in our laboratory (Giusti and Wrolstad, 1996; Rodriguez-Saona et al., 1998) and have been proposed as potential natural food colorants.

Depending on the HPLC conditions and the complexity of the anthocyanin profile, the length of the experimental run may range from several minutes to up to an hour. Usually, for practical reasons, the approach is to develop a systematic methodology that could be applied to a wide variety of different commodities under the same experimental conditions, and that implies the use of a longer HPLC program and in many cases also implies a decrease in peak resolution. Even then, a 30-60 min duration run is relatively short and easy as compared to former methodologies applied for the separation of anthocyanin such as paper chromatography or thin layer chromatography (TLC). For separation of commodities ranging from non-acylated to acylated anthocyanins, we developed a 30 min program, that could also allow for separation of the 6 aglycons commonly found in nature.

A limitation of the HPLC methodology is that the retention times and resolution obtained will be very dependent on the instrument and conditions used, and changes in mobile phase composition may result in drastic changes in separation and even in the elution order of the pigments. In the case of red cabbage, the literature agrees on the presence of cyanidin derivatives, but there is no agreement on the number of pigments present, ranging from 6 to 15 anthocyanins reported. The use of photodiode array

detector allows for the analysis of spectral characteristics that give information about acylation and glycosylation patterns (Hong and Wrolstad, 1990). However, this method will not be able to discriminate among pigments with similar retention times if they have similar spectral characteristics. That was the case with radish anthocyanins, where we separated 4 pigments, but only 2 different patterns of spectral characteristics were found, suggesting the presence of isomeric forms of the same pigments. We used mass spectroscopic analysis as an additional tool for anthocyanin characterization (Giusti and Wrolstad, 1996).

ES-MS anthocyanin profile

ES-MS proved to be a powerful method for anthocyanin identification. Figures 5.1 through 5.5 show a comparative pigment profile obtained with HPLC and ES-MS. No HPLC separation was performed prior to MS analyses, and sample purification was the same used for HPLC, using C-18 Sep-Pak cartridge purification with or without the use of ethyl acetate as a sequential eluting solvent.

ES-MS uses low voltage and atmospheric pressure, and is very versatile as a ionization technique. Anthocyanins are positively charged at low pH values and are very soluble in water and alcohol, with molecular weights ranging from a few hundreds to a few thousands for simple (e.g. pelargonidin-3-glucoside (Pgd-3-glu), from strawberries, with MW 433) and poly-acylated ones (e.g. acylated anthocyanins in *Clitoria ternatea* flowers, MW 2,107, reported by Terahara et al., 1990), respectively. These characteristics of anthocyanins would make it difficult to work with methodologies that require volatility of the compounds. The ES-MS delivers the

sample in a liquid phase and sprays it into a chamber where a dry gas flows opposite to the mist and a low voltage is applied, causing the disintegration of the drops into charged droplets which become smaller as the solvent vaporizes (Hesse et al., 1997). In that way ES-MS allows analysis of polar compounds from aqueous solution without derivatization (Black and Fox, 1995). Molecules that are inherently charged in solution by virtue of their chemical structure, weak association with other charged species or by chemical reactions occurring in the solution eventually leave the liquid phase and become gas phase ions in the atmospheric region of the ion source (Covey, 1995). The positive charge of anthocyanins at low pH values permits their easy detection using low voltages since other potentially interfering compounds are usually not ionized.

A chromatogram with only the base peak for every mass spectrum provides a more readily interpretable data because of less interference peaks (Sagesser and Deinzer, 1996). ES-MS produces primarily intact molecular ions although fragmentation can be accomplished by varying ionization conditions (Black and Fox, 1996). Under the conditions used in this experiment, clean and clear profiles were obtained, with the presence of intact molecular ions and very little fragmentation.

Molecular weights of common anthocyanins and typical glycosylating and acylating groups are presented in Table 5.1.

The ES-MS of Roselle clearly showed the molecular ions for the 2 major anthocyanins (Fig. 5.1). The m/z of 580.8 and 596.8 corresponded to cyanidin-3-xylosyl-glucoside (cyd-3-xyl-glu) and delphinidin-3-xylosyl-glucoside (dpd-3-xyl-glu), as reported in the literature (Pouget et al., 1990).

Table 5.1. Molecular weight of anthocyanidins, anthocyanins and acylating groups commonly found in nature. pgd: pelargonidin; cyd: cyanidin; pnd: peonidin; Dpd: delphinidin; ptd: petunidin; mvd: malvidin; Acd: anthocyanidin; hex: hexose; pent: pentose.

Anthocyanidins	**Pgd**	**Cyd**	**Pnd**	**Dpd**	**Ptd**	**Mvd**
	271	**287**	**301**	**303**	**317**	**331**
Hexose (hex)	180.2	180.2	180.2	180.2	180.2	180.2
Hex - H_2O	162.2	162.2	162.2	162.2	162.2	162.2
Acd + 1hex	433.2	449.2	463.2	465.2	479.2	493.2
Acd + 2 hex	595.4	611.4	625.4	627.4	641.4	655.4
Acd + 3 hex	757.6	773.6	787.6	789.6	803.6	817.6
Pentose (pent)	150.0	150.0	150.0	150.0	150.0	150.0
Pent - H2O	132.0	132.0	132.0	132.0	132.0	132.0
Acd + 1pent	403.0	419.0	433.0	435.0	449.0	463.0
Acd + 1hex + 1pent	565.2	581.2	595.2	597.2	611.2	625.2
Rhamnose	164.2	164.2	164.2	164.2	164.2	164.2
Rutinose	326.2	326.2	326.2	326.2	326.2	326.2
Rutinose - H20	308.2	308.2	308.2	308.2	308.2	308.2
Acd + rutinose	579.2	595.2	609.2	611.2	625.2	639.2
Acd + rutinose + 1hex	741.4	757.4	771.4	773.4	787.4	801.4
Acd + rutinose + 1pent	711.2	727.2	741.2	743.2	757.2	771.2
Common acylations						
p-coumaric acid	164.2	164.2	164.2	164.2	164.2	164.2
- H_2O	146.2	146.2	146.2	146.2	146.2	146.2
caffeic acid	180.2	180.2	180.2	180.2	180.2	180.2
- H_2O	162.2	162.2	162.2	162.2	162.2	162.2
ferulic acid	194.2	194.2	194.2	194.2	194.2	194.2
- H_2O	176.2	176.2	176.2	176.2	176.2	176.2
sinapic acid	224	224	224	224	224	224
- H_2O	206	206	206	206	206	206
acetic acid	82	82	82	82	82	82
- H_2O	64	64	64	64	64	64
propionic acid	96.1	96.1	96.1	96.1	96.1	96.1
- H_2O	78.1	78.1	78.1	78.1	78.1	78.1
malonic acid	104.1	104.1	104.1	104.1	104.1	104.1
- H_2O	86.1	86.1	86.1	86.1	86.1	86.1
succinic acid	118.1	118.1	118.1	118.1	118.1	118.1
- H_2O	100.1	100.1	100.1	100.1	100.1	100.1

In the cases of Concord grape, the anthocyanin profile was very complex due to the presence of 5 different aglycons: malvidin (mvd), cyanidin (cyd), peonidin (pnd), petunidin (ptd) and delphinidin (dpd), and different patterns of substitution. Good HPLC resolution is usually difficult to obtain, however, the ES-MS highly simplified the results because of the similar pattern of substitutions for all anthocyanidins. Figure 5.2 clearly showed the pattern of anthocyanin distribution in Concorde grapes. The first group of molecular ion peaks corresponded to the 5 aglycons that were probably produced by the ionization process. A second group corresponded to the 5 non-acylated mono-glucosylated anthocyanins, followed by the 5 mono-glucosylated pigments acylated with *p*-coumaric and finally the 5 acylated di-glucosylated anthocyanins. Peaks assignments were done using as reference the results reported by Baublis et al. (1994), but using the ES-MS information we were able to make further tentative assignments of some minor peaks, that were not identified by those researchers.

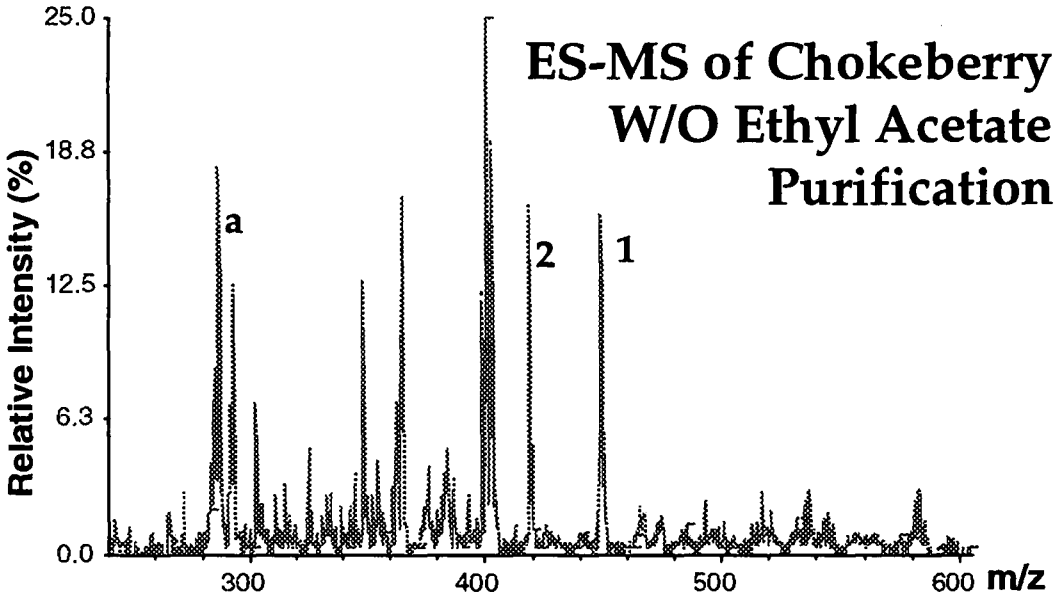
Red cabbage ES-MS gave m/z ratios corresponding to the different cyanidin derivatives reported for this commodity (Ichi et al., 1996; Mazza and Miniati, 1993), with the presence of acylated and non-acylated pigments (Fig. 5.3).

In the case of radish anthocyanins (Fig. 5.4), HPLC coupled with photodiode array detector separated the 4 major pigments, but did not detect spectral differences between peaks 1 and 3 and peaks 2 and 4. It was only after ES-MS that we were able to detect the presence of an additional acylating group on peaks 3 and 4, malonic acid, as evidenced by the additional molecular weight of 86 units (Giusti and Wrolstad, 1996).

ES-MS may also be useful to detect other charged molecules in the sample. That was the case with potato extracts, where the ES-MS revealed the presence of toxic positively charged alkaloids (Fig. 5.5). The clean profile obtained with intact molecular ions facilitated the process of anthocyanin and alkaloid identification.

The use of mass spectroscopic analyses for characterization of anthocyanins has been increasing over the last decade, with most of those reports using HPLC coupled to a MS detector or isolating individual pigments prior to the mass spectroscopic analysis (Baublis et al., 1994; Bakker et al., 1997; Saito, et al., 1996; Shi et al., 1993). The ionization technique more often reported has been fast atom bombardment (FAB) and reports on the use of ES-MS on anthocyanins have been limited.

Some advantages of the use of ES-MS ionization and the direct injection of the anthocyanin extracts on the mass spectrometer include the simple and easy sample preparation, short run time (a few minutes), very consistent anthocyanin profiles, and the generation of results which are more reproducible since no column separation or mobile phase gradients are required, eliminating the variability due to those sources. Mass spectroscopic techniques does not differentiate between different diasteroisomeric forms of sugars, and therefore it will not provide information regarding the exact glycosidic substitution other than the number of carbons or presence of methylations in the sugars. That was the case for chokeberry anthocyanins (Fig. 5.6), where the ES-MS only showed 2 peaks corresponding to two different



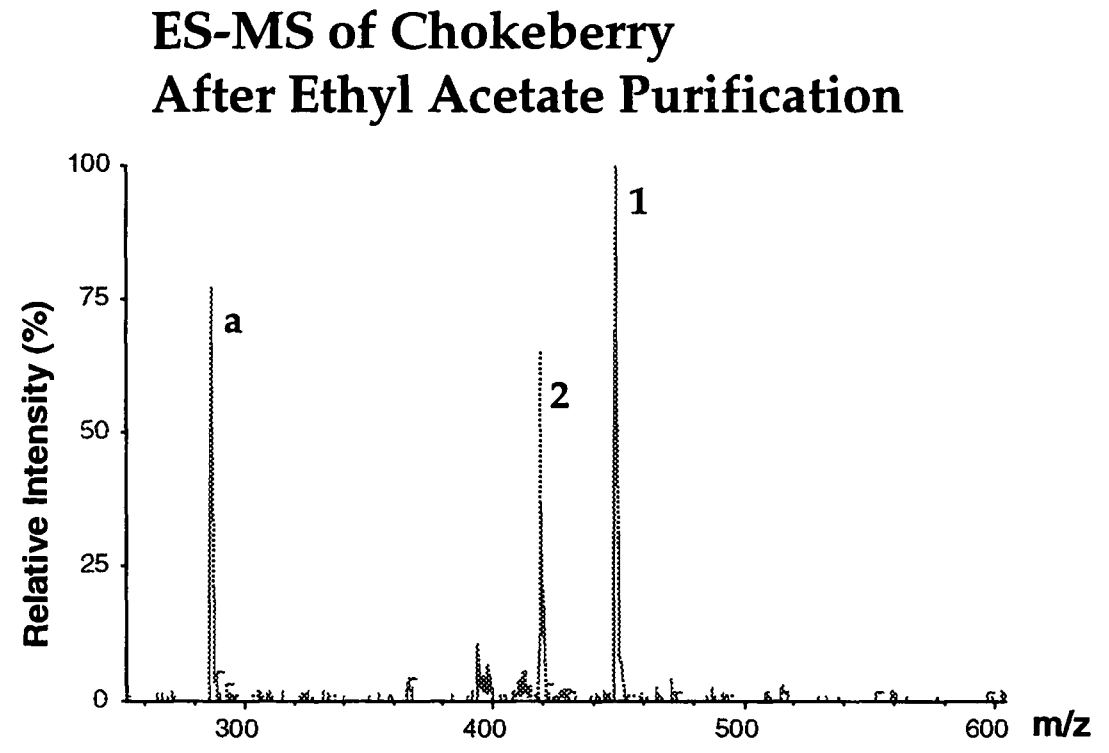


Figure 5.6. ES-MS spectra of chokeberry (*Aronia melanocarpa*) anthocyanins with and without the use of ethyl acetate purification. Tentative peaks assignment, a: cyd, 1: cyd-3-arab and cyd-3-xyl.2: cyd-3-gal and cyd-3-glu.

anthocyanins each: cyd-3-arabinoside and cyd-3-xyloside for one, and cyd-galactoside and cyd-glucoside for the other. This could represent a limitation in some instances, however, it could become an advantage when analyzing samples with a very complex anthocyanin pattern for authenticity by simplifying the spectra obtained.

