Isolation and Identification of Anthocyanins

from *Nesaea Crassicaulis*

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August 2008
Preface

Anthocyanins in *Nesaea Crassicaulis* have been deduced to play a role in protection against photoinhibition by Nielsens’ group in Roskilde University (Nielsen S.L. & Nielsen H.D., 2006). This project is part of that *Nesaea Crassicaulis* analysis, including isolation, identification of anthocyanins and test of their antioxidative properties.

This report is divided into three main parts: Introduction, Problem formulation and Experimental part. Introduction gives the background knowledge about anthocyanins and the plant *Nesaea Crassicaulis*; Experimental part consists of four topics: plant growth; isolation and primary identification of anthocyanins, 1D and 2D NMR (Nuclear magnetic resonance spectroscopy) and evaluation of antioxidative property of anthocyanins based on electrochemical detection.

The project took six months (from Feb. 2008 to Jul. 2008) with the help of many people in our group: Professor Søren Laurentius Nielsen, Professor Torben Lund and the technicians Rita Buch, Annette Christensen, Jacob Krake. I deeply appreciate their kind participation. Finally I wish to dedicate this report to my dear supervisor- Poul Erik Hansen, who provides me careful and outstanding instructions, and continuing encouragement and engagement during this study.

Jing Wang
Aug. 1, 2008
Abstract

Anthocyanins were extracted from leaves of Nesaea Crassicaulis by 2.5% HCOOH in acetone, pre-purified by reverse-phase C18 columns and cation exchange columns, and then fractionized by semi-preparative HPLC (high performance liquid chromatography). The molecular structures were identified using LC-MS (liquid chromatography-mass spectrometry) and 1D and 2D NMR (nuclear magnetic resonance spectroscopy). Seven anthocyanins were identified. Four of them were assigned to be delphinidin-3,5-di-β-D-glucopyranose (M.W.=627), cyanidin-3,5-di-β-D-glucopyranose (M.W.=611), peonidin-3,5-di-β-D-glucopyranose (M.W.=625) and cyanidin-3-β-D-glucopyranose (M.W.=449). Three of them were deduced to be Peonidin-3-β-D-glucopyranose (M.W.=463), Petunidin-3,5-di-β-D-glucopyranose (M.W.=641) and Malvidin-3,5-di-β-D-glucopyranose (M.W.=655). Cyanidin-3,5-di-β-D-glucopyranose was the main anthocyanin, which took up about 50% of the total amount of anthocyanins in Nesaea Crassicaulis.

The electrochemical properties of the former five anthocyanins (627, 611, 625, 449 and 463) were analyzed by electrochemical detector (ECD). All of them were oxidized in several steps at different oxidation potentials. (627), (611) and (449) had the same first oxidation potential 450mV (150mV vs ferrocene). (463) and (625) were more resistant to oxidation and had a first oxidation potential at 700mV (400mV vs ferrocene). (463) and (449) which had one glucose less than the others underwent gradual oxidation after the first oxidation potential and had no apparent second oxidation potential.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANS</td>
<td>anthocyanidin synthase</td>
</tr>
<tr>
<td>C4H</td>
<td>cinnamate 4-hydroxylase</td>
</tr>
<tr>
<td>CHI</td>
<td>chalcone isomerase</td>
</tr>
<tr>
<td>4CL</td>
<td>4-coumaroyl:CoA ligase</td>
</tr>
<tr>
<td>CHS</td>
<td>chalcone synthase</td>
</tr>
<tr>
<td>DFR</td>
<td>dihydroflavonol 4-reductase</td>
</tr>
<tr>
<td>ECD</td>
<td>Electrochemical detector</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>F3H</td>
<td>flavanone 3-hydroxylase</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilic-Lipophilic-Balanced reverse-phase column</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear correlation through Multiple Quantum Coherence</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ID</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MCX</td>
<td>Mixed-Mode Cation-eXchang reversed-phase column:</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>M.W.</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PAL</td>
<td>phenylalanine ammonia-lyase</td>
</tr>
<tr>
<td>TFA</td>
<td>Tri-fluro acidic acid</td>
</tr>
<tr>
<td>UFGT</td>
<td>UDP glucose-flavonoid 3-O-glucosyl transferase</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultraviolet ray/visible light</td>
</tr>
<tr>
<td>1D</td>
<td>one dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
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1. Introduction

1.1. Nesaea crassicaulis

Nesaea Crassicaulis are beautiful and widely used aquarium plants. They belong to the Lythraceae family and originally come from Africa. Enough sunlight and very soft media are necessary for their growth. Even though they need very strict growth conditions, they are very easy to propagate by site shoots or cuttings1.

In different growth stages they can have green or red colored thin leaves (fig.1.1). Since anthocyanins are the basis of red to blue colors of plants (Davies K. et al., 4, 2004), the red leaves may result from accumulation of anthocyanins. And Nesaea Crassicaulis have been reported to produce high concentrations of anthocyanins when they are grown submerged with high CO2 availability (Nielsen S.L. & Nielsen H.D., 2006). But the dissolved CO2 in the aquaria can decrease the water pH to be acidic in the following equilibriums:

\[ \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \quad \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \quad \text{HCO}_3^- \rightleftharpoons \text{H}^+ + \text{CO}_3^{2-} \]

So if extra CO2 is pumped to the aquaria, the CO2 flow should be adjusted by a pH controlling system in order to maintain a permitted pH range (from 5.5-8.5).

1 www.tropica.com
1.2. Background of anthocyanins

1.2.1. Anthocyanin Distribution

Anthocyanins together with other pigments (chlorophylls, carotenoids, betalains etc.) distribute widely in the plant kingdom, giving the plants various and attractive colors (Davies et al. Chap4/ Stintzing et al. 2006).

Different kinds of plants have been analyzed for their anthocyanins, including fruits (various berries etc.), flowers (roses etc.), vegetables (pigmented potatoes, onions etc.) and even grasses (Fossen et al., 2002). Generally speaking, fruits and vegetables have relatively simpler anthocyanins than flowers (Cabrita et al. 2000). The latter one also have more complicated color variety: yellow to dark red roses, blue Limonium Sinuata, black Datura and so on. Even for a single flower, the anthocyanin distribution is not the same in anthers, pollens, perianthes etc. (Nakayama et al. 1999/2004).

![Fig.1.2 Indication of different anthocyanin distribution in a flower (M. Nakayama et al. 2004)](image)

A: Flower ‘Ben van Zanten’; B: a-perianth, b-perianth bottom, c-anther and pollen, d-pistil

In a given plant, the distribution of anthocyanins varies according to different growth stages. It could be easily found in the garden that many young leaves are more deeply colored than the older ones, which might indicate the protection role of anthocyanins for the small plants when they are not strong enough. It has been proved in Basil that the less acylated forms of anthocyanins begin to appear as plants mature (Phippen & Simon, 1998).

So, analysis of anthocyanin distribution can be helpful in elucidating functions of
different anthocyanins as well as some important biological pathway in plant growth, such as photosynthesis and photoinhibition (Nielsen & Nielsen 2006).

1.2.2. Structure

About 600 naturally occurring anthocyanins have been reported (Torskangerpoll & Andersen 2005/Jordheim et al., 2007). Anthocyanidin (aglycone), sugar and acyl groups are the three basic constituents of anthocyanins, which produce different anthocyanins by different constitution.