Usefulness of ethyl acetate purification

Sample purification using C-18 cartridges is a very simple and fast procedure that provides efficient separation of aromatic compounds in only a few minutes. Sugars and acids have no affinity for the column and can be washed away by passing the sample through the C-18 cartridge in an aqueous solution. Anthocyanins, phenolics and other compounds with a non-polar character will be separated from other compounds due to their affinity to the matrix. These compounds can be recovered using methanol. However, the use of ethyl acetate as an intermediate step will remove from the column the less-polar compounds such as phenolic acids and flavonoids, separating them from anthocyanins, our target compounds, which are subsequently recovered with acidified methanol.

Figure 5.6 shows an example of the drastic improvement in the ES-MS obtained when ethyl acetate is used as compared to the simpler C-18 procedure. Adding the ethyl acetate step does not add much time to the purification procedure but contributes substantially to the cleanliness of the MS output. According to our experiments, samples with anthocyanins with multiple glycosylations or acylations (m/z > 600) did not show much of a problem in the absence of ethyl acetate purification, since most interfering peaks appeared at low m/z ranges. However, the use of ethyl

acetate would still be recommended since it does not take much time and could allow for the detection of lower molecular weight anthocyanins present.

Tandem mass spectrometry

Tandem mass spectrometry experiments, also known as MS-MS, use two mass analyzer regions within the same instrument for the collision induced decomposition of a chosen mass-to-charge ratio ion. The first region is used as a separation device before inducing fragmentation in a collision cell followed by analysis of the fragment ion in the second (McLafferty and Turecek, 1993; Jennings, 1996; Lawson, et al., 1996). Ions of a chosen mass-to-charge ratio (m/z) generated in the ionization process collide with an inert gas, leading to the formation of dissociation fragment ions (Jennings, 1996) in a energy transfer normally thought as involving primary vibrational energy transfer. This process provides structural information on the components of a mixture, which lead to the unambiguous identification of fragmentation pathways (Lawson, et al., 1996).

MS-MS of individual peaks were performed (Table 5.2) and resulted in clear and characteristic fragmentation patterns. All glycosidic substitutions at positions C3 and C5 of the pyrilium ring were cleaved. The MS-MS resulted in cleavage of glycosidic bonds only between the flavilium ring and the sugars directly attached to it. Tandem MS of 3,5 glycosylated anthocyanins produced fragments that corresponded to the aglycon, the C3-substituted anthocyanins, and the C5-substituted anthocyanins, in addition to the molecular parent ion. In figure 5.7 the cleavage of non-acylated Pg-3-soph-5-glu produced the fragments corresponding to Pg-3-soph, Pg-5-glu, and Pgd.

Table 5.2. Anthocyanin fragmentation patterns obtained with tandem mass spectrometry. Dpd: delphinidin; cyd: cyanidin, pnd: peonidin; ptd: petunidin, mvd: malvidin; pgd: pelargonidn; xyl: xyloside; glu: glucoside; soph: sophoroside; rut: rutinoside; + p-coumaric, + ferulic, + sinapic: acylated with p-coumaric ferulic or sinapic acids, respectively.

Peak	Identification	Fragments
Roselle (*Hibiscus sabdariffa* L.)		
1	Dpd-3-xyl-glu	597.2 (M+) , 303.2 (dpd)
2	Cyd-3-xyl-glu	581.2 (M+) , 287.2 (cyd)
Concord grape		
2	Pnd-3-glu	462.8 (M+), 301.2 (pdn)
3	Dpd-3-glu	465.2 (M+), 303.2 (dpd)
6	Cyd-3-glu + p-coumaric	595.2 (M+), 287.2 (cyd)
7	Dpd-3-glu + p-coumaric	610.8 (M+), 303.2 (dpd)
9	Ptd-3-glu + p-coumaric	624.8 (M+), 317.2 (ptd)
13	Dpd-3-glu-5-glu + p-coumaric	773.2 (M+), 610.8 (M+ - glu), 464.8 (dpd-glu), 303.2 (dpd)
14	Ptd-3-glu-5-glu + p-coumaric	787.2 (M+), 624.8 (M+ - glu), 478.8 (ptd-glu), 317.2 (ptd)
15	Mvd-3-glu-5-glu + p-coumaric	801.2 (M+), 639.2 (M+ - glu), 492.8 (mvd-glu), 331.2 (mvd)
Red cabbage		
6	Cyd-3-di-glu-5-glu + sinapic	979.6 (M+) , 817.6 (M+ - glu), 449.2 (cyd-glu), 287.2 (cyd)
10	Cyd-3-di-glu-5-glu + ferulic	1155.2 (M+), 993.6 (M+ - glu), 449.2 (cdn-glu), 287.2 (cyd)
11	Cyd-3-di-glu-5- (2) sinapic	1185.6 (M+), 1023.6 (M+ - glu), 449.2 (cyd-glu), 287.2 (cyd)
Red radish		
1	Pgd-3-soph-5-glu	757.2 (M+), 595.2 (M+ - glu), 433.2 (pgd-glu), 271.2 (pgd)
2	Pgd-3-soph-5-glu + p-coumaric	903.2 (M+), 741.2 (M+ - glu), 433.2 (pgd-glu), 271.2 (pgd)
3	Pgd-3-soph-5-glu + ferulic	933.2 (M+), 771.2 (M+ - glu), 433.2 (pgd-glu), 271.2 (pgd)
4	Pgd-3-soph-5-glu + p-coumaric and malonic acids	989.6 (M+), 741.2 (M+ - glu-mal), 518.8 (pgd-glu-mal), 271.2 (pgd)
5	Pgd-3-soph-5-glu + ferulic and malonic acids	1019.2 (M+), 771.2 (M+ - glu-mal), 518.8 (pgd-glu-mal), 271.2 (pgd)
Red-fleshed potato		
1	Pgd-3-rut-5-glu	741.4 (M+), 578.8 (M+ - glu), 433.2 (pgd-glu), 271.2 (pgd)
2	Pgd-3-rut	579.2 (M+), 433.2 (M+ - rham), 271.2 (pgd)
3	Pgd-3-rut-5-glu + p-coumaric	887.2 (M+), 725.2 (M+ - glu), 433.2 (pgd-glu), 271.2 (pgd)
4	Pgd-3-rut-5-glu + ferulic	919.6 (M+), 755.2 (M+ - glu), 433.2 (pgd-glu), 271.2 (pgd)
5	Pgd-3-rut + p-coumaric	725.4 (M+), 433.2 (pgd-glu), 271.2 (pgd)

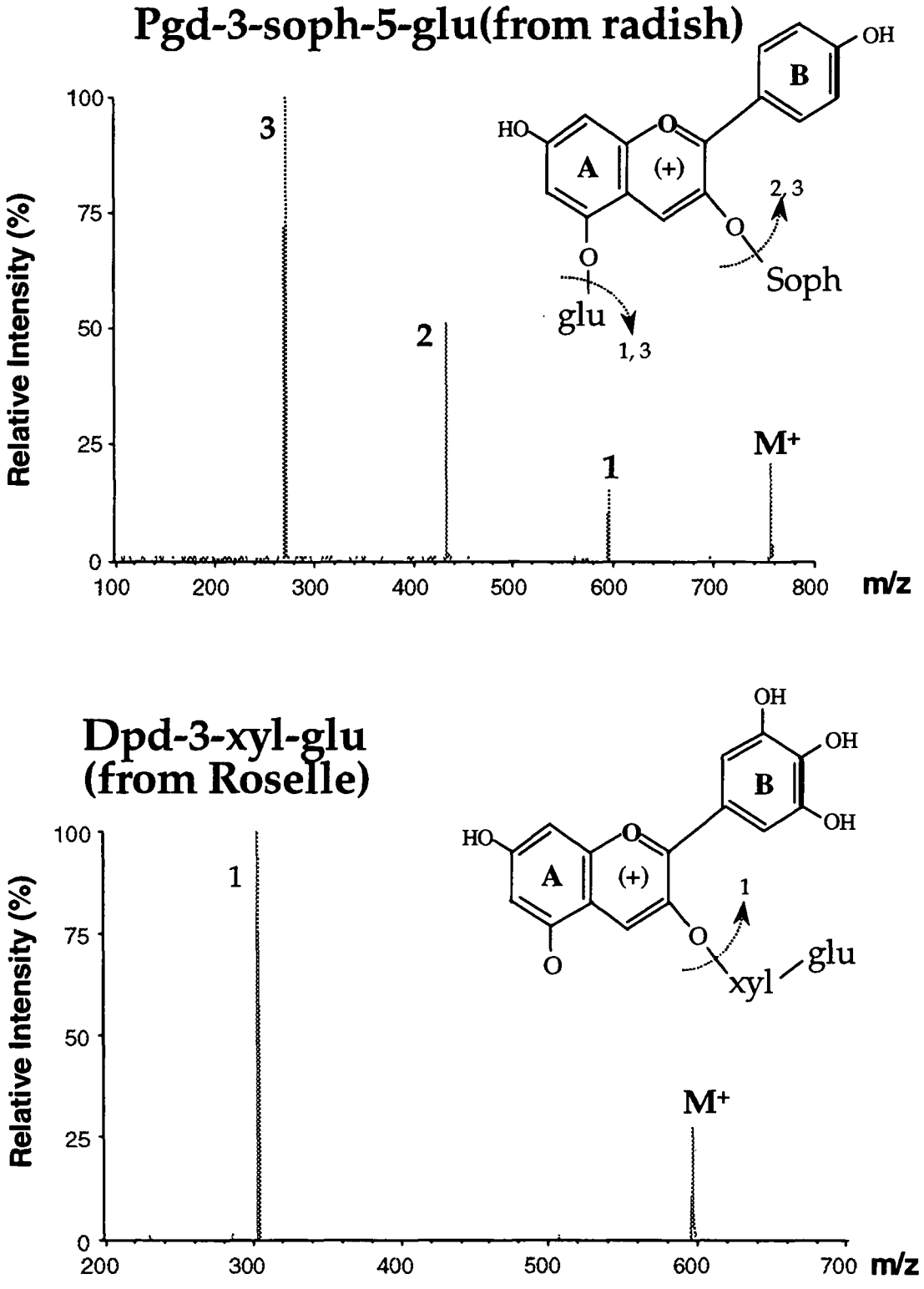


Figure 5.7. Fragmentation pattern of non-acylated anthocyanins, e.g. pgd-3-soph-5-glu and dpd-3-xyl-glu.

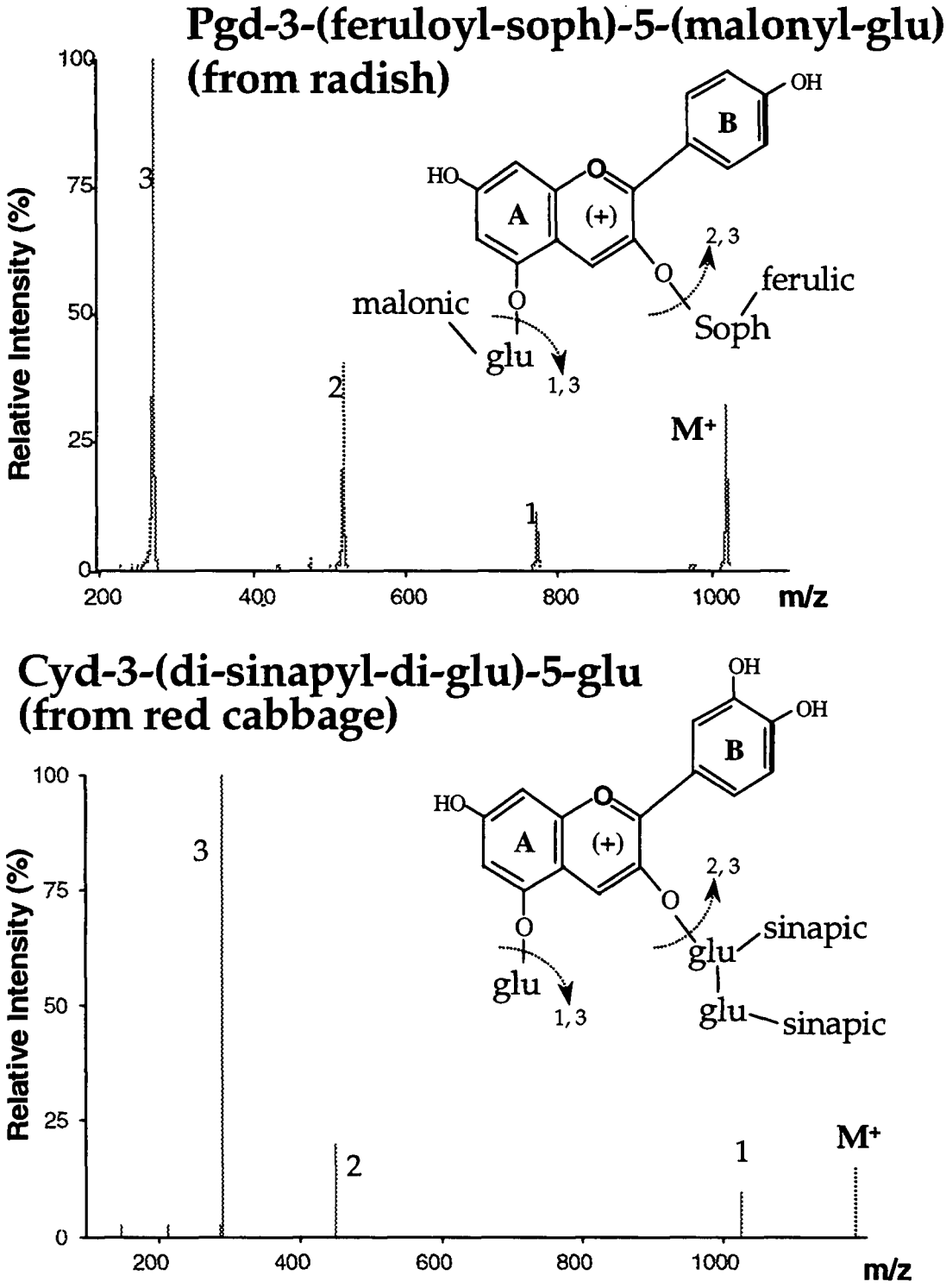


Figure 5.8. Fragmentation pattern of acylated anthocyanins, e.g. pgd-3-(feruloyl-soph)-5-(malonyl-glu) and cyd-3-(di-sinapyl-di-glu)-5-glu.

In the case of acylated anthocyanins (Fig. 5.8), this fragmentation pattern may allow for a rough determination of the location of the acylating groups. In the case of radish anthocyanins, the fragments produced are consistent with the presence of one cinnamic acid acylating group attached to the C3 glycosidic substituent while the other, malonic acid, is attached to the sugars of C5. In the case of red cabbage anthocyanins, both acylating groups are attached to the sugars of C3, resulting in the formation of a large fragment (m/z 1023.6) corresponding to a fragment with the 2 acylating groups, and other fragments corresponding to the aglycon and the monoglucosylated cyanidin. This same pattern of fragmentation was evidenced with most anthocyanins analyzed (Table 5.2).

The fragmentation of C3-substituted anthocyanins produced only one fragment, corresponding to the m/z of the aglycon, and that was the case for the di-glycosylated anthocyanins of Roselle. Pigments 1 and 2 are reported as dpd-3-sambubioside and cyd-3-sambubioside, and the fragments obtained with MS-MS were only dpd and cyd, respectively (Table 5.2 and Fig. 5.7). The only exception to this pattern were the C3 substituted anthocyanins from potato, reported as pgd-3-rutinoside (pgd-3-rut) and pgd-3-rut acylated with p-coumaric acid. The stability of the linkage 1-6 between rhamnose and glucose allows for free rotation and more accessibility of the gas used to produce the fragmentation. In the case of the other anthocyanins analyzed the disaccharides present were either sambubiose (xylose 1-2 glucose), or sophorose (glucose 1-2 glucose) with more stable sugar bonds, and the Tandem spectroscopy analysis did not cause its cleavage at that bond.

No cleavage of ester linkages was obtained with MS-MS on any of the acylated anthocyanins analyzed (Table 5.2).

The internal energy of the fragmenting ion is determined both by the initial ionization process and by the internal energy gained in the collision, and the reproducibility of the experiment depends on the control of the experimental conditions (Jennings, 1996). When experimental conditions are controlled, differences of MS-MS spectra are indicative of differences in the structures of the ions undergoing collision. Different voltages were used to evaluate the fragmentation pattern with MS-MS. Under the conditions used for this study, high reproducibility was obtained, and the fragmentation patterns obtained with anthocyanins were always the same, regardless of the energy used for the experiment (in the range of 15-25 eV). The only variations found were on the proportions at which these fragments were present. The use of energies higher than 25 eV ($\geq$ 30 eV) resulted in complete fragmentation of the parent ion, with the production of only one fragment corresponding to the m/z of the aglycon.

The coupling of reversed phase liquid chromatography to MS allows the molecules to be characterized by retention time, UV-visible response and mass spectral information for the individual components and fragments (Hutton and Major, 1995) and is becoming a technique gaining popularity over the last years. The development of electrospray ionization as a sensitive technique, as well as a specific and versatile detector for liquid sample introduction has contributed enormously to the establishment of liquid chromatography LC-MS as an analytical technique for mixture analysis (Gaskell, 1996). ES-MS allows analysis of polar compounds from aqueous solution without derivatization (Black and Fox, 1995) which makes it well suited for in-line

analysis in conjunction with liquid chromatography. Combined information of HPLC-UV-Vis spectra and mass spectra is a very powerful tool for anthocyanin identification and characterization.

Our findings indicate that the use of ES-MS and MS-MS are powerful and rapid techniques for screening and characterization of anthocyanins in samples where some background information is available, or where a first compositional overview is required. Electrospray mass spectroscopy may be used as a fast procedure for identification of anthocyanins, requiring minimal sample preparation. ES-MS was fast and effective detecting molecular ions of anthocyanins and anthocyanin extracts. Tandem mass spectroscopy has extraordinary capabilities for the structural analysis of individual components of complex mixtures (Gaskell, 1996), with the first MS working as the separation device and the second one used for chemical structure determination, and showed to be very effective for structural analysis of anthocyanins.

In combination with HPLC, ES-MS and MS-MS could be a useful tool for monitoring the authenticity of anthocyanin-containing fruit juices and vegetable extracts.

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CHAPTER 6

ANTHOCYANIN PIGMENT COMPOSITION OF RED RADISH CULTIVARS AS POTENTIAL FOOD COLORANTS

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ABSTRACT

Red radish (*Raphanus sativus* L.) cultivars were evaluated with respect to qualitative and quantitative anthocyanin (ACN) pigment content. Radishes were grown at 2 locations (Corvallis and Hermiston, OR) and harvested at 2 maturity stages. Pigment content was dependant on cultivar, root weight and location, higher amounts being obtained at Hermiston. Spring cultivars (n=22) had pigmentation in the skin, ranging from 39.3 to 185 mg ACN/100g skin. Red-fleshed Winter cultivars (n=5) had pigment content ranging from 12.2 to 53 mg ACN/100g root. ACN profiles were similar for different cultivars, the major pigments being pelargonidin-3-sophoroside-5-glucoside, mono- or di-acylated with cinnamic and malonic acids; individual proportions varied among cultivars. Estimated pigment yields ranged from 1.3 to 14 kg/ha.

Key words: Anthocyanins, red radish, *Raphanus sativus* L., cultivars, pigment profile.

INTRODUCTION

Radish (*Raphanus sativus* L.) is an easy vegetable to grow and can be harvested within 4 to 6 wks (Hartmann et al., 1988). Radishes occur in a variety of shapes: global, oval, long, flattened and pear-shaped roots; and colors: white, red, purple, yellow and black (George and Evans, 1981).

The anthocyanin (ACN) pigments responsible for red and purple radishes have been characterized by different researchers (Ishikura and Hayashi, 1962 and 1965; Harborne 1963; Fuleki, 1969), and the presence of pelargonidin- and cyanidin-derivatives was reported in red and purple radishes, respectively. Red radish ACN were

characterized as pelargonidin-3-sophoroside-5-glucoside (Pg-3-soph-5-glu, or raphanusin) derivatives, with cinnamic acids attached to the glycosidic moieties. The presence of this basic structure has been confirmed in red radish cultivar (cvs) Fuego and an additional acylating group on this basic structure was also detected and identified as malonic acid (Giusti and Wrolstad 1996a).

Interest in ACN has increased because they are potential natural alternatives to artificial colorants. The use of red radish ACN extract for coloring maraschino cherries was evaluated (Giusti and Wrolstad, 1996b) and the color characteristics of radish-colored brined cherries were extremely close to those with FD&C Red No. 40 for $\approx$ 6 mo storage at room temperature. The high color and pigment stability were attributed to the presence of acylating groups attached to the ACN moiety.

Our objective was to determine which red radish cvs may be more appropriate for potential colorant production. We analyzed different cvs to determine those with higher pigment contents and examined the qualitative pigment composition to make inferences regarding hue and chroma. The effect of planting location and maturity on pigment yield were also evaluated by comparing results from 2 locations, and by harvesting at 2 maturity stages. Based on radish yield obtained and published data, we calculated potential yields of pigment/ha and pigment/ha-day, to determine the viability of pigment production on a commercial scale.

MATERIALS AND METHODS

Plant material

A total of 26 red radish cvs were evaluated. Seeds were obtained from Daehnfeldt Inc. (Albany, OR); Nunhems International (Haelen, Holland); W. Atlee Burpee Co. (Warmenster, PA); Rogers Seed Co. (Boise, ID); Stokes Seeds Inc. (Buffalo, NY); Known-You Seed Co. (Kaohsiung, Taiwan); Tokita Seed Co. (Nakagawa, Japan); American Takii Inc. (Salinas, CA); Sakata Seed America (Morgan Hill, CA). Additional seeds were provided by Mah Dong King (Tianjin Processing Center of Agricultural Products, Tianjin, P.R. China) (Table 1).

During the first year (Summer 1994) 23 red radish cvs were planted at the Oregon State University (OSU) Lewis-Brown Horticultural Farm (Corvallis, OR) and screened for pigment content and profile and from those, 11 were selected for second year (Summer 1995) analyses. For the second year trials, radishes from the selected cvs were planted at 2 locations: the OSU Lewis-Brown Horticultural Farm (Corvallis, OR) and the OSU Hermiston Agricultural Research and Extension Center (Hermiston, OR). Two cvs that showed high pigment content in previous years, as well as 4 additional Chinese cvs were planted only at Corvallis and analyzed during a third year (Summer 1996).

Radishes were harvested, leaves manually cut, and roots washed with cold water using a vibratory spray washer (A.B. McLauchlan, Co., Salem, OR) to eliminate extraneous matter. Radishes were weighed and refrigerated at 1°C until analyzed. Sample size was 1 kg root or 4 units, whichever was larger. Radish cvs with pigment

only in the skin were manually peeled, the epidermal tissue was frozen in liquid nitrogen and stored at -23°C until analyzed. Radish cvs with pigmentation in the flesh were cut into small cubes (about 1 cm^3 each), the cubes were mixed, and a sample of 200 g was frozen and stored at -23°C until analyzed.

Pigment extraction

The extraction followed the procedure described by Giusti and Wrolstad (1996b). Frozen epidermal tissue (for skin pigmented radishes) was liquid nitrogen powdered using a stainless steel Waring Blendor. Powdered samples were blended with 1 volume of acetone and filtered on a Buchner funnel using Whatman #1 paper. For radishes with red flesh, frozen radish cubes were directly blended with 1 volume of acetone and filtered as described. The filter cake residue was re-extracted with aqueous acetone (30:70 v/v) until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with 2 volumes of chloroform and stored overnight at 1/C. The aqueous portion was collected and placed on a Büchi rotavapor at 40°C until all residual acetone was evaporated (5 to 10 min) and brought to a known volume with distilled water.

Monomeric anthocyanin content

Monomeric ACN content was determined using the pH-differential method (Wrolstad et al., 1982). A Shimadzu 300 UV spectrophotometer and 1 cm pathlength disposable cells were used for spectral measurements at 510 and 700 nm. Pigment content was calculated as pelargonidin-3-glucoside, using an extinction coefficient of 31,600 L cm^{-1} mol^{-1} and a molecular weight of 433.2 g mol^{-1} (Wrolstad, 1976).

Anthocyanin purification

The aqueous extract was passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the mini-column; sugars, acids and other water soluble compounds were removed with 2 volumes of 0.01% aqueous HCl and ACN were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotavapor at 35°C and pigments were dissolved in distilled deionized water containing 0.01% HCl.

Alkaline hydrolysis of anthocyanins

Purified pigment (ca. 10 mg) was hydrolyzed (saponified) in a screw-cap test tube with 10 mL of 10% aqueous KOH for 8 min at room temperature in the dark, as described by Hong and Wrolstad (1990). The solution was neutralized using 2N HCl, and the hydrolysate was purified using a C-18 Sep-Pak cartridge, as described.

High performance liquid chromatography (HPLC)

Apparatus: A high performance liquid chromatograph Perkin-Elmer Series 400, equipped with a Hewlett-Packard 1040A photodiode array detector, Gateway 2000 P5-90 computer with a Hewlett-Packard HPLC^{2D} ChemStation software and a Beckman 501 autosampler with a 50 :L loop was used, with simultaneous detection at 280, 310 and 520 nm. The spectra (detection wavelengths from 250 to 600 nm) were recorded for all peaks.

Columns and Mobile Phase. System I: a PLRP-S column (5 μ) 250 x 4.6 mm i.d. (Polymer Labs, Amherst, MA), fitted with a Polymer Labs, 1.5 cm x 4.6 mm i.d. guard column was used. The solvents used were A: 100% HPLC grade acetonitrile and B: 4% phosphoric acid (aqueous). **System II**: a Supelcosil LC-18 column (5), 250 x 5 mm i.d. (Supelco, Inc., PN), fitted with an ODS-10, 4 cm x 4.6 mm i.d., Micro-Guard column (Bio-Rad Laboratories) was used. The solvents used were A: 100% HPLC grade acetonitrile and B: 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v:v:v) in water. Flow rate: 1 mL/min. Solvents and samples were filtered through a 0.45 :L Millipore filter type HA (Millipore Corp., Bedford, MA).

Conditions for anthocyanin analysis

Radish ACN and saponified radish ACN were separated using Systems I and II. System I used a linear gradient from 12 to 22% A in 50 min. System II used a linear gradient from 0 to 30% A in 30 min. The identity of ACN was verified by the 2 different HPLC systems.