1.2.2.1. Aglycone

Figure 1.3 shows two kinds of elementary structures of the aglycones: the one on the left is quite common among anthocyanins, while the one on the right is very rare, named pyranoanthocyanins (Andersen & Markham, chap.10). The aglycones vary based on different substitutions on the aromatic rings by hydroxyl, methyl and probably acyl groups. Even though many kinds of anthocyanidins have been identified, around 90% of all anthocyanins are derived from only six of them. They are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Table 1.1). Among all the six ones, cyanidin is the most commonly used aglycone (Andersen & Markham, chap.10/Wu & Prior 2005).
1.2.2.2. Other substitutions

The diversity of anthocyanins is mainly based on secondary modifications, such as hydroxylation, glycosylation, methylation and acylation. Almost all substitutions connect to the aglycones by O-linkage; however, a natural C-glycosylanthyocyanin has been identified by Saito’s group (Saito et al., 2003).

The saccharides on anthocyanidins could be mono-, di- or trisaccharides, and the number of saccharides that each anthocyanin contains also varies. Anthocyanins with one, two or three monosaccharides are very common. The saccharide groups are always located at carbons 3, 3’, 7, 3’ and 5’ of the aglycones (Kosir et al., 2004/Cooney et al., 2004). But substitution in the 4’ position also exists (Fossen et al.
2003). Among all the saccharides, glucose is most commonly found in substitution (Davies et al. Chap4/ Wu & Prior 2005). Generally speaking, if an anthocyanin contain only one saccharide, it would be in the 3-position; if it has two, 3, 5-position is normal (Wu & Prior 2005). Figure 1.4 is a summary of common saccharides in anthocyanins.

Acylation refers to the addition of acid (acyl) groups to the saccharides residues of anthocyanins. This kind of modification plays important roles in co-pigmentation of anthocyanins, which also functions in stabilization and has a blueing effect (Mikanagi et al. 2000/ Torskangerpoll & Andersen 2005). More than 34% of the reported anthocyanins have been found to be acylated (Fossen et al. 2007).

The substitutions can have various effects on the colors of anthocyanins. Besides blueing effect of acylation, Methylation and the 6-hydroxylation have a reddening effect (Davies et al. Chap4/ Cabrita et al. 2000). The saccharides themselves do not affect the colors of anthocyanins, but the position of this substitution is important, and it can also affect subsequent modifications (Davies et al. Chap4).
1.2.3. Anthocyanin Biosynthesis

Anthocyanins belong to the flavonoid group. Flavonoids are polyphenolic compounds that come from the metabolic pathway in plants (Bloor & Abrahams 2002). As is shown in Fig.1.5, anthocyanin comes later in the flavonoid synthetic pathway. The flavanones first undergo hydroxylation to produce dihydroflavonols. The next step catalyzed by DFR (dihydroflavonol 4-reductase) is quite important for its determinant role in anthyocyanin types, thus, it should be of potential importance in the control of flower colors. The anthocyanins then undergo some secondary modifications, such as hydroxylation, glycosylation, methylation and acylation, to produce various anthocyanins (Winkel-Shirley 2001/ Jaakola et al., chap.1/ Davies et al., Chap. 4).

Fig.1.5 Biosynthesis pathway of anthocyanins (Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP glucose-flavonoid 3-o-glucosyl transferase) --B. Winkel-Shirley 2001
1.2.4. Stability

Modifications of anthocyanins, including acylation, inter-/intro- molecular co-pigmentation, influence the stability of anthocyanins. The pH of the solvent is also a crucial element, because anthocyanins undergo structure changes at different pH.

Acylated anthocyanins:

- It is well known that acylated (especially with aromatic acyl groups) anthocyanins are more stable than the non-acylated ones (Phippen & Simon, 1998/ Mikanagi et al. 2000/ Wu & Prior 2005). The acylated anthocyanins are more resistant to hydration (Fossen et al., 1998/ Torskangerpoll & Andersen 2005), besides, they influence in inter-/ intro- molecular co-pigmentation (Torskangerpoll & Andersen 2005). In addition to stabilization, the intermolecular co-pigmentation also has a blueing effect and increases the intensity of the color of anthocyanins. Intramolecular co-pigmentation was reported to stabilize more complex anthocyanins, for example, the ones polyacylated with aromatic acids (Goto & Kondo, 1991). Besides, anthocyanins also co-pigment with other aromatics like flavones and cinnamic acids (Fossen et al. 2007).

Conformation change of anthocyanins related to pH:

- For the relatively simple anthocyanins (e.g. anthocyanidin-3-glucosides), it is generally accepted that they undergo structural changes in different pH: at pH=3 or lower, they exist mainly in orange or red colored flavylium cation form; As pH goes up, hydration and proton transfer reaction of acidic aromatic hydroxyl groups take place. The former reaction results in a colorless carbinol pseudo-base, while the later one gives rise to quinonoidal bases (Cabrita et al. 2000/ Fossen et al., 1998). However, most anthocyanins with aromatic acyl groups are much more stable referring to pH differences (Jordheim et al., 2007).

- The structural feature for a more complicated anthocyanin has been analyzed
by T. Fossen group recently (Fossen et al. 2007). It is about an anthocyanin-flavone C-glycoside. In the NMR solvent (CD$_3$OD-CF$_3$COOD; 95:5; v:v), this compound undergoes equilibrium of different forms (Fig. 1.6). The freshly made sample mainly constitutes of the flavylium cations. After longer time of storage, the amount of hemiacetals rose, together with the flavylium cations, became the main constituents.

![Fig.1.6 structure of the anthocyanidins of the equilibrium forms of malvidin 3-O-(6-O-α-rhamnopyranosylAIV-β-glucopyranosideAII)-5-O-β-glucopyranosideAIII] detected in CF$_3$COOD-CD$_3$OH (5:95; v:v) at 300K. 1d, 1e: quinonoidal bases; 1a is the flavylium cation; 1b, 1c stands for the hemiacetal forms.

Generally speaking, it is good to do the anthocyanin analysis in a proper acidic solution and use fresh-made samples. For the simple anthocyanins (the ones with only one or two sugar substitutions on the aglycones), about 70% were kept in their initial form after 60 days at pH 1-3 and 10°C (Cabrita et al. 2000).

### 1.2.5. Function

#### 1.2.5.1. Pigmentation

The most apparent and well-known function of anthocyanins is pigmentation.
They absorb light at the longest wavelength, and contribute to the red, pink, orange, purple to blue pigments in nature. In the plant kingdom, such attractive color is a very important feature. Beautifully colored flowers draw the attention of pollinators, while the colored fruits attract seed dispersers who might eat the fruits and disperse the seeds.

Color is an important element in the food industry, and such natural occurring colorants as anthocyanins definitely have a much higher priority than the synthetic ones, let alone the good water solubility. They have been used widely to produce the attractive color of juices, jams, preserves etc. But the stability of anthocyanins could always be a big limitation. As is described in 1.2.2, different functional groups have different contributions to different colors, they always act together with inter-/intro-molecular co-pigmentation to produce the color variety (Kosir et al., 2004/ Pazmino-Duran et al., 2001).