Conditions for phenolic acid analysis

Phenolic acids, obtained from radish ACN saponification, were separated using the 2 different HPLC systems. System I used a linear gradient from 10 to 15% A in 15 min, a linear gradient from 15 to 25% A in 15 min and isocratic at 25% A for 5 min. System II was run isocratic at 5% A, with simultaneous detection at 280 and 320 nm. The identity of acyl groups was confirmed by the 2 different HPLC systems. Retention times and spectra were compared to pure standards. Caffeic acid, ferulic acid and *p*-coumaric acid standards were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

The data were analyzed using regression analyses and analysis of variance (ANOVA) as a complete randomized block (location) design. Significance of differences was defined at $p \leq 0.05$.

RESULTS AND DISCUSSION

Monomeric anthocyanin content of radish cultivars

Different red radish cvs (23) were screened during the first year for pigment content (Table 6.1) and other horticultural characteristics. Radish cvs are often grouped according to their time to mature and season when grown. We examined both Spring and Winter radish types for cvs that could provide high ACN concentration. Rapid-growing and quick-maturing Spring cultivars are commonly used for salads, usually small, globally shaped with mild pungency, but a few Spring cultivars have larger and longer roots (Hartmann et al., 1988). All Spring cvs analyzed showed red pigmentation only on the skin, and most were round and small, with exception of cvs Flamboyant Sabina, Jumbo, Long Red and Picolo that had an elongated shape. Later-maturating Winter radish varieties are usually more pungent, larger, and well adapted for storing, with considerable variations in size, color (internal and external) and shape (Hartmann et al., 1988). Winter cvs analyzed included red-skinned radishes (cvs Joyce) and those with red pigmentation in the flesh (white/green epidermal color), all with large size and round shape.

Table 6.1. Monomeric anthocyanin content in red radish cvs (mg ACN/100 g). Source: 1=Daehnfeldt; 2=Nunhems; 3=Burpee; 4=Rogers; 5=Stokes, 6=Known-You; 7=Tokita; 8=Takii; 9=Sakata; 10=Mah Dong King.

	Cultivars	**1994**		**1995**		**1996**		**Average**	
Source	**Spring cvs**	**skin**	**root**	**skin**	**root**	**skin**	**root**	**skin**	**root**
1	Arabic Red	98.7	16.7					98.7	16.7
2	Arista	158	33.3	149	33.3			154	33.3
3	Cherry Bomb	119	25.0					119	25.0
3	Crimson Giant	113	20.9					113	20.9
1	Crimson Giant	122	29.0	146	32.0			134	30.5
1	Danra	122	22.6					122	22.6
3	Easter Egg	62.3	11.6					62.3	11.6
1	Flamboyant Sabina	127	23.7	92.3	16.5			110	20.1
4	Fuego	161	32.0	193	42.6	202	41.9	185	38.8
5	Jumbo	113	22.3					113	22.3
2	Leda	127	24.4					127	24.4
1	Long Red	91.2	25.1	65.7	17.4			78.5	21.3
2	Picolo	42.6	11.1					42.6	11.1
4	Red Baron	163	37.5	179	39.2			171	38.4
4	Red Pak	131	30.2					131	30.2
4	Red Prince	126	29.3					126	29.3
2	Robijn	144	32.0	128	30.3			136	31.2
1	Saxa "Korto"	163	30.7	116	29.5			140	30.1
4	Scarlet Knight	102	21.9					102	21.9
10	Red Harvest #1					39.3	4.7	39.3	4.7
10	Wu Ying Shui					77.8	8.6	77.8	8.6
10	Cherry Radish					91.4	15.3	91.4	15.3
	Winter cvs	**skin**	**root**		**root**		**root**	**skin**	**root**
6	Joyce	52.8	12.2					52.8	12.2
7	Chinese Round Red Cored		48.9		36.0				42.5
8	Chinese Red Meat		42.8		76.9		39.3		53.0
9	Misato Rose Flesh		60.4		28.1				44.3
10	Man Tang Hong						48.7		48.7

The different cvs (Table 6.1, 1994) showed marked differences in monomeric ACN content, ranging from 11.1 to 60.4 mg ACN/100g root. Spring cvs Arabic Red, Easter Egg and Picolo, and Winter cvs Joyce showed the lowest pigment content, < 20 mg ACN/100g root. The highest pigment content per total root weight, > 42 mg ACN/100g root, was found in Winter flesh pigmented cvs. The Red Baron, Arista and Fuego cvs showed the highest pigment content among Spring cvs with monomeric ACN content > 32 mg ACN/100g root.

From the 23 cvs screened, we selected 8 which showed higher pigment content and 3 based on size, shape or growing characteristics: Crimson Giant because of larger size (see Table 6.2), Flamboyant Sabina because of smooth elongated shape, and Long Red because of large and elongated root - for further analyses of pigment content and ACN profile.

Anthocyanin content of different cultivars as related to planting seasons

During the second season (Table 6.1, 1995) cvs were planted at 2 different locations. Results showed good consistency with the previous year results (1994) for Spring cvs, but were more variable on the results obtained for Winter cvs. Lower ACN content was found for Misato Red and Chinese Round Red Cored and higher ACN content for Chinese Red Meat. The ACN content per 100g of epidermal tissue of Spring cvs ranged from 65.7 to 193 mg ACN/100g skin, and the ACN content based on total root weight ranged from 17.4 to 76.9 mg ACN/kg root. The highest pigment content was obtained with Spring cvs Fuego and Winter cvs Red Meat, and the lowest pigment content corresponded to cvs Long Red.

Table 6.2. Comparing skin and root weight and ACN content of red radishes harvested at 2 maturity stages. *Same planting (1994) harvested at 2 maturity stages: 4 and 7 wks after seeding. **Average root weight for 1 kg (small roots) to 3 kg (large roots) samples of radish. ***Winter cv Joyce with pigmentation in the skin only was not included for this average. Source: same as Table 6.1.

Source	Cultivar	4 wks				7 wks			
		root** wt (g)	g skin/ kg root	mg ACN/ 100g skin	**mg ACN/ 100g root**	root** wt (g)	g skin/ kg root	mg ACN/ 100g skin	**mg ACN/ 100g root**
	Spring cvs								
1	Arabic Red	27.5	169.3	98.7	**16.7**	250.0			
2	Arista	22.8	210.9	158	**33.3**	61.1			
3	Cherry Bomb	17.0	209.4	119	**25.0**	69.1			
3	Crimson Giant	34.1	185.3	113	**20.9**	148.2	203.0	106.0	**21.6**
1	Crimson Giant	28.4	238.5	122	**29.0**	204.4	206.4	103	**21.3**
1	Danra	25.0	185.3	122	**22.6**	181.9			
3	Easter Egg	25.0	186.2	62.3	**11.6**	159.1			
1	Flamboyant Sabina	37.3	187.2	127	**23.7**	191.1	132.2	90.3	**11.9**
4	Fuego	19.2	198.6	161	**32.0**	54.0	134.3		
5	Jumbo	26.3	196.8	113	**22.3**	159.2	115.5	117	**13.6**
2	Leda	17.1	192.5	127	**24.4**	143.0	164.6		
1	Long Red	46.4	275.4	91.2	**25.1**	435.0	246.5	53.5	**13.2**
2	Picolo	26.5	260.4	42.6	**11.1**	172.1	234.4	58.0	**13.6**
4	Red Baron	23.8	230.0	163	**37.5**	73.0			
4	Red Pak	18.4	230.6	131	**30.2**	48.6	236.3	96.4	**22.8**
4	Red Prince	21.3	232.4	126	**29.3**	66.2			
2	Robijn	28.6	221.4	144	**32.0**	136.1			
1	Saxa "Korto"	23.8	188.6	163	**30.7**	127.5	210.7	109	**22.9**
4	Scarlet Knight	14.6	215.8	102	**21.9**	64.2			
	Winter cvs								
6	Joyce					71.4	231.3	52.8	**12.2**
7	Chinese Round Red Cored					129.2			**48.9**
8	Chinese Red Meat					125.9			**42.8**
9	Misato Rose Flesh					152.7			**60.4**
	Average of Spring cv.s	**25.4**	**211.3**	**120.3**	**25.2**	**144.4**	**188.4**	**91.7**	**17.6**
	Standard deviation	7.7	28.0	32.2	**7.1**	91.9	48.1	23.6	4.9
	Average of Winter red flesh cv.s***					**135.9**			**50.7**
	Standard deviation					14.6			8.9

A third year planting (Table 6.1, 1996) of Fuego and Red Meat cvs showed good reproducibility for Fuego as compared with the previous years, while high variability was found with cvs Red Meat. During this season, additional Chinese cvs were evaluated. Among those, all Spring cvs had a pigment content much lower than that of cvs Fuego. The Winter cvs Mah Tong Hong showed pigment content higher than that obtained from Chinese Red Meat during the same season.

Comparing the pigment content at two maturity stages

Spring cvs radishes from the first planting were harvested at 2 maturity stages: 4 or 7 wks after seeding (Table 6.2). Changes in radish weight were monitored for all cvs, and changes in pigment content were monitored in 8 cvs randomly selected to evaluate the effects of maturity on radish and pigment yield. All cvs showed an increase in weight when grown for an additional 3 wks (avg. 5.7 times increase), but not all showed the same weight increments with time. The root weight of cvs Arista, Fuego, and Red Pak increased 2.6 - 2.7 times, while cvs Arabic Red and Long Red increased in weight almost 10 times. Cultivars Crimson Giant, Danra, and Leda also showed a large root weight increment during the additional 3 wks in the field, with weights between 7.2 - 8.5 times the original weight.

The skin pigment content did not significantly change (p-value > 0.1) with maturity. The average pigment content / kg root decreased when radishes were left in the field for a longer time, with exception of Crimson Giant and Picolo cvs, where pigment contents slightly increased.

The average pigment content / kg root of Spring cvs was reduced by 30% during this time (Table 6.2). If the average root weight increased 5.7 times and the pigment content / kg root decreased 30%, there would be a 4-fold increase in total pigment from the same number of radishes after allowing them to grow for 3 additional wks. The changes in weight and pigment content observed in the Spring radish cvs suggest that a more efficient pigment yield would result from allowing the roots to grow for a longer period.

Effect of growing location on pigment yield

During the second year Spring (8) and Winter cvs (3) were planted at 2 locations and the monomeric anthocyanin content was evaluated (Table 6.3). Since maturity affected monomeric anthocyanin content, the weight of the radishes was also recorded at time of harvesting. Planting location and weight of radishes had a significant effect on pigment content (p-values < 0.01), which was also dependant on cultivar (p-value < 0.01). Planting location (Corvallis and Hermiston, OR) had a significant effect on total pigment content and the highest amounts of ACN from either Spring or Winter cvs sample were from those harvested at Hermiston. However, the effect of location was more clear for Winter cvs which showed a higher pigment content in those grown at Hermiston. Winter cvs Chinese Red Meat showed monomeric ACN content substantially higher when grown at Hermiston, as compared to Corvallis, and much higher than the other two red-flesh cvs (Table 6.3b). We also observed that pigment content showed high variability within red meat cvs.

Table 6.3. Monomeric anthocyanin content in red radish cvs grown at 2 different locations in Oregon (1995). Corvallis I and II: Two different plantings at the Lewis Brown Horticultural Farm, Corvallis, OR; Hermiston: Grown at the OSU Hermiston Agricultural Research & Extension Center, Hermiston, OR. Source: same as Table 6.1.

a) Anthocyanin content in red radish epidermal tissue (mg ACN/100g skin)

Source	Cultivar	I	II	Hermiston	Average
	Spring cvs				
2	Arista	123	131	195	150
1	Crimson Giant	139	149	151	146
1	Flamboyant Sabina	103	84.6	88.9	92.2
4	Fuego	164	206	210	193
1	Long Red	76.0	56.1	64.9	65.7
4	Red Baron	161	186	192	180
2	Robijn	120	131	133	128
1	Saxa "Korto"	133	119	94.5	116

b) Anthocyanin content in whole red radish roots (mg ACN/100g root)

Source	Cultivar	I	II	Hermiston	Average
	Spring cvs				
2	Arista	31.3	28.6	40.1	33.3
1	Crimson Giant	37.4	30.0	28.7	32.0
1	Flamboyant Sabina	19.7	15.6	14.1	16.5
4	Fuego	40.5	36.0	51.2	42.6
1	Long Red	21.8	16.0	14.3	17.4
4	Red Baron	36.6	31.0	50.0	39.2
2	Robijn	33.5	26.4	30.8	30.3
1	Saxa "Korto"	31.6	23.8	33.0	29.4
	Winter cvs (red flesh)				
7	Chinese Round Red Cored	16.2	30.4	37.8	28.1
8	Chinese Red Meat	37.7	25.1	168	76.9
9	Misato Rose Flesh	26.1	16.0	66.1	36.1

Several factors affecting differences in radish and pigment yields from different locations may include differences in temperature, illumination and soil characteristics. Soil levels of phosphorous have a great effect on root yields of radish (Sanchez et al., 1991). Wilcox and Pfeiffer (1990) determined that temperature had a significant effect on seed germination, growth and root development of radishes, when at 10°C or lower. The two locations evaluated were close geographically but had differences in latitude (44°37' and 45°49', respectively) and elevation (70 and 189 m above sea level, respectively). Typically, the Hermiston location has higher temperatures and more growing days (at 10°C) than the Corvallis area (Oregon Climate Service, 1997). These factors may have been important for higher radish yields from Hermiston as compared with Corvallis. For Summer 1995, Hermiston maximum temperatures during the day (29.7°C) were 3.8 degrees higher and minimum temperatures (13.1°C) 2.4 degrees higher than Corvallis (Oregon Climate Service, 1997). Anthocyanin biosynthesis is a photomorphogenetic phenomenon controlled by phytochromes (Guruprasad and Laloraya, 1980). The effects of light on the light-mediated synthesis of many compounds, like anthocyanins, depends on the intensity and quality of absorbed light (Buschmann and Lichtenthaler, 1982). Differences in number of light hours and temperatures may have caused some of the differences in pigment contents.

Anthocyanin profiles

We used the ACN profile of cvs Fuego as a reference since it had been previously described (Giusti and Wrolstad, 1996a). The ACN profiles were very similar for different red radish cvs, the major pigments being *Pg*-3-soph-5-glu (raphanusin),

mono- or di-acylated with cinnamic acids (ferulic, *p*-coumaric and caffeic acids) and malonic acid (Fig. 6.1).

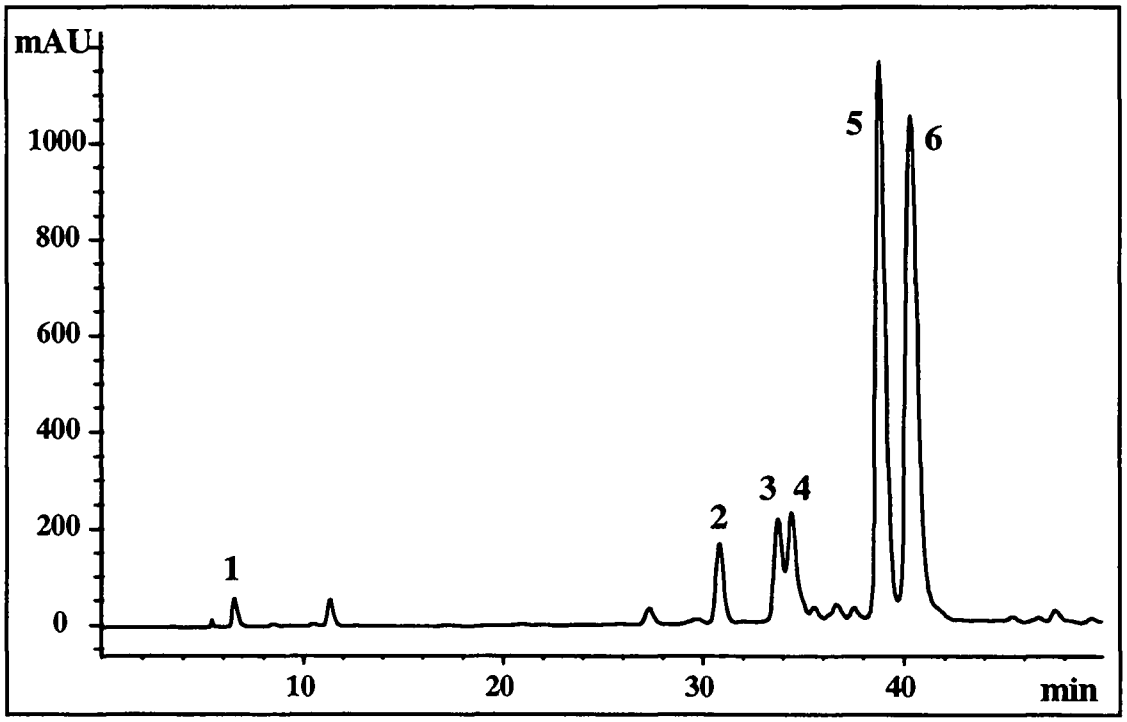

Figure 6.1. HPLC separation of red radish anthocyanins. Polymer Labs PLRP-S, 250 x 4.6 mm i.d. column. Solvent A: 100% acetonitrile, B: 4% phosphoric acid. Linear gradient from 12 to 22% A in 50 min. Flow rate: 1 mL/min. Injection volume: 50 µL. Peak assignments: 1: Pg-3-soph-5-glu; 2: same, acylated with caffeic acid; 3: same as 1, acylated with *p*-coumaric acid; 4: same as 1, acylated with ferulic acid; 5: same as 3, with additional malonic acid acylation; 6: same as 4 with additional malonic acid acylation.

The relative amounts of pigments (Table 6.4) after deacylation with subsequent HPLC analysis of saponified ACN showed that all cvs had the raphanusin structure. The non-acylated raphanusin structure (pigment 1) was present in low levels that ranged from 0.6 to 2.5% of the total pigment area, while acylated ACN (pigments 2-6) represented the major proportion of ACN in all cvs analyzed. The proportions of

mono- and di-acylated ACN (cinnamic acid only, or cinnamic plus malonic acids) were different. For the Spring cvs Arista, Flamboyant Sabina, Fuego, and Saxa'Korto' di-acylated ACN (Pks 5 and 6) represented 60 - 80% of the total ACN content (based on HPLC peak area). Winter cvs showed a lower proportion of these di-acylated ACN, representing 52 - 59% of total pigment. Additional unidentified di- or poly-acylated raphanusin derivatives, represented between 1.8 to 27 % of the total area at 520 nm. Those ACN were present in larger amounts in all Winter cvs and Spring cvs Long Red.

The analysis of phenolic acids from ACN saponification revealed the presence of *p*-coumaric, caffeic and ferulic acids (Table 6.5). The major phenolic acids present as acylating groups in Fuego cvs ACN were *p*-coumaric and ferulic acids, in similar concentrations, and caffeic acid was present in low levels. Flamboyant Sabina and Arista cvs showed a much higher proportion of *p*-coumaric acids as the acylating group. Cultivar Crimson Giant showed the highest amount of caffeic acid (14.8%) as the acylating group, while other cvs showed concentrations between 4.6 and 8.7% of caffeic acid.

Many studies have reported (Francis, 1989; Lu et al., 1992, Dangles et al., 1993; Giusti and Wrolstad, 1996b) that acylation improved color and pigment stability of anthocyanins. Acylation with aromatic acids also caused a bathochromic shift to higher wavelengths of maximum absorbance, which caused differences in perceived color (Dangles et al., 1993). The differences found in the acylation pattern of ACN from the various red radish cvs may have an effect on the color and pigment stability of the extracts when evaluated as potential natural red food colorants.

Table 6.4. Relative anthocyanin composition* of red radish cultivars. * % peak area at 520 nm. 1: Pg-3-soph-5-glu. 2, 3 and 4: same as 1, acylated with caffeic, p-coumaric and ferulic acids, respectively. 5 and 6: same as 3 and 4, with additional malonic acid, respectively. ( ): Standard deviations. Source: same as in Table 6.1.

Source	Cultivar	Pigment						
		1	2	3	4	5	6	Others
	Spring cvs							
2	Arista	0.6	7.2	6.7	2.0	59.7	20.8	3.2
		(0.3)	(0.6)	(3.7)	(1.3)	(7.3)	(2.1)	(4.5)
1	Crimson Giant	1.3	14.8	4.9	5.4	26.2	33.9	13.5
		(0.3)	(0.7)	(0.6)	(0.9)	(3.5)	(0.8)	(3.2)
1	Flamboyant Sabina	0.7	7.0	6.9	2.0	62.5	17.2	3.9
		(0.9)	(2.5)	(4.9)	(0.7)	(7.7)	(0.1)	(3.8)
4	Fuego	0.6	4.6	5.5	5.5	39.1	41.4	3.5
		(0.6)	(0.6)	(0.6)	(2.8)	(2.8)	(1.6)	(2.2)
1	Long Red	1.1	7.4	4.9	3.6	29.4	32.5	21.3
		(0.6)	(2.3)	(0.5)	(1.3)	(6.9)	(5.0)	(7.2)
4	Red Baron	0.9	7.7	7.9	4.8	41.0	31.1	6.7
		(1.0)	(2.5)	(7.4)	(3.5)	(1.6)	(12.7)	(4.9)
2	Robijn	1.0	8.7	7.1	2.1	52.8	25.1	3.5
		(0.8)	(3.7)	(4.9)	(0.4)	(6.9)	(11.4)	(2.2)
1	Saxa "Korto"	0.8	8.0	5.3	3.5	56.7	24.0	1.8
		(0.5)	(2.9)	(2.0)	(3.1)	(1.7)	(2.8)	(1.8)
	Winter cvs							
7	Chinese Red Round Cored	2.1	7.3	9.1	7.0	22.5	33.4	18.7
		(0.3)	(3.3)	(2.3)	(1.3)	(7.6)	(6.5)	(13.6)
8	Chinese Red Meat	2.0	7.7	5.1	5.9	18.3	33.8	27.4
		(0.3)	(0.1)	(0.8)	(2.5)	(0.8)	(5.7)	(8.0)
9	Misato Rose Flesh	2.5	7.0	8.0	7.4	26.8	31.8	16.6
		(0.5)	(0.5)	(1.3)	(2.8)	(1.1)	(2.5)	(0.4)

Table 6.5. Acylating groups* on anthocyanins of red radish cultivars. Expressed as % of total peak area at 520 nm. Source: same as in Table 6.1. ( ): Standard deviation.
* Acylating groups: % of total acylated anthocyanins that are acylated with that acid.
** Acylated Acns: % of total anthocyanins corresponding to acylated anthocyanins

Source	Cultivar	acylated ACN	caffeic acid	p-coumaric acid	ferulic acid	malonic acid
	Spring cvs					
2	Arista	99.6	7.2	66.7	22.9	80.8
		(3.3)	(0.6)	(5.5)	(1.7)	(4.7)
1	Crimson Giant	85.2	17.4	36.5	46.1	70.5
		(1.6)	(0.7)	(2.1)	(0.9)	(2.2)
1	Flamboyant Sabina	95.6	7.3	72.6	20.1	83.4
		(3.3)	(2.5)	(6.3)	(0.4)	(3.9)
4	Fuego	96.1	4.8	46.4	48.8	83.8
		(1.8)	(0.6)	(1.7)	(2.2)	(2.2)
1	Long Red	77.8	9.5	44.1	46.4	79.6
		(3.9)	(2.3)	(3.7)	(3.2)	(6.0)
4	Red Baron	92.5	8.3	52.9	38.8	77.9
		(5.4)	(2.5)	(4.5)	(8.1)	(7.2)
2	Robijn	95.8	9.1	62.5	28.4	81.3
		(4.9)	(3.7)	(5.9)	(5.9)	(9.2)
1	Saxa "Korto"	97.5	8.2	63.6	28.2	82.8
		(2.4)	(2.9)	(1.9)	(3.0)	(2.3)
	Winter cvs					
7	Chinese Red Round Cored	79.3	9.2	39.8	50.9	70.5
		(5.8)	(3.3)	(5.0)	(3.9)	(7.1)
8	Chinese Red Meat	70.8	10.9	33.1	56.1	73.6
		(3.0)	(0.1)	(0.8)	(4.1)	(3.3)
9	Misato Rose Flesh	81.0	8.6	43.0	48.4	72.3
		(1.4)	(0.5)	(1.2)	(2.7)	(1.8)

Red radish ACN yields

The potential of food plants as commercial sources of anthocyanins is generally limited by availability of raw material and economic considerations (Jackman and Smith, 1996). From an economic perspective, the best potential commercial sources of anthocyanins are those from which the pigment is a byproduct of manufacture of other value-added product, e.g. grape and grape skin extract. The availability of highly pigmented low cost crops such as red cabbage makes viable the use of these crops as possible sources for commercial food colorants (Jackman and Smith, 1996). We evaluated the potential yield and availability of radish pigment and compared it to those estimated for red cabbage.

The yield of anthocyanins from red radishes was estimated using average values from experimental and published data (Table 6.6). Yields of radish obtained in the OR experimental stations for radishes harvested 4 or 7 wks (28 and 39.4 MT/ha, respectively) after seeding were considerably higher than those reported for marketable radishes produced in Florida (4.3 MT/ha). This is probably due to the weight increase produced with longer growing times and very low levels of discarded material. Sanchez et al. (1991) reported marketable yields of radishes cvs Red Baron between 6 and 10 MT/ha when using phosphorus fertilization, and losses between 2 and 47%. Radish losses would be much lower when used for pigment production since size and cracks would not be criteria for selection.

Radishes harvested at a commercial stage would yield about 1.4 kg ACN/ha. However, radishes left in the ground for longer time resulted in higher pigment yields. Cultivar Fuego could produce between 9 and 16 kg ACN/ha when they were grown for

4 or 7 wks, respectively. Chinese Red Meat cvs showed an estimated yield of anthocyanin of 7.8 kg ACN/ha. Similar calculations were used to determine the potential pigment yield from a commodity already used for pigment production, red cabbage. Calculations based on data for red cabbage yields in Florida (Florida Agricultural Statistics, 1996), and reported pigment yield (Timberlake, 1988) showed that 14 kg ACN/ha could be obtained. The estimated yields for radishes and red cabbage suggests similar potentials for pigment production.

Table 6.6. Estimated radish pigment yield. *: reported radish yield (Florida Agricultural Statistics, 1996) and experimental pigment content for radish at commercial maturity. **: based on experimental data for row spacing, radish yield, and pigment content. ***: reported cabbage yield (Florida Agricultural Statistics, 1996) and pigment content (Timberlake, 1988)

	Radish				Red
		Fuego		Red Meat	cabbage
	3 wks*	4 wks**	7 wks**	7 wks**	14 wks***
Row area (m^2)		16.7	16.7	50.2	
Rows/ha		598	598	199	
yield (Kg/ha)	4300	28000	39400	19500	56000
Pigment content (mg ACN/100g)	30	32	40	40	25
Pigment yield (kg/ha)	1.3	9.0	15.8	7.8	14.0
Pigment yield (kg/ha-day)	0.06	0.32	0.32	0.16	0.14

The maturing times of these crops should be considered to make a more valid comparison. Typical growing periods for commercial radishes are 3 wks, and we also used 4 and 7 wks to increase pigment yields. Typical growing periods for cabbage are in the order of 12-15 wks, depending on climate and horticultural practices (Hunt and Bortz, 1986). Another way of comparing yields for commodities with different

maturing times is to report yields/ha per day of growing period. For radishes grown for 4 and 7 wks yields on that basis were the same, and more than twice as much as that obtained for red cabbage (Table 6.6). Estimated values would vary depending on different horticultural practices and growing conditions, but pigment production from radishes at a commercial scale should be feasible.