Anthocyanins were also used in the trans-genetic modification of plant color, which was normally done by preventing pigment formation. Since the anthocyanin biosynthetic pathway has been known (fig.1.5), prevention of a key synthetic enzyme can significantly prevent the anthocyanin formation, generating white or less-colored phenotype. As pH has an influence on coloration as well, plant color could also be changed chemically by changing the pH of their growth condition.

1.2.5.2. Protection

Anthocyanins act as a UV-protectant for plants, which seems to be a sun-screen by absorbing blue to green and UV-light to prevent plant cell from high light damage (Stintzing et al. 2006/ Phippen & Simon, 1998). It is quite common in autumn leaves that chlorophylls are masked by anthocyanins to give yellow or red color. This phenomenon was suggested as a reduction of photo-oxidative damage to the leaves (Bjøry et al., 2007).

Anthocyanins can inhibit pathogens after infection. It has been reported that sorghum synthesized 3-deoxyanthocyanidins after fungal infection, which act as
phytoalexins (Lo & Nicholson, 1998). Beside UV radiation and pathogen attack, stimulation of anthocyanin synthesis can also be stimulated by other factors, such as low temperature (Christie et al., 1994).

The antioxidant activity of anthocyanins has been a hot topic these years, which also indicates their potential clinical use, such as anti-inflammatory, cancer chemoprevention. But the mechanism of their therapeutic properties is still not elucidated.
2. Problems formulation

2.1. Aim of the project

Anthocyanins in *Nesaea Crassicaulis* have been initially analyzed by Nielsen’s group (Nielsen, 2006). And they suspected the *Nesaea* anthocyanins have a protential role in protection against photoinhibition. The aim of the project is to find the different kinds of anthocyanins in *Nesaea Crassicaulis*, including plant growth, isolation and identification. The second aim is to analyze the antioxidant properties of these anthocyanins based on the electrochemical properties.

2.2. Challenges of the project

![Fig.2.1 summary of the experiment design](image)

The chart is a brief description of the experiment. There are several problems to be dealt with.
✧ How to maintain the stability of anthocyanins during the whole experiment?
✧ How to produce anthocyanins efficiently in plant growth?
✧ How to pre-purify the extract in order to make it pure enough for semi-preparative HPLC?
✧ How to set a proper gradient program for the HPLC systems?
✧ What kind of structure information can be obtained from primary identification of HPLC-UV/Vis-MS? Are there any similar compounds that have been identified by other researcher?
✧ Does the 1D-NMR contain enough clues to assign the structures after comparison with the known ones in articles?
✧ Is it possible to analyze the antioxidant properties by electrochemical methods?

In the following experiment part, all the challenges will be dealt with in detail.
3. Experimental part

3.1. Plant Growth

3.1.1. Principles

$^{13}$C enrichment

During plant growth, Ca$^{13}$CO$_3$ was used as a source to enrich the $^{13}$C content in anthocyanins.

1.08% of all carbons in nature are carbon-13 atoms. So it is unlikely that a molecule will contain more than one $^{13}$C atom. Thus a $^{13}$C-NMR spectrum is built up from a collection of molecules. The low natural abundance causes the difficulty to obtain a good $^{13}$C-NMR spectrum. Nowadays, carbon-13 can be enriched in the synthesis steps by addition of the commercially available $^{13}$C-compounds, such as Ca$^{13}$CO$_3$.

In the slightly acidic solvent, Ca$^{13}$CO$_3$ gradually release $^{13}$CO$_2$, which then take part in photosynthesis. The $^{13}$C enriched glucose from photosynthesis then goes to different biological pathways,

\[
\begin{align*}
Ca^{13}CO_3 & \xrightarrow{H^+} ^{13}CO_2 \xrightarrow{\text{photosynthesis}} ^{13}C\text{-enriched glucose} \\
\text{different biological pathways} & \\
\text{Fig.3.1. a probable travel of } ^{13}\text{C in plant after addition}
\end{align*}
\]

The sources of $^{13}$C-enrichment for anthocyanins could be the $^{13}$C-enriched glucose, phenylalanine, malonyl-CoA (fig.1.5) and other substituent including methoxyl and acyl groups.

If the compound were successfully enriched with $^{13}$C, the intensities of $^{13}$C-NMR
The enrichment could also be reflected by 1D $^1$H spectra from intensity of satellite signals (fig.3.2). The major isotope ($^{12}$C) is not NMR active so very little of the proton signal is coupled. The coupled signal (one bond coupling $^{13}$C-$^1$H) appears as small satellite signals on either side of the main uncoupled signal. The coupling constants are between 115 and 250 Hz (usually 125 to 160 Hz). If anthocyanins were enriched successfully with $^{13}$C, the intensities of the satellite peaks should be higher than the non-enriched ones.

![Satellite Peaks Illustration](image)

**Fig.3.2** An illustration of satellite peaks from proton NMR of CHCl$_3$; the peaks marked with the red arrows are the satellite peaks of the proton ($\delta=8.0$ppm) from one bond coupling of $^{13}$C to $^1$H

### 3.1.2. Methods

*Nesaea Crassicaulis* were bought from Tropica Company (No. 033B). The young plants were grown submerged in aquaria under an irradiance of 170 $\mu$mol m$^{-2}$ s$^{-1}$ (photosynthetically active radiation) in 16h: 8h light/dark cycle at 22°C. Commercially available slow release fertilizers (ASB-Grünland, Vallensbæk Strand, Denmark) were added to the rooting medium at regular intervals to ensure sufficient nutrient supply.

The water in the aquaria was a 20: 80 mixture of tap water and demineralized water, which was replaced gradually according to the turbidity. The pH of the water was maintained between 7.5 and 7.8.
aquaria was controlled to be 6.5 by additional CO$_2$ supply. 8ml of 3% H$_2$O$_2$ was added to 50ml of water to reduce the amount of algae, which was a big problem in aquaria planting.

When the leaves started to become red, 5g Ca$^{13}$CO$_3$ (Sigma-Aldrich, 492027) was added to enrich the $^{13}$C in anthocyanins. CaCO$_3$ had poor water solubility so that 200ml 0.1% HCl was used as solvent. The aquarium was first sealed using plastic sheets, leaving only a placket to put CaCO$_3$ in. All air pumps and CO$_2$ pumps were removed. CaCO$_3$ solvent was added to the aquarium immediately after dissolved in 0.1% HCl. Then the aquarium was left completely sealed for three days.

Leaves were harvested as soon as they are red and stored in -20°C until extraction.

### 3.1.3. Results and Discussion

- **Algae problems**

  Algae have been a big problem in the aquaria growth. But if the growth condition was set properly, this problem could be solved out. At first, a relatively simple setting was used: air supply by an air pump; without pH control by CO$_2$ and without H$_2$O$_2$ in the aquaria. After a few days of growing, algae became a big problem, which covered the whole plant and destroyed the leaves as well as the plant growth. At last, we chose to make the water acidic by CO$_2$ but in the permitted range of pH (5.5-8.5). It has been reported that a proper amount of H$_2$O$_2$ did no harm to the aquaria plants and fishes, but controlled algae\(^1\) efficiently. Anyway both acidity and H$_2$O$_2$ were efficient ways to reduce algae.

- **Influence of CO$_2$ and H$_2$O$_2$ addition**

  According to my experiment, CO$_2$ and H$_2$O$_2$ addition itself did not change either the kinds or the amount of anthocyanins. It might have influence on chlorophyll production. For same amount of leaves collected before or after CO$_2$ and H$_2$O$_2$ addition, the overnight acetone extract of the former was light red, while the one of

the latter was totally green. The bigger amount of sticky and poor water-soluble chlorophyll adds to the difficulty of anthocyanin purification.

- **$^{13}$C enrichment**

As was stated in the methods, the aquarium has been sealed for three days mainly with carbon from Ca$^{13}$CO$_3$ as the carbon source. If the $^{13}$C compound can be uptaken by the plants, the $^{13}$C atoms will then go to any relevant biological pathway besides the anthocyanin synthesis pathway (fig.1.5).

Because of the algae problem, only a small amount of leaves were saved after addition of Ca$^{13}$CO$_3$. From these leaves, two relatively pure fractions were obtained, Cyanidin-3,5-di-$\beta$-D-glucopyranose (1.2 mg) and Cyanidin-3-$\beta$-D-glucopyranose (less than 1mg). In the NMR experiments, $^{13}$C spectra could be recorded after overnight running, and the peaks had good intensities. In proton spectra, satellite peaks could be identified with a coupling constant around 165 Hz ($^{13}$C-$^1$H). But if the intensities were compared quantitively, the ratio was about 1%. So the $^{13}$C enrichment might not be very successful. And anthocyanins were not efficiently enriched with $^{13}$C. However, good results were obtained in the carbon spectra from small amount of compounds.

$^{13}$C atoms were uptaken by the plant in the form of CO$_2$ which was a gas and could easily evaporate. And the plants were incubated only for three days after addition of Ca$^{13}$CO$_3$. So a stable form of $^{13}$C enriched compound instead of a gas may be a better choice. The carbon sources for plant can be CO$_2$ for photosynthesis and carbons from fertilizers. If the slow release fertilizers are made $^{13}$C enriched, the plants may uptake them gradually through the whole growing process. But $^{13}$C atoms can not only go to anthocyanins in either enrichment method. We need to find the more efficient way.
3.2. Isolation and Primary Identification

3.2.1. Principles

3.2.1.1. Extraction

Anthocyanins achieve higher stability in acidified solutions in lower temperature (1.2.4), thus, it is crucial to choose the right solvent and acid for extraction and avoid high temperatures in operation. Several solvents have been used in extraction, such as acidified aqueous, methanol, acetone and acetonitrile, and acetone has the priority in low temperature required for concentration (Garcia-Viguera et al., 1998). Milder acids, including TFA, formic acid and acetic acid, can be used. Strong acids, such as HCl, have more risk in hydrolysis and deacylation (Garcia-Viguera C. et al., 1998).

2.5% HCOOH in acetone was used for the plant extraction overnight in room temperature. The filtrated raw extract was concentrated with a rotary evaporator under vacuum at 30°C.

As was stated in 3.1, chlorophyll influences the isolation. In bigger amount of extraction, additional partition step (e.g. water against ethyl acetate) needs to be included (Fossen et al., 1998). But in small amount of extraction, the amount of chlorophyll is also small. After the raw extract is concentrated from rotatory evaporation, chlorophyll becomes a black green layer floating on the surface of anthocyanin solution (mainly water as solvent). This layer can be removed efficiently by filtration. And the remaining chlorophyll can be washed out with a big amount of methanol and acetone in the ion exchange chromatography.

3.2.1.2. Quantification of total anthocyanins (Giusti et al., 2000)

The concentration of anthocyanins can be estimated depending on the absorbance change of different anthocyanins structures in different pH: the colored flavylum form exists at pH 1.0, while the colorless hemiketal form dominates at pH 4.5. The
flavylium form absorbs light at 520nm while the hemiketal form has no absorption at that wavelength. So the absorbance difference at 520nm is proportional to the pigment concentration.

The absorbances of the samples were measured on a Spectronic Genesys 5 detector at 520nm and 700nm. Results were expressed based on cyaniding-3-glucoside (M.W. 449.2 g/mol; molar extinction coefficient $\varepsilon$ 26 900 mol$^{-1}$cm$^{-1}$). And the calculation was performed using the following equations:

The absorbance A:

$$A = (A_{520} - A_{700})_{pH1} - (A_{520} - A_{700})_{pH4.5}$$

According to the Beer-Lambert law ($\varepsilon$-extinction coefficient, c-concentration in mol/L, l-optical path length in cm, l is always 1cm):

$$A = \varepsilon \cdot c \cdot l$$

And the molar concentration (mol/L) can be transferred to mass concentration (g/L), if molecular weight (M.W.) is known. So the anthocyanin concentration is:

$$\text{Anthocyanin concentration (g/L)} = (A \cdot \text{M.W.}) \cdot \varepsilon^{-1} \cdot l^{-1}$$

3.2.1.3. Primary purification by HLB and MCX

Two kinds of columns were used in primary purification: HLB (Hydrophilic-Lipophilic-Balanced reverse-phase column) and MCX (Mixed-Mode Cation-exchange reversed-phase column). Figure 3.3 is a simple illustration of their binding parts. Both of them have hydrophilic and lipophilic parts, but the MCX have an additional cation binding part-sulphonic group, which can bind the positively charged anthocyanins strongly.
Anthocyanins have lipophilic aromatic parts and hydrophilic parts (hydroxyl groups and sugar moieties). They bind to the column together with other non-polar compounds.

In the HLB chromatography, polar water soluble compound like sugar and acids can be removed by washing with aqua eluent; anthocyanins and other non-polar compounds will stick to the column and be recovered by methanol elution.

In the MCX chromatography, other non-positive charged compounds can be washed out by methanol and water. The positive charged $H^+$ ions compete with anthocyanins for the binding part, so that the anthocyanins will be eluted using acid with a proper concentration. About 2% hydrochloric acid was used here.

### 3.2.1.4. HPLC-UV/Vis-MS¹ (Pavia et al., 2001/ Synder et al., 1983)

HPLC-UV/Vis-MS system was used for primary identification of anthocyanins in the extract. Unlike semi-preparative HPLC, which is used in purification (3.2.1.5), HPLC-UV/Vis-MS is an analytical system. Fig.3.4 shows a simplified composition of the HPLC. The analyte is injected by the injector and goes with mobile phase to the column. Different components in the analyte have different interactions with stationary phase in the column and the mobile phase. For example, the components which have a stronger interaction with the mobile phase than stationary phase will be eluted faster. So the solutes are eluted from the system in the order of their increasing distribution coefficients with respect to the stationary phase. The eluted solutes then individually pass through detectors and are analyzed separately. In the experiment, we used two kinds of detectors: UV-Vis (UV and visible light) detector and MS (mass spectrometer).

In the following descriptions, detailed information will be stated about the HPLC instrument as well as the detection methods used in UV-Vis and MS detectors.

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¹ E-books from [http://www.library4science.com/eula.html](http://www.library4science.com/eula.html)
Fig. 3.4 simplified HPLC-UV/Vis-MS systems - the solutions of the analyte are injected onto an HPLC column. Compounds are separated based on their relative interaction with stationary phase and mobile phase. Components eluting from the chromatographic column are first introduced to UV-VIS detector and then to MS spectrometer.