CONCLUSIONS

Radish cvs showed different monomeric ACN contents after accounting for factors such as planting location, maturity stage and root weight. Chinese Red Meat cvs had the highest pigment content (53 mg ACN/100g root). High variability within Winter cvs and between planting locations was found. Cultivar Fuego had the highest pigment content (39 mg ACN/100g root) among Spring cvs. All radish cvs showed similar ACN profiles: pelargonidin-3-sophoroside-5-glucoside with 1 or 2 acylating groups. Differences among pigment profiles of cvs were in the relative amounts at which the different acylating groups were found. Estimation of pigment yields suggest that production of pigment at commercial scale could be viable.

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CHAPTER 7

SPECTRAL CHARACTERISTICS AND MOLAR ABSORPTIVITY OF ACYLATED AND NON ACYLATED PELARGONIDIN DERIVATIVES AND THEIR IMPACT ON COLOR ATTRIBUTES

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ABSTRACT

Acylated and non-acylated pelargonidin derivatives were isolated to compare the effect of glycosylation and acylation on spectral characteristics, molar absorptivity and color attributes. Pigments were obtained from strawberry, radish, potato and from partially hydrolyzed radish pigments. Individual pigments were isolated using semi-preparative HPLC. Spectral and color (CIEL*ch) attributes of purified pigments were measured. Molar absorptivity ranged from 15,600 to 39,590 for pelargonidin-3-glucoside (pg-3-glu) and pg-3-rutinoside-5-glucoside acylated with p-coumaric acid, respectively. The presence of cinnamic acid acylation showed great impact on spectral and color characteristics causing a bathochromic shift on λmax. Sugar substitution also showed an important role, with an hypsochromic shift caused by the presence of glycosylation. Pg-3,5-diglu and pg-3,5-triglu showed higher hue angle (>40°) than the other pg-derivatives when dissolved in buffer pH 1.0, corresponding to the yellow-orange region of the solid of color. Acylation with malonic acid did not show effect on λmax and showed little effect on color characteristics. The solvent system had an effect not only on molar absorptivity, but also on the visual color characteristic of the pigments.

Key words: Pelargonidin-derivatives, acylation, molar absorptivity, color.

INTRODUCTION

Anthocyanins are being considered as potential natural red colorants. Anthocyanin based colorants which are commercially available, e.g. grape skin and red cabbage extracts, exhibit a red-purple hue at pH above 3. Under acidic conditions the

color of non-acylated or mono-acylated anthocyanins is determined largely by the substitutions in the B ring of the aglycon (Jackman and Smith, 1996, Mazza and Miniati, 1993). However, anthocyanins bearing the same chromophore can give rise to different colors, depending on physical and chemical factors (Figueiredo et al., 1996a,b). The presence of additional acylation with cinnamic acids produce a bathochromic shift in the λmax of the pigment, with a slight blueing effect (Jackman and Smith, 1996; Dangles et al., 1993; Harborne, 1967).

Chemical structure of anthocyanins, including sugar substitution, hydroxylation and methoxylation of the B ring, and presence of acylating groups has been correlated with pigment stability. However, not much information is available regarding the effect of sugar substitutions on color characteristics of anthocyanins.

Quantitative determination of anthocyanin content is chiefly used in research and quality control. To evaluate anthocyanin sources is necessary to evaluate pigment content, qualitative anthocyanin composition and the color characteristics they provide. This requires determination of the identity of the pigments as well as their molar absorptivity in the solvent used. Availability of an efficient method for separation of anthocyanins, such as HPLC, combined with a list of absorption coefficients should simplify the quantitative estimation of individual anthocyanins (Francis, 1989). However, through the years there has been lack of uniformity on the values of absorptivity reported, mainly due to the difficulties of preparing crystalline anthocyanin, free from impurities, in sufficient quantities to allow reliable weighing under optimal conditions (Fuleki and Francis, 1968; Francis, 1982).

Our objective was to determine the role of glycosylation and acylation with both cinnamic and organic acids on the molar absorptivity and color characteristics of pelargonidin-based anthocyanins in aqueous or methanolic solutions.

MATERIALS AND METHODS

Sources of pigments

Pelargonidin-3-glucoside (pg-3-glu) was extracted from strawberry. Four acylated Pg-derivatives were obtained from radish: Pelargonidin-3-sophoroside-5-glucoside (pg-3-soph-5-glu, also known as raphanusin) acylated with *p*-coumaric acid, raphanusin acylated with ferulic acid, raphanusin acylated with *p*-coumaric and malonic acid and raphanusin acylated with ferulic and malonic acids. Pelargonidin-3-rutinoside-5-glucoside (pg-3-rut-5-glu) acylated with *p*-coumaric acid was extracted from potato. Other pg-derivatives: pg-3-soph-5-glu, pg-3-soph, pg-3-glu-5-glu, and pg-5-glu, pg aglycon, were obtained from partial hydrolysis of radish anthocyanins. Anthocyanins were extracted from strawberry, radish and red potato using acetone, and partitioned with chloroform as described by Giusti and Wrolstad (1996).

Anthocyanin semi-purification

Semi-purification was carried out using C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the mini-column; sugars, acids and other water soluble compounds were removed with 2 volumes of 0.01% aqueous HCl. Less polar phenolics were removed from the

mini-column by washing with 2 volumes of ethyl acetate and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotovapor at 35°C and pigments were dissolved in distilled deionized water containing 0.01% HCl.

Alkaline and acid hydrolysis of anthocyanins

Approximately 30 mg of purified pigment was hydrolyzed (saponified) in a screw-cap test tube with 10 mL of 10% aqueous KOH for 8 min at room temperature in the dark, as described by Hong and Wrolstad (1990). The solution was neutralized using 2N HCl, and the hydrolysate was purified using semi-preparative HPLC and C-18 Sep-Pak cartridge, as previously described.

Fifteen mL of 2N HCl was added to ca. 1 mg of purified saponified pigment in a screw-cap test tube, flushed with nitrogen and capped. The pigment was hydrolyzed for different times, ranging from 15 to 30 min at 100°C, then cooled in an ice bath. The hydrolysate was purified using a C-18 Sep-Pak cartridge, as previously described. The partially hydrolyzed pigments were separated using semi-preparative HPLC.

Pigment isolation

Semi-purified pigments were isolated using semi-preparative HPLC. Individual pg-derivatives were collected and further purified by passing them through a C-18 Sep-Pak cartridge as previously described. Pigments were recovered from the cartridge with 90% methanol and 10% acidified methanol (0.01% HCl methanol). The methanol was evaporated in a Büchi rotovapor at 35°C and pure methanol added and evaporated again to facilitate the removal of water remaining in the sample. This procedure was

repeated 3 times. The flask containing dried pure anthocyanins was cooled for 1-2 hr in a desiccator in the dark, and the weight recorded.

Pigment isolation was performed in triplicate, but only the extract with the highest purity level was used for further analyses. Purity of isolated pigments was checked using analytical HPLC.

High performance liquid chromatography (HPLC)

Apparatus: A semi-preparative Dynamax Rainin Model SD-300 High Performance Liquid Chromatograph was used, equipped with a Hewlett-Packard 1040A photodiode array detector and a Gateway 2000 P5-90 computer with a Hewlett-Packard HPLC^{2D} ChemStation software. A 1 mL injection loop was used.

Columns and mobile phase. A Microsorb™ C-18 column (5μ), 250 x 21.4 mm i.d. fitted with a 50 x 21.4 mm i.d. guard module (both from Rainin Instrument Co., Inc., Emeryville, CA) was used. The solvents used were A: 100% HPLC grade acetonitrile and B: 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v:v:v) in water. Flow rate: 20 mL/min. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA).

HPLC conditions for anthocyanin separation and isolation

Radish anthocyanins and saponified radish anthocyanins were separated using isocratic conditions at 10% A. The identity of anthocyanins was verified by collecting data at 280, 310, and 520 nm and collecting peak spectra (from 260 to 600 nm) of all peaks at 520 nm.

Molar absorptivity

Purified dried pigments were dissolved in 10 mL of 100% HPLC grade methanol. An exact aliquot of that solution was diluted in methanol containing 0.1% HCl and in pH 1.0 buffer (0.2N KCl), by duplicate, to obtain a final concentrations of 1-5 x 10^{-5} mol/L. Spectral characteristics of known dilutions were recorded on a Shimatzu 300 UV spectrophotometer using 1 cm pathlength quartz cells. For molar absorptivity calculations, the molecular weight used included the weight of a chloride counter ion and a water molecule of hydration.

Color analyses

Color parameters (Hunter CIEL*ch) were obtained with a ColorQuest Hunter colorimeter (HunterLab, Hunter Associates Laboratories Inc., Reston, VA). Solutions containing purified anthocyanins were placed in a 1 cm pathlength optical glass cell (Hellma, Germany) and CIEL*ch values along with percent haze were measured by duplicate in the total transmission mode using Illuminant C and 10° observer angle.

RESULTS AND DISCUSSION

Molar absorptivity and spectral characteristics

The use of semi-preparative HPLC allowed for collection and purification of considerable amounts of individual pelargonidin derivatives, ranging from 8 to 65 mg for pg-derivatives extracted from strawberry, radish and potato. Pigments obtained from the acid hydrolysis of radish anthocyanins were not produced in sufficient amounts to go through all purification steps and obtain reproducible weight

measurements. Therefore, only spectral and color characteristics will be reported for pg-5-glu, pg-3-glu-5-glu and pg-3-soph and no absorptivity coefficients are reported for them. Purity of isolated pigments was checked by analytical HPLC, monitoring the chromatogram at 520, 320 and 280 nm, as well as spectral characteristics. Purity of pigments isolated was higher than 90% as calculated by the % peak area at 280 nm.

The absorptivity is constant for a particular compound and is more commonly expressed as the molar absorptivity at an absorption band maximum (Kemp, 1991). The calculated absorption coefficients (Table 7.1) were expressed as molar absorptivities and as the absorption of a 1% solution ($E^{1\%}$).

The solvent system and the presence of sugars and acylating groups played an important role on the absorption coefficients of pg-derivatives.

EFFECT OF SOLVENT SYSTEM:

Spectral characteristics as well as molar absorptivity were recorded in two different solvent systems. Pigment purity, concentration and pH were exactly the same in the aqueous (pH 1.0 buffer) and alcoholic (0.1% HCl in methanol) solutions for each individual pigment, permitting the analysis of the effect of solvent system without interference of other confounding effects.

The position and intensity of an absorption band may shift when the spectrum is recorded in different solvents. The use of a solvent with increased polarity may cause bathochromic or hypsochromic changes in absorption bands and these changes are dependent on the nature of the chemical group and the molecular orbitals involved (Kemp, 1991).

Table 7.1. Absorptivity Coefficients (L cm^{-1} mol^{-1}) of different pelargonidin derivatives

Anthocyanin	Weight (mg)	Conc. mg/L	Conc. (mol/L)x10^{-5}	MW	Lambda max		Molar Absorptivity		$E_{1\%}$	
					MeOH	Buffer	MeOH	Buffer	MeOH	Buffer
Pg aglycon	10.2	2.5	0.77	325.3	524	505	19785	18423	608	566
Pg-3-glu	21.2	8.56	1.76	487	508	496	17330	15600	356	320
Pg-3-soph-5-glu	24.2	24.2	2.98	810.5	506	497	30695	25370	379	313
Pg-3-soph-5-glu + p-coumaric	8.3	16.6	1.74	956.5	508	506	34889	28724	365	300
Pg-3-soph-5-glu + ferulic	18.1	27.2	2.76	986.5	507	506	29636	24137	300	245
Pg-3-soph-5-glu + p-coumaric & malonic	64.1	25.6	2.46	1042.5	508	508	39785	33015	382	317
Pg-3-soph-5-glu + ferulic & malonic	17.5	35	3.26	1072.5	508	508	39384	31088	367	290
Pg-3-rut-5-glu + p-coumaric	38.5	15.4	1.64	940.9	511	504	39591	32076	421	341

Absorption coefficients obtained for pg-derivatives were consistently higher in acidified methanol than in the pH 1.0 buffer solutions, in the order of 20% higher in all cases except for the aglycon where the difference was only 7%.

Solvent effects were dependant on the pigment chemical structure. Non-acylated pigments showed the highest solvent effect on spectral characteristics, with a drastic shift not only on the dimension of the molar absorptivity but also with a marked change in the λmax. In acidified methanol, absorption at the 490-520 nm band was substantially higher than in aqueous solution. Methanol also caused a bathochromic shift of about 10 nm in the λmax as compared to the same pigment in buffer solution (Fig. 7.1).

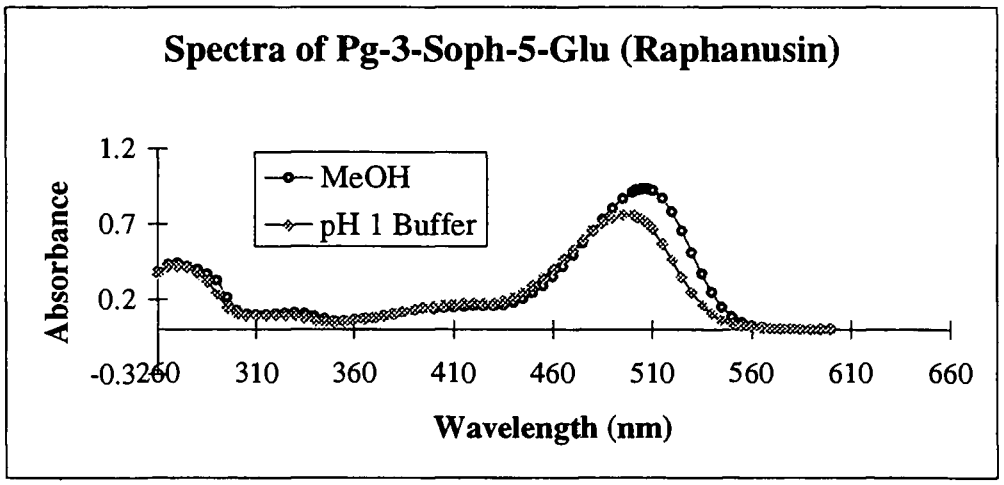

Figure 7.1. UV-visible spectra of pg-3-soph-5-glu in methanolic (0.1% HCl in MeOH) and aqueous solutions (pH 1.0 buffer).