➤ Mobile Phase

Mobile phase in a HPLC refers to the solvent being continuously applied to the whole system. The mobile phase always consists of a combination of different solvents such as water, methanol, acetonitrile etc. There are different types of mobile phase; constant or gradient. But the constant mobile phase always results in poor resolution of the spectrum (Synder, L.R.et al., 1983). So nowadays, gradient mobile phase is most commonly used.

In the gradient elution of reverse phase chromatography (discussed below), the amount of organinc solvent (less polar fraction) in mobile phase is increased gradually. At the time of injection, a weaker mobile phase (with less organic fraction) is applied to the system. The strength of the mobile phase is then raised by increasing the amount of organic fractions, which subsequently causes the elution of the retained components by the stationary phase.

➤ Columns

HPLC column comprises a narrow stainless steel tube packed with the stationary phase. There are a variety of stationary phases commercially available. Different stationary phases result in different mechanisms in elution. There are several more commonly used ones:
- Size exclusion; the stationary phases are porous beads. The compounds are separated depending on their size. The larger ones can not enter the interior of the beads and will be eluted first. The smaller ones will be retained by the beads and eluted depending on their ability to escape from the beads.

- Normal phase chromatography is operated depending on the hydrophilicity and lipophilicity. The stationary phase is polar while the mobile phase is less polar. The hydrophobic compounds are eluted faster than the hydrophilic ones.

- Ion exchange; like the MCX column discussed in 3.2.1.3, the sample is retained by replacing the counter-ions of the stationary phase with its own ions. The sample is eluted from the column by changing the properties of the mobile phase. The mobile phase will now displace the sample ions from the stationary phase (ie. changing the pH).

- Affinity chromatography; this kind of technique is normally used to separate bioactive compounds such as enzymes. The stationary phase is immobilized biochemicals which has a specific affinity to the compound of interest.

- Reverse phase chromatography; it is also operated on the basis of hydrophilicity and lipophilicity. But it uses non-polar stationary phase and relatively polar mobile phase. The reverse phase columns are the most commonly used ones. The stationary phase normally consists of silica compounds like RMe₂SiCl₁, where R stands for an alkyl groups such as C₁₈H₃₇ or C₈H₁₇. The non-polar compounds have a better affinity towards the stationary phase and thus have a longer retention time. The retention time can also be modified by adjusting the polarity of the mobile phase. For example, the retention time is increased by adding a polar solvent to the mobile phase.

The columns used for the HPLC in this project are all reverse phase columns with different internal diameter (ID). Large ID column has a large loading capacity and

1 http://en.wikipedia.org/wiki/HPLC
was used in semi-preparative HPLC to purify anthocyanins. The analytical columns used in HPLC-UV/Vis-MS and electrochemical analysis had smaller internal diameters.

- **UV-Vis detector**

  The detector is equipped with a UV lamp (normally deuterium lamp, 190-360 nm) and Vis lamp (optional tungsten lamp, 360-800 nm). Thus the compounds that absorb light in the range of 180 to 800nm can be detected by the UV/Vis detector.

  The eluted components from the column pass through a short cylindrical cell of the sensor. The UV/Vis light passes the cell to a photo-electric array. If the analyte can absorb light at a certain wavelength, the photo-electric array will detect the change. The output from the photo-electric array then passes to a modifying amplifier and to a recorder and data acquisition system.1

  The intensity of the light (I) transmitted through the cell can be proportioned to the concentration of the solute according to the Beer-Lambert law (ε-extinction coefficient, c-concentration in mol/L, l-optical path length in cm, l is always 1cm, I₀ is the intensity of the light entering the cell):

  \[ A = \varepsilon \cdot c \cdot l \]

  \[ A = -\ln \left( \frac{I}{I_0} \right) \]

  Anthocyanins have two light absorptions at around 280nm and 530nm. So the UV/Vis detectors were used in all the HPLC systems. In semi-preparative HPLC, the UV/Vis detector was the only detector with a detecting wavelength at 530nm. The component which had absorption at 530nm could be detected and thus collected as a fraction. In the analytical HPLC, two kinds of detector were used like UV/Vis with MS and UV/Vis with ECD. The MS detector will be introduced below, while the information about ECD will be given in 3.4.

- **MS detector**

  Generally speaking, a component undergoes three different steps in the MS spectrometer:

  First, the molecules are bombarded by a stream of high energy electrons,

  

1. [http://www.library4science.com/eula.html](http://www.library4science.com/eula.html)
converting the molecules to ions. The molecular ions are then accelerated in an electric field.

Second, in a magnetic or electric field, the accelerated ions can be separated according to their mass-to-charge ratios.

Finally, the ions which have a particular mass to charge ratio are detected. The output of the detector is amplified and recorded by the recorder.

The full name of the MS detector used for anthocyanin analysis was positive mode ESI-tandem mass spectrometry. ESI (electrospray ionisation) stands for the ionization method.

As is shown in fig. 3.5, the analyte from LC or syringe pump passes the spray needle and forms charged droplets at the end of the needle. An uncharged nitrogen flow is used to nebulizer the liquid and to help evaporate the solvent in the droplets. As the solvent evaporates, the analyte molecules are forced closer, repel each other and then the droplets are broken up. This process is driven by repulsive coulombic forces between ions. The broken-up will not stop until all the analytes are free of solvent and are lone ions. Then the ions are detected. Anthocyanins are already a positive ion, so they do not need ionization and go directly to the mass spectrometer after solvent evaporation step. The process can be shown as following (M stands for anthocyanin, l=liquid phase, g= gas phase):

\[ M^+ (l) \rightarrow M^+ (g) \]

Tandem mass experiment refers to multiple steps of mass spectrometry selection.
with some form of fragmentation occurring in between the stages (fig.3.6). In tandem mass experiment, more information about the structure can be found through fragments in MS² and MS³. Take anthocyanins as an example: sugar moieties will be removed one by one. Thus, depending on the loss of molecular weight, the sugar can be identified primarily (e.g. hexose or pentose). Eventually the molecular weight of the aglycone will be known after removing sugar moieties. The type of aglycone can then be identified by comparison with the known ones.

Fig.3.6. an illustration of tandem mass method which produces a fragment ion of the selected ion from MS¹

### 3.2.2. Materials and Methods

#### 3.2.2.1. Materials

- **Chemicals**

  All the chemicals including acetone, methanol, formic acid, hydrochloric acid are HPLC-grade. MilliQ water was used to make the aqueous solution throughout the experiment.

  Buffers for quantification of anthocyanins:

  - pH=1  1.86g KCl in 1L H₂O (adjust pH by HCl)
  - pH=4.5  54.43g CH₃CO₂Na·3H₂O in 1L H₂O (adjust pH by HCl)
The total amount of solution is 1L, so adjust pH before adding water to 1L (960ml water may be firstly added).