All acylated pigments showed increased absorptivity in all major absorption bands, the 280, 320 and 500 nm regions (Fig. 7.2), when they were dissolved in acidified methanol. However, a decrease in absorption was observed in the 400-440 nm

region, decreasing the $A_{400-440}/A_{max}$ ratio. Only a small bathochromic shift was observed on the 280 nm band due to the solvent system. No shifts were observed on the λmax on the other bands and this was consistent for all acylated derivatives (Table 7.1 and Fig. 7.2).

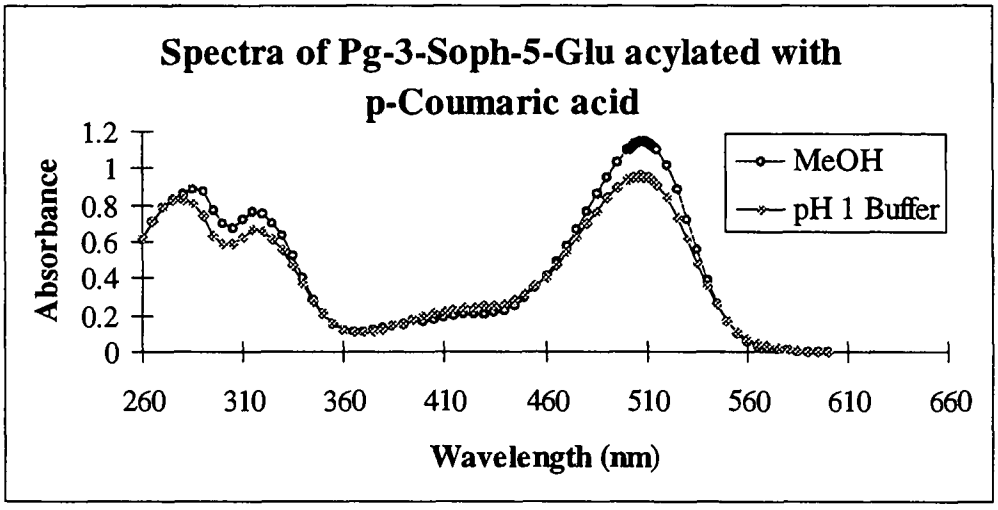

Figure 7.2. UV-visible spectra of pg-3-soph-5-glu acylated with p-coumaric and malonic acids in methanolic (0.1% HCl in MeOH) and aqueous solutions (pH 1.0 buffer).

The effects of solvent system on anthocyanins will determine the quaternary structure of the molecule, and it is believed to have a strong impact on the color of the primary, secondary or tertiary structures (Brouillard and Dangles, 1993).

EFFECT OF GLYCOSYLATION AND ACYLATION:

The lowest molar absorptivity coefficients (Table 7.1) obtained corresponded to the pg aglycon and pg-3-glu, with values for methanol and buffer systems that were almost half of those obtained with the other pg-derivatives. However, it is important to highlight that the absorptivity of the aglycon was almost twice as much as that of the

other pigments when they were compared on a weight basis instead of in a molar one. Other molar absorptivity coefficient values reported in the literature are presented on Table 7.2.

Glycosylation at the 3 and 5 positions of the anthocyanidin molecule has an important impact on the spectral characteristics and extensive research has been published in that area (Harborne, 1967, Hong and Wrolstad, 1990, Giusti and Wrolstad, 1996). Anthocyanins with glycosidic substitutions at position 3 only, exhibit a ratio of absorbance at 400-440 nm to the absorbance at the maximum visible wavelength that is almost twice as much as that of anthocyanins with glycosidic substitution at position 5 or both, positions 3 and 5 (Table 7.3).

Inconsistent information is available with respect to the role of sugars on anthocyanin absorptivity and color characteristics. The nature of the glycosyl group has been reported (Jackman and Smith, 1996) as having no apparent influence in anthocyanin coloration, although increased glycosyl substitution and/or substitution at the C-7 hydroxyl is believed to increase color intensity. It has also been reported (Figueiredo et al., 1996a and 1996b, Elhabiri et al., 1995) that molecules possessing a disaccharide as a substituent group in position 3 of the chromophore exhibit a large drop in their absorptivity. Cyanidin-3,5-triglycoside, delphinidin-3-gentiobioside and cyanidin-3-rutinoside showed a marked hypochromic effect when compared to the corresponding 3-glucosides and 3,5-diglucosides with similar substitution pattern (Figueiredo et al. 1996a and 1996b and Elhabiri et al., 1995).

Table 7.2. Reported molar absorptivity of anthocyanins

Anthocyanin	MW	Solvent system	Lambda max (nm)	E mol	Reference
Pg	324.5 *	0.1% HCl in EtOH	504.5	17800	Schou, 1927
Pg-3-glu	486.5 *	1% HCl in H2O	496	27300	Jorgensen et al., 1955
	433			36600	Wrolstad et al., 1970
		1% HCl	513	22390	Swain, 1965
		1% HCl in MeOH	516	31620	Swain, 1965
Pg-3,5-diglu		HCl in MeOH	510	32360	Swain, 1965
Pg-3-soph-5-glu		aqueous pH 0.8	498	18000-20000	Dangles et al., 1993
Caffeoyl derivatives of Pg-3-soph-5-glu		aqueous pH 0.8	498	18000-20000	Dangles et al., 1993
Pg-3-(dicaffeoylglu)-soph-5-glu		aqueous pH 0.8	512	28000	Dangles et al., 1993
Cyd	340.5 *	0.1% HCl in EtOH	510.5	24600	Schou, 1927
	340.5 *	0.1% HCl in EtOH	547	34700	Ribereau-Gayon, 1959
Cyd-3-glu	449	0.1N HCl	520	25740	McClure, 1967
	502.5 *	1% HCl in MeOH	530	34300	Siegelman et al., 1958
Cyd-3-gal	502.5 *	0.1% HCl in MeOH	530	34300	Siegelman et al., 1958
	502.5 *	0.1N HCl : EtOH (15:85)	535	44900	Sakamura and Francis, 1961
	502.5 *	0.1N HCl : EtOH (15:85)	535	46200	Zapsalis and Francis, 1965
	502.5 *	0.1N HCl : EtOH (15:85)	535	46230	Fuleki and Francis, 1968
		HCl in MeOH	530	30200	Swain, 1965
Cyd-3-ara	472.5 *	0.1N HCl : EtOH (15:85)	538	44400	Zapsalis and Francis, 1965
	472.5 *	0.1N HCl : EtOH (15:85)	535	44460	Fuleki and Francis, 1968
Cyd-3,5-diglu		0.1N HCl	520	30175	Niketic-Aleksic and Hrazdina, 1972
		Methanolic HCl	508.5	35000	Brouillard and El Hague Chahine, 1980
Cyd-3-rut		aqueous pH 0.9	510	7000	Figueiredo et al., 1996a
		1% HCl	523	28840	Swain, 1965
Cyd-3-soph-5-glu		Methanolic HCl	524	37150	Hrazdina et al., 1977
Cyd-3-soph-5-glu + malonic		Methanolic HCl	528	32360	Hrazdina et al., 1977
Cyd-3-soph-5-glu + sinapic		Methanolic HCl	528	37150	Hrazdina et al., 1977
Cyd-3-soph-5-glu + di-sinapic		Methanolic HCl	530	38020	Hrazdina et al., 1977
Cyd-3-soph-5-glu + ferulic		Methanolic HCl	528	32360	Hrazdina et al., 1977
Cyd-3-soph-5-glu + di-ferulic		Methanolic HCl	530	34670	Hrazdina et al., 1977

Continued.....

Anthocyanin	MW	Solvent system	Lambda max (nm)	E mol	Reference
Cyd-3-soph-5-glu + p-coumaric		Methanolic HCl	526	38020	Hrazdina et al., 1977
Cyd-3-soph-5-glu + di-p-coumaric		Methanolic HCl	528	32360	Hrazdina et al., 1977
Cyd-3-sam-5-glu		aqueous pH 0.9	522	3600	Figueiredo et al., 1996a
Cyd-3-sam-5-glu + sinapic + ferulic		aqueous pH 0.9	528	15100	Figueiredo et al., 1996a
Cyd-3-sam-5-glu + sinapic + p-coum + malonic		aqueous pH 0.9	536	19000	Figueiredo et al., 1996a
Cyd-3-sam-5-glu + sinapic + caffeic + malonic		aqueous pH 0.9	538	21200	Figueiredo et al., 1996a
Cyd-3-sam-5-glu + sinapic + ferulic + malonic		aqueous pH 0.9	538	20100	Figueiredo et al., 1996a
Pnd	354.5 *	0.1% HCl in EtOH	511	37200	Schou, 1927
	354.5 *	0.1N HCl : EtOH (15:85)	532	40800	Sakamura and Francis, 1961
Pnd-3-glu	516.5 *	0.1% HCl in MeOH	536	11300	Somers, 1966
Pnd-3-gal	516.5 *	0.1N HCl : EtOH (15:85)	532	48400	Sakamura and Francis, 1961
	516.5 *	0.1N HCl : EtOH (15:85)	532	48400	Zapsalis and Francis, 1965
	516.5 *	0.1N HCl : EtOH (15:85)	531	48340	Fuleki and Francis, 1968
Pnd-3-ara	486.5 *	0.1N HCl : EtOH (15:85)	532	46100	Zapsalis and Francis, 1965
	486.5 *	0.1N HCl : EtOH (15:85)	532	46070	Fuleki and Francis, 1968
Pnd-3,5-diglu		0.1N HCl	520	36654	Niketic-Aleksic and Hrazdina, 1972
Dpd	356.5 *	0.1% HCl in EtOH	522.5	34700	Schou, 1927
Dpd-3-glu	518.5 *	1% HCl in MeOH	543	29000	Asen et al., 1959
Ptd-3-glu	532.5 *	0.1% HCl in MeOH	546	12900	Somers, 1966
Ptd-3,5-diglu		0.1N HCl	520	33040	Niketic-Aleksic and Hrazdina, 1972
		HCl in MeOH	535	23440	Swain, 1965
Mvd	400.5 *	0.1% HCl in EtOH	520	37200	Schou, 1927
	400.5 *	0.1% HCl in EtOH	557	36200	Ribereau-Gayon, 1959
Mvd-3-glu	562.5 *	0.1% HCl in MeOH	546	13900	Somers, 1966
	562.5 *	0.1% HCl in MeOH	538	29500	Koeppen et al., 1966
	529	0.1N HCl	520	28000	Niketic-Aleksic and Hrazdina, 1972
	529	Methanol pH 1.0	535	36400	Metivier et al., 1980
Mv-3,5-diglu	724.5 *	0.1% HCl in EtOH	519	10700	Schou, 1927
	724.5 *	0.1% HCl in EtOH	545	10300	Ribereau-Gayon, 1959
		0.1N HCl	520	37700	Niketic-Aleksic and Hrazdina, 1972
Mvd-3-glu + p-coum	718.5 *	0.1% HCl in MeOH	536	30200	Koeppen et al., 1966

Table 7.3. Color attributes of purified non-acylated pg-derivatives

Anthocyanin	lambda max	lambda max 400-440	A440-440/ Amax	L*	hue	chroma	haze
Pg aglycon	513	423	0.38	81.43	18.84	35.68	0.88
Pg-5-glu	509	407	0.18	83.57	26.83	35.85	1.22
Pg-3-glu	503	429	0.45	83.60	37.97	34.46	0.92
Pg-3-soph	503	427	0.46	82.93	37.37	35.82	1.06
Pg-3-glu-5-glu	500	417	0.2	85.08	41.26	35.41	1.03
Pg-3-soph-5-glu	500	417	0.22	85.03	40.72	35.29	1.02

In this study, we found a slight drop in molar absorptivity when one sugar was present, as compared to the aglycon. The addition of more glucose units to the molecule seemed to have a hyperchromic effect of the pigment, since pg-3-soph-5-glu showed a molar absorptivity substantially higher than the corresponding monoglucoside.

When comparing the values obtained with other values reported in the literature (Table 7.2) we must consider that most of the values available dated from the 70's or before, and that the analytical techniques have advanced throughout the years. Difficulties of preparing crystalline anthocyanin, free from impurities, in sufficient quantities to allow reliable weighing under optimal conditions (Fuleki and Francis, 1968, Francis, 1982) have limited the consistency of the reported data. The more common effect of the presence of impurities in the sample would be an underestimation of the absorptivity coefficient due to the presence of compounds that contribute to the weight but not to the color. Impurities, however, may also have an hyperchromic effect on anthocyanins due to copigmentation of anthocyanins with other non-colored phenolic compounds (Lenoble et al., 1996, Mazza and Brouillard, 1990, Goto and

Kondo, 1991), causing an overestimation of the absorptivity coefficients. It is interesting that most of the values reported more recently (Brouillard and El Hache Chahine, 1980; Dangles et al., 1993; Figueiredo et al., 1996a) are more conservative (35000 or lower) than many of the values reported in the 60's, > 40,000. The values we report for pg aglycon are very comparable to those reported by Schou in the late 20's (Table 7.2). However, the absorptivity coefficients obtained for pg-3-glu were lower than values previously reported (Jorgensen et al., 1955; Swain, 1965), while the molar absorptivity of pg-3-soph-5-glu in aqueous solution (25,370) was higher than the previously reported (Dangles et al., 1993).