- **Instrumentation**
  - **Columns for pre-purification**
    - Oasis HLB: Hydrophilic-Lipophilic-Balanced reverse-phase column: Part# 186000118
    - Oasis MCX: Mixed-Mode Cation-eXchang reversed-phase column: Part #186000255 and 186000776
    - Both kinds of columns were purchased from Waters.
  - **HPLC-UV/Vis-MS**
    - The HPLC was a TSP spectra system and equipped with an AS3000 auto sampler, P4000 gradient pump, the analytical column was a 150mm Luna 3U C18 100R column from Phenomenex with an i.d. of 2.0mm. The UV detector was TSP UV6000; The mass detector was LCQ-Deca ion trap instrument from ThermoFinnigan equipped with an electrospray ionization interface (ESI).
  - **Semi-preparative HPLC**
    - The HPLC system was from Gilson. Two pump 306 and 307 connected with Dynamix mixer 811C. The column used was a 250mm Gemini 5u C18 110R column from Phenomenex with an i.d. of 10mm. The detector was Gilson UV/Vis-151.
3.2.2.2. Methods

**Extraction**

About 60g wet leaves were used for each time of extraction. Liquid N₂ was poured onto the leaves and the frozen leaves were grinded in a mortar with a pestle to fine powder. The plant powder was transferred to an Erlenmeyer flask. Acetone (500 ml) with 2.5% HCOOH was added and left overnight at room temperature under continuous rotation.

Then extract was filtrated through filter paper and concentrated on a rotary evaporator under vacuum at 30 °C. After that the extract was filtered again because of a floating dark green layer (chlorophyll). Then 200ml H₂O was added to the anthocyanin extract.

**Pre-purification by HLB and MCX columns**

The raw extract was purified by HLB and MCX column. In order to get rid of HCl (in eluent of MCX), sample was passed through HLB again. The sample used for each loading on to the column was vacuum evaporated to remove organic solvents and re-dissolved in 2.5% HCOOH in H₂O.

**HLB**: Column was pretreated twice with 10ml methanol and twice with 10ml 0.5% HCl in H₂O. After sample loading, the column was washed with 2.5% HCOOH in H₂O. Samples were then eluted with 2.5% HCOOH in MeOH.

**MCX**: Column was pretreated twice with 10ml methanol and twice with 10ml H₂O. After sample loading, the column was washed with enough MeOH and a
little bit of acetone to get rid of chlorophyll and polyphenols, then anthocyanins were eluted with MeOH: H₂O: HCl (25:25:1; v:v:v).

**HPLC-UV/Vis-MS**

The gradient program was set in 55min with solvent A: H₂O+2.5%HCOOH; solvent B: Acetonitrile; solvent C: MeOH. Table 3.1 was the setting used.

<table>
<thead>
<tr>
<th>Time/min</th>
<th>Flow (ml/min)</th>
<th>A%</th>
<th>B%</th>
<th>C%</th>
</tr>
</thead>
<tbody>
<tr>
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<td>95</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>0.33</td>
<td>0.18</td>
<td>95</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>10.00</td>
<td>0.18</td>
<td>85</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>26.00</td>
<td>0.18</td>
<td>75</td>
<td>10</td>
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<td>0.18</td>
<td>65</td>
<td>15</td>
<td>20</td>
</tr>
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<td>50</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
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<td>0.18</td>
<td>40</td>
<td>30</td>
<td>50</td>
</tr>
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<td>30</td>
<td>50</td>
</tr>
<tr>
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<td>0.20</td>
<td>20</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>45.30</td>
<td>0.18</td>
<td>95</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>55.00</td>
<td>0.18</td>
<td>95</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.1 gradient program for HPLC-UV/Vis-MS

The scan wavelength of UV6000 was set to 190-800nm, while the mass range of the LCQ Deca MS spectrometry was set to 120.00-1200.00.

**Semi-preparative HPLC**

Semi-preparative HPLC was used to separate different kind of anthocyanins in the extract.

Eluent A: 5% MeOH, 2.5% HCOOH in MilliQ H₂O

Eluent B: MeOH: Acetonitrile: HCOOH= 300: 150:10 (v:v:v)

Table 3.2 shows the gradient program:

<table>
<thead>
<tr>
<th>Time/min</th>
<th>Flow (ml/min)</th>
<th>A%</th>
<th>B%</th>
</tr>
</thead>
<tbody>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>18.00</td>
<td>6.5</td>
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<td>29.20</td>
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<td>75</td>
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</tr>
<tr>
<td>30.10</td>
<td>7</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
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<td>8</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>32.10</td>
<td>10</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>32.18</td>
<td>6</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.2 Gradient program for semi-preparative HPLC
Wavelength range 520-540nm was specific for anthocyanins (table 3.4), so the detection wavelength of UV/Vis detector was set to 530nm. The peak level of the fraction collector and the sensitivity needed to be adjusted according to the sample.

### 3.2.3. Results

#### 3.2.3.1 Evaluation of solid phase extraction

After three-step primary purification of raw anthocyanin by HLB-MCX-HLB, the efficiency of this method was evaluated by anthocyanin quantification and HPLC-UV/Vis-MS.

- **Anthocyanin quantification**

  ![Graph showing quantification of anthocyanins](image)

  Fig. 3.8 Evaluation of solid phase extraction by anthocyanin quantification after each step

  In the X axis, 1 stands for the raw extract; 2: after HLB; 3: after HLB-MCX and 4: after HLB-MCX-HLB

As was shown in figure 3.8, there would be a decrease of the amount of anthocyanins after each step of purification. However, about 45%-65% anthocyanins were kept compared with the ones in the raw extract. That is about 1mg anthocyanins can be extracted from 10g wet leaves in this way.

- **HPLC-UV/Vis-MS evaluation**

  In table 3.3 below, it was apparent that after purification by cation exchange column (MCX), the anthocyanins were nicely purified (proved by mass spectrum, Table 3.4). It was a four main peak pattern. Compared this pattern with the chromatogram of HLB-MCX-HLB, there was not much difference. They showed the same peak patterns except for the slightly difference in some small peaks. However,
an additional HLB-purification was greatly recommended, because hydrolyses and deacylation of anthocyanins have been reported in HCl acidified solvent (Garcia-Viguera et al., 1998). Besides, the eluent for HLB was 2.5% HCOOH in MeOH and had a very low melting point (MeOH, m.p. -97.8°C). And low temperature storage helped to keep the solute stable. Anthocyanins dissolved in this eluent were stored at -20°C in liquid form and could be used directly without thaw.

The chromatogram of HLB was almost as complicated as that of the raw extract, which was definitely not proper for semi-preparative HPLC.
Table 3.3 Evaluation of solid phase extraction by LC-UV/Vis-MS

The peaks in all four figures were from UV/Vis detector in total scan with a wavelength range from 190-800nm, anthocyanins were eluted from 15-30min.
3.2.3.2. HPLC-UV/Vis-MS

HPLC-UV/Vis-MS was also used to primarily identify different anthocyanins in *Nesaea Crassicaulis*. Seven anthocyanins were identified (Table 3.4, Fig.3.9) based on the fragmentation in the mass spectrum and the comparison with the known anthocyanins in the former literatures. In the following discussions, the molecular weight was used as a symbol for different anthocyanins.

![Table 3.4 Seven different anthocyanins in Nesaea Crassicaulis](image)

The references used for comparison were: 1. F.C. Stintzing et al., 2006; 2. T. Mas et al., 2000; 3. T. Fossen et al., 2003; 4. M.T. Escribano-Bailon et al., 2006; 5. Wang et al., 2003; 6. Y. Mikanagi et al., 2000; the chromatograms from UV and MS detector of each anthocyanins was in Appendix 3.
The compound represented by peak 1 (fig.3.9, table3.4) was a delphinidin derivative containing two hexose moieties. The major signal at the full mass spectrum was \(m/z\) 627, which corresponded to the molecular ion. In the MS\(^2\) analysis, two major fragments were generated from the molecular ion: \(m/z\)465 and \(m/z\)303. The \(m/z\) 465 fragment corresponded to the loss of a hexose moiety from \(m/z\)627 (-162 amu), while the \(m/z\) 303 fragment resulted from the loss of another hexose group (-162 amu). The MS\(^3\) analysis generated one main fragment: \(m/z\)303, which fitted the molecular weight of delphinidin (table1.1). So peak 1 was deduced to be delphinidin with two hexose moieties (627).