It has been reported as a general characteristic that an increase in the number of substitutions is accompanied by an increase in the molar absorptivity of the flavylium cation form when compared to their parent mono- and di-glucosides (Figueiredo et al., 1996a). Our results showed a general hyperchromic effect of acylation of pg-derivatives as compared to the parent pg-3-soph-5-glu, in particular, when 2 acylating groups were present. In the case of pg-3-rut-5-glu acylated with p-coumaric acid, we also obtained a relatively large molar absorptivity (Table 7.1), but the molar absorptivity of the parent non-acylated compund was not calculated. When comparing the effect of the different acylating groups, p-coumaric and malonic acids seemed to contribute to a greater extent to the absorptivity in the visible band than ferulic acid. The effect of malonic acid addition on spectral characteristic is shown in Fig. 7.3. The absorbance was normalized at the 280 nm band to compare the effect of malonic acid acylation on the visible range. No shift in the wavelength of maximum absorbance was observed as a result of the presence of malonic acid, the main spectral difference being

the higher absorption of the major visible band. A similar effect was obtained with additional acylation with malonic acid on the ferulic acid acylated pg-derivative.

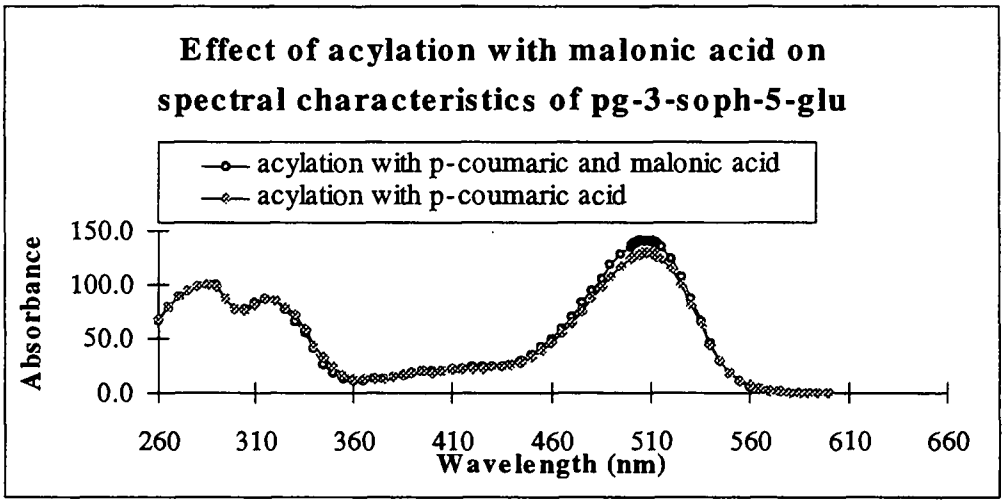

Figure 7.3. Effect of acylation with malonic acid on spectral characteristics of acylated pg-3-soph-5-glu.

Color characteristics

For comparison of color characteristics of the same pigment in different solvent, the weight and molar concentrations were exactly the same. However, when making comparisons among the different pigments we need to consider that they were prepared under different concentrations. Color of non-acylated pg-derivatives presented in Table 7.3 use a uniform level of chroma since no quantitation of those pigments was possible due to the low amounts available. Color characteristics presented in Table 4 use similar molar concentrations, between 1.5 x 10^{-5} to 2.7 x 10^{-5} mol/L, which correspond to concentrations ranging from 5 to 29 mg/L. An additional data point is included for a higher pigment concentration of pg-3-soph-5-glu only to illustrate the effect of pigment concentration on color characteristics. It is also important to mention that extensive work is available on spectral characteristics of anthocyanins and color inferences have

been made based on these characteristics. However, little has been reported on how color parameters correlate to those characteristics.

EFFECT OF SOLVENT SYSTEM:

The solvent system had a clear impact on color characteristics of all pg-derivatives. In most cases the effect of the methanolic solution was not only hyperchromic, with a higher chroma, but also produced lower hue values (Table 7.4). Solvent effect was more marked on non-acylated pigments and on more diluted solutions. No sensory analysis was performed, but visual observations were recorded, and the differences registered by the ColorQuest on non-acylated pg-derivatives were clearly evidenced on the visual appearance of the solutions. We also found solvent effects on the color characteristics of acylated pigments (Table 7.2), but were not large enough to produce noticeable visual differences.

EFFECT OF GLYCOSYLATION AND ACYLATION:

For comparisons of the effect of glycosidic substitution on color of pg-derivatives we used dilutions of the different pigments at the same level of chroma. These pigments were obtained from partial hydrolysis of acylated pg-3-soph-5-glu, and due to the limited amounts purified they were only analyzed in one solvent, the mobile phase from HPLC (15% acetonitrile, 10% acetic acid, and 1% phosphoric acid in water).

Table 7.3 shows the increasing trend in hue obtained with increasing number of glucose units. The aglycon was the pigment with the lowest hue, in the red region. Position of glycosylation also showed impact on hue, when compared at the same level

Table 7.4. Color attributes of purified pg-derivatives

Anthocyanin	Conc. mg/L	Conc. (mol/L)x10^{-5}	MW	MeOH L*	MeOH hue	MeOH chroma	Buffer L*	Buffer hue	Buffer chroma
Pg aglycon	5.0	1.54	271.8	87.09	357.25	22.14	89.99	22.67	16.68
Pg-3-glu	8.56	1.76	433.2	88.11	17.62	20.15	90.27	44.01	17.63
Pg-3-soph-5-glu	15	1.85	757	91.76	15.47	13.5	89.95	41.02	20.34
	24.2	2.98	757	78.36	39.2	53.41	81.8	55.97	53.50
Pg-3-soph-5-glu + p-coumaric	16.6	1.73	903	85.32	16.36	30.27	86.38	23.32	26.8
Pg-3-soph-5-glu + ferulic	27.2	2.76	933	82.82	19.48	36.45	83.37	24.13	33.71
Pg-3-soph-5-glu + p-coumaric & malonic	25.6	2.46	989	83.43	18.29	35.35	86.78	21.65	25.88
Pg-3-soph-5-glu + ferulic & malonic	29	2.70	1019	82.63	20.45	36.99	83.06	22.12	33.85
Pg-3-rut-5-glu + p-coumaric	18.3	1.94	887.4	87.22	11.01	25.7	87.26	23.05	24.78

of chroma: pg-3-glu and pg-3-di-glu (pg-3-soph) showed lower hue than the corresponding pg-5-glu and pg-3,5-di-glu, respectively. Pg-derivatives with glycosidic substitution at both, the 3 and 5 positions of the pyrylium ring showed the highest hue level, with yellowish appearance.

The ability of flavonoids to associate with numerous biological molecules is of theoretical and practical importance. Anthocyanins are know to form molecular complexes with other anthocyanins (self-association), with other colorless compounds (intermolecular copigmentation) and, in the case of acylated anthocyanins, intramolecular copigmentation (Brouillard and Dangles, 1994). Anthocyanin copigmentation accounts for the diversity of colors that are produced by anthocyanin-containing solutions as well as for color stabilization. Anthocyanin-3-glucosides have been reported to exhibit weaker self-association as compared to the 3,5-diglucosides, pointing out the importance of the residue at 5-position in self-association. Hoshino and coworkers (1981) reported bathochromic and hypsochromic shifts in the visible absorption band with various anthocyanins, and suggested vertical stacking in a right or left handed screw axis.

A comparison between pg-3-soph-5-glu acylated with p-coumaric acid and pg-3-rut-5-glu acylated also with p-coumaric acid illustrates the effect of the nature of the glycosidic substitution and position of acylation. Both pigments are triglycosylated, with a disaccharide at position 3 of the pyrylium ring, and both are acylated with p-coumaric acid. However, the difference in the nature of the sugar (sophorose vs rutinose) and a difference in the position where the cinnamic acid is attached to the sugar, are enough to impart different color characteristics to the molecule. Pg-3-rut-5-

glu showed lower chroma in both solvent systems, even though the molar absorptivity was higher. It also showed lower hue in methanolic solution.

The bathochromic shift caused by cinnamic acid acylation translated to higher chroma for a similar molar concentration of pigment (Table 7.4) in the two different solvent systems evaluated. Hue was clearly decreased by acylation in the aqueous buffer solutions, going from the orange-yellow hue to a more reddish color, consistent with the bathochromic shift at the visible band. Lightness of the samples decreased with addition of cinnamic acid acylation to the anthocyanin pigment, also consistent with the increased absorptivity obtained. The addition of a malonic acid acylation had little effect in chroma and lightness. Malonic acid acylation, however, showed an opposite effect on hue in the different solvent systems, increasing the hue in the methanolic solution and decreasing it in the aqueous buffer solution which results in closer hue values between the two solvent systems. Therefore, pigments acylated with malonic acid did not show much of a solvent effect on color attributes.

Several studies (Brouillard, 1981; Goto, 1987; Goto and Kondo, 1991; Figueiredo et al., 1996a and 1996b) have suggested that the intramolecular copigmentation or interaction within anthocyanins may play an important role in the increased stability of acylated anthocyanins (Fig. 7.4). The flexible saccharide chains can act as linkers allowing the folding of the acyl aromatic rings over the planar pyrylium ring Formation of a sandwich type complex has been proposed for anthocyanins with two cinnamic acid acylating groups. This stacking phenomenon excerpts a protective effect on anthocyanins but also contributes to the color stabilization of the system. Cinnamic acid residues stack parallel with the

anthocyanidin nucleus protecting the chromophore against water nucleophillic attack (Brouillard and Dangles, 1994).

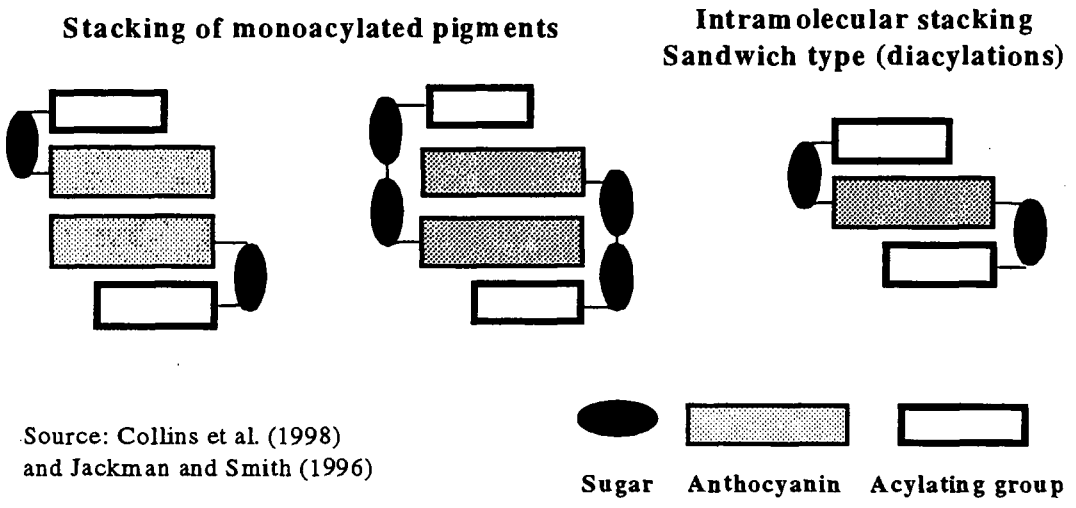

Figure 7.4. Stabilization mechanisms of anthocyanins.

Copigmentation has also been reported to have an impact on the anthocyanin spectral characteristics (Mazza and Brouillard, 1990) causing a hyperchromic and bathochromic effect, increasing the color intensity as well as the wavelength of maximum absorbance of the pigment. No clear mechanism has been proposed for the intramolecular association of aliphatic acid acylation, but the zwitterionic character of the pigments would make likely an ionic attraction between the positively charged portion of the oxonium form, and the negatively charged free carboxylic group from malonic acid

CONCLUSIONS

Small differences in anthocyanin chemical structure may have a critical impact on color and tinctorial strength of anthocyanin extracts. The nature and position of the glycosylation and acylation played a role in spectral and color characteristics. Findings also showed that the environment where anthocyanins are dissolved need to be considered when quantifying and characterizing anthocyanins.

LIST OF ABBREVIATIONS

pg : pelargonidin

cyd : cyanidin

glu : glucoside

rut : rutinoside

soph : sophoroside

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CHAPTER 8. SUMMARY

Eight anthocyanins were separated and characterized from red radish. HPLC and UV-visible spectral analyses of radish anthocyanins confirmed previous identifications of the pigments as Pg-3-soph-5-glu derivatives. Two novel zwitterionic anthocyanins di-acylated with a cinnamic and a malonic acid were found by MS and their molecular structure and configuration elucidated using 1-D and 2-D NMR techniques. Folding of the molecule was evidenced by correlation between the cinnamic acid acylating group and the anthocyanidin moiety as revealed by NOESY techniques. Intramolecular copigmentation may play an important role in the high pigment stability previously reported for these pigments.

Our findings showed electrospray and tandem mass spectrometry (ES-MS and MS-MS) as powerful and rapid techniques for screening and characterization of anthocyanins in samples where some background information is available, or where a first compositional overview is required. ES-MS required minimal sample preparation, and was fast and effective detecting molecular ions of anthocyanins and anthocyanin extracts. Tandem mass spectrometry showed extraordinary capabilities for the structural analysis of individual anthocyanins of complex mixtures. In combination with HPLC, ES-MS and MS-MS could be a useful tool for monitoring the authenticity of anthocyanin-containing fruit juices and vegetable extracts.

Comparison of the quantitative and qualitative anthocyanin pigment composition of 27 red radish cultivars showed differences in monomeric anthocyanin content after accounting for factors such as planting location, maturity stage and root

weight. Chinese Red Meat cvs had the highest pigment content (53 mg ACN/100g root). High variability within Winter cvs and between planting locations was found. Cultivar Fuego had the highest pigment content (39 mg ACN/100g root) among Spring cvs. All radish cvs showed similar ACN profiles: pelargonidin-3-sophoroside-5-glucoside with 1 or 2 acylating groups. Differences among pigment profiles of cvs were in the relative amounts at which the different acylating groups were found. Estimation of pigment yields suggest that production of pigment at commercial scale could be viable.

Small differences in anthocyanin chemical structure may have a critical impact on color and tinctorial strength of anthocyanin extracts. The nature and position of the glycosylation and acylation played a role in spectral and color characteristics. Findings also showed that the environment where anthocyanins are dissolved need to be considered when quantifying and characterizing anthocyanins.

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