The other six anthocyanins (from peak 2 to 7 in table 3.4) showed similar fragmentation pattern as that of peak 1. They were deduced correspondingly to be Cyanidin+2Hexose (611); Petunidin+2Hexose (641); Peonidin+2Hexose (625); Malvidin+2Hexose (655); Cyandin+Hexose (449) and Peonidin+Hexose (463).

As was shown in figure 3.9, five of the seven anthocyanins were separated very well using the gradient program (Table 3.1). The anthocyanin with a molecular weight 641 (deduced to be Petunidin+2Hexose) was eluted a little bit later than Cyanidin+2Hexose (611), giving a small shoulder for peak 611. This also meant that the amount of 641 was much smaller than 611. The peak 655 was greatly overlapped with peak 625 according to the UV/Vis spectrum. But in the mass spectrum where more information is given about the compound each peak stands for, these two peaks were confirmed to overlap at the tail part of peak 625. And the amount of 655 was much smaller than 625 in relations to the peak intensity of molecular ion (data not shown). So at least a relatively pure 625 could be obtained in semi-preparative HPLC.
3.2.3.3. Semi-preparative HPLC

All the fractions that were collected were shown in fig.3.10. Five relatively pure anthocyanins were obtained from fractions 1, 3, 5, 7 and 9. According to the analysis of MS, fraction 1 was 627 (deduced to be Delphinidin+2Hexose); fraction 3 was 611 (Cyanidin+2Hexose); fraction 5 was 625 (Peonidin+2Hexose); fraction 7 was 449 (Cyandin+Hexose); fraction 9 was 463 (Peonidin+Hexose).

The anthocyanin mixture that was loaded onto semi-preparative HPLC was quite pure after purification by HLB-MCX-HLB (table 3.3). So the anthocyanins (641 and 625) that were overlapped might be obtained by collecting the eluent between fractions. Upon MS results, fraction 4 was a mixture of 641 (Petunidin+2Hexose) and 625 (app. 5:3 in amount) with a little 611; fraction 6 was mainly 655 (Malvidin+2Hexose) with a little 625.

Fractions from semi-preparative HPLC were first concentrated with a rotary evaporator under vacuum at 30°C. But it took quite a long time to evaporate H₂O at
that temperature. So in order to avoid anthocyanin degradation in a longer time of evaporation at higher temperature, samples were then passed through HLB column. This operation could concentrate samples as well as purify.

The concentrated fractions of anthocyanins were then freeze dried for later use in NMR.

### 3.2.4. Discussion

**Extraction**

2.5% HCOOH in acetone worked well in the extraction. The only disadvantage is that chlorophylls were extracted as well. As a result of the poor water solubility of chlorophylls, they added a little difficulty in the purification step. But chlorophylls can be easily dissolved in organic solvent, such as ethanol, acetone, ethylether, chloroform etc. So researchers always choose to do partition using water against ethyl acetate. Chlorophylls will go to the organic phase while anthocyanins stay in water phase. There is also one group who used aqueous acetonitrile containing TFA to extract anthocyanins (Fukui et al. 1997). This kind of extraction solvent with water may reduce the amount of chlorophylls that will be extracted.

Anyway, acetone could be easily evaporated with rotary evaporator at a low temperature (30°C). That avoided degradation of anthocyanins efficiently. It was a big advantage.

**Purification**

Anthocyanins are positive charged and can be retained by cation exchange columns. The MCX columns used here are mix-mode columns, which also have affinity based on the hydrophobicity and hydrophilicity. On one side, they will retain the compounds which are not positive charged, but these unwanted compounds can be washed out by water and proper organic solvent. On the other side, the positive charged anthocyanins bind strongly to the column and purified efficiently. After purification by HLB-MCX-HLB, a quite pure anthocyanin mixture could be obtained (the four-main-peak pattern in table 3.3). And in our purification steps, no
significant degradation was observed according to the MS spectrum.

The anthocyanin mixture was fractionized by semi-preparative HPLC. But the sensitivity of the collection system was not very high, and not all peaks could be recognized as individual pure fractions. That meant the overlapped anthocyanins could not be purified. This problem could be solved out simply by collection between peaks (fraction 4 and 6 in fig.3.10). In that case, the anthocyanin mixture needed to as pure as possible before used in the semi-preparative HPLC. Even the sample obtained in that case was not pure either, but the anthocyanin wanted was the main fraction.

The overlapped anthocyanins may be separated using a much more gradual gradient program. But different gradient programs have been tried in semi-preparative HPLC; in the end the one in table 3.5 was used. If the gradient was changed more gradually, it would be too time consuming. And a gradient program in 33 min worked all right in the isolation, the samples could be used in NMR for structure identification.

- **Anthocyanins in small fractions**

Seven anthocyanins have been distinguished according to the UV/Vis and MS spectrum. There was only one main anthocyanin (611: Cyanidin+2Hexose) in *Nesaea Crassicaulis*, while the others were in relatively small amount. Maybe there will be more anthocyanins in *Nesaea Crassicaulis* in very small fractions, which may also be new compounds.

The new compounds refer to the ones that have not been identified by former researchers. And the amount of new compounds is always very little so that it is not easy to notice their existence. Thus it is always good to do big amount of extraction. But the leaves used here were grown in green house and there were limitations including space, light, temperature and other growth conditions. As a result the amount used was not very high (about 500mg wet leaves) and they were not obtained under completely natural conditions.

For example, 463 (Peonidin+Hexose) is not very easy to be purified through semi-preparative HPLC (a very small peak in fig.3.10), and it might disappear
during the purification steps.

3.3. NMR

3.3.1. Background

Nuclear magnetic resonance spectroscopy (NMR) is used to make a final identification of anthocyanin structures. $^1$H-NMR spectra have been recorded from all the anthocyanin fractions from semi-preparative HPLC. However, the natural abundance of $^{13}$C atoms is very low, only 1.08% of the carbon atoms are C-13 atoms. So it is more difficult to obtain carbon-13 spectra than proton spectra. In the case of anthocyanins, carbon-13 enrichment is not very successful as mentioned in 3.1, besides, the concentrations of several anthocyanins like 463 (Peonidin+Hexose) are pretty small. So carbon-13 NMR spectra were only collected from the ones with bigger concentrations of anthocyanins ($\geq$2mM).

All proton chemical shifts for relevant anthocyanins have been found (Appendix 1). So we can make assignments by comparison with the known ones. But from the limited information in 1D spectrum, it is difficult to decide the glycosidic linkage, where hexose moieties connect to the aglycone.

So HMQC (Heteronuclear correlation through Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) experiments were carried out. Fig 3.11 shows the pulse sequences of HMQC and HMBC. Both of them are inverse detection, whereby $^{13}$C responses are observed in the $^1$H spectrum (acquisition at $^1$H channel). HMQC deals with one-bond couplings between carbon and proton while HMBC will give longer range couplings (e.g. 2 to 4 bonds). The major difference is the duration of the initial delay after the first 90° $^1$H pulse: HMQC has a fixed $\Delta_1$ value that is very small ($\Delta_1=1/2J$, $^1$J of $^{13}$C-$^1$H is around 110 to 270Hz); $\Delta$ value for HMBC is bigger because long range coupling constant J is small (around 0 to 15Hz).

As a result, the carbonds of anthocyanins that have directly attached protons can be identified by HMQC, and HMBC will help to assign the quaternary carbons
according to long range coupling between $^1$H and $^{13}$C.

1D $^1$H, $^{13}$C and 2D HMQC, HMBC NMR experiments were successfully done on four purified anthocyanins: 627 (Delphindin+2Hexose); 611 (Cyanidin+2Hexose); 625 (Peonidin+2Hexose) and 449 (Cyandin+Hexose). Because the concentrations for the rest three ones are very low, only $^1$H NMR was obtained. They are: 463 (Peonidin+Hexose), mixed 641 (mainly Petunidin+2Hexose) and mixed 655 (mainly Malvidin+2Hexose).

In the following result part, detailed assignment will be given.

### 3.3.2. Materials and Methods

#### Materials

The freeze dried anthocyanins were dissolved in CF$_3$COOD: CD$_3$OD (5: 95/ v:v).

Both CF$_3$COOD and CD$_3$OD were purchased from Sigma-Aldrich with CAS # 599-00-8 and 811-98-3.

#### Methods

All the NMR experiments were performed at 25º C. The softwares used for analyze NMR spectra were MestReC 4.9.9.6 and MestReNova 5.2.3-3833.

- **Proton decoupled $^{13}$C-NMR**

  The 1D $^{13}$C spectra were recorded on Varian Mercury 300 MHz spectrometer.
equipped with a 5mm broadband probe, at $^{13}$C resonance frequency 75.464 MHz. The spectral width was 20000 Hz with 128000 data points; the number of scans was 80000. The recycling time was about 6 s per scan.

The 1D $^1$H and 2D $^1$H-$^{13}$C HMQC, $^1$H-$^{13}$C HMBC spectra were recorded on Varian Unity Inova 600 MHz spectrometer with a 5mm triple resonance ($^1$H, $^{13}$C and $^{15}$N) gradient probe, at proton frequency 599.844MHz.

- **$^1$H-NMR**
  
  The spectral width was 8604 Hz with 64530 data points; the number of scans was 2048. The recycling time was about 5 s per scan.

- **HMQC and HMBC**

  Tables 3.5 give the parameters for 2D HMBC and HMQC.

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Spectrometer Frequency/ MHz</th>
<th>Spectral Width/Hz</th>
<th>Taumb(Δ value/s)</th>
<th>Number of Scans</th>
<th>Number of points</th>
<th>Spectral Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{1}$H, $^{13}$C</td>
<td>$^{1}$H, $^{13}$C</td>
<td>$^{1}$H, $^{13}$C</td>
<td>$^{1}$H, $^{13}$C</td>
<td>$^{1}$H, $^{13}$C</td>
<td></td>
</tr>
<tr>
<td>HMQC</td>
<td>(599.84, 150.84)</td>
<td>(8604.0, 24000.0)</td>
<td>1/280</td>
<td>128</td>
<td>(5036, 768)</td>
<td>(4096, 2048)</td>
</tr>
<tr>
<td>HMBC</td>
<td>(599.84, 150.85)</td>
<td>(8604.0, 45000.0)</td>
<td>0.055</td>
<td>128</td>
<td>(5036, 768)</td>
<td>(4096, 2048)</td>
</tr>
<tr>
<td>HMBC</td>
<td>(599.84, 150.84)</td>
<td>(8000.0, 20000.0)</td>
<td>1/280</td>
<td>64</td>
<td>(8192, 512)</td>
<td>(4096, 1024)</td>
</tr>
<tr>
<td>HMBC</td>
<td>(599.84, 150.84)</td>
<td>(8000.0, 20000.0)</td>
<td>0.15</td>
<td>320</td>
<td>(8192, 512)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5 Parameters for HMQC and HMBC; the numbers on the left in the bracket stand for $^1$H parameters while the one on the right stand for $^{13}$C

### 3.3.3. Delphinidin-3,5-di-β-D-glucopyranose (627)

- **$^1$H NMR (Appendix2, 627)**

  Table 3.6 shows the data from the 1D $^1$H-NMR spectrum. The $^1$H signals are designated by letters in decreasing chemical shift order from A to S (skipping H to avoid confusion),
There are four aromatic proton signals from A to D, representing the aglycone protons. The integration of B is 2, which means overlapped proton signals. As is predicted in the LC-MS, the aglycone of 627 was delphinidin (fig.3.12). Because of the symmetry of the B ring, the chemical shifts for H2’ and H6’ must be the same. So, δH2’=δH6’=δB.

H6 and H8 look like α and β protons in naphthalene (fig.3.13). Very small long range couplings (like 5J) have been measured in this aromatic system of naphthalene\(^1\). For example, the coupling constant of Hα1 and Hα2 is 0.82Hz, while the one of Hβ1 and Hα2 is 0.23Hz. The four-bond coupling constant between Hβ1 and Hα1 is larger

---

\(^1\) Spectra database of Organic Compound (SDBS), No. 1350HPM-02-953, [http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi](http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi)
(1.24Hz\(^1\)). \(H_D\) and \(H_C\) fit this pattern well: there are four-bond couplings between them with a coupling constant 2.4Hz. And \(H_8\) has a five-bond coupling with \(H_4\) \((J=0.6Hz)\). So \(H_6= H_C\ (7.064, d 2.4Hz)\) and \(H_8= H_D\ (7.05, dd 0.6/2.4Hz)\). And the rest \(H_4=H_A\ (9.081, brs)\).

The vicinal coupling constant depends on the bond angle of the coupling partners (Pavia et al., 2001). For example, the coupling constant between two axial (a) hydrogens, Jaa, is normally 10 to 14Hz (bond angle \(\alpha=180^\circ\)), whereas the vicinal coupling between an axial hydrogen and an equatorial (e) hydrogen, Jae, is generally 4 to 5Hz \((\alpha=60^\circ)\). As a result, the type of the hexose was identified based on the coupling constant information.

\(H_E\) and \(H_F\) stand for the anomeric protons \((HI)\) of the hexose with a coupling constant of 7.8Hz (fig.3.14, the red labels are specific for hexose carbons and protons). All observed vicinal coupling constants are from 7.8 to 12Hz. So the hexoses in 627 fit with the structure of \(\beta\)-D-Glucopyranose (fig.3.14).

\(H_K\) and \(H_M\) have doublet of doublet (dd) peaks with a coupling constant 7.8 and 9 Hz. Compared with the coupling constant of anomeric protons \((7.8Hz)\), \(H_K\) and \(H_M\) are assigned to be protons of carbon II. \(H_G, H_I, H_J\) and \(H_L\) are the protons from \(C_{VI}\), the two protons of which have germinal couplings between each other \((J=12Hz)\) and also couple to \(H_V\). Since \(H_V\) couples to \(H_{IV}\) and two protons of \(C_{VI}\), it always shows a multiplet with different \(J\) values. So \(H_N\) and \(H_O\) stand for \(H_V\). Signals \(H_P\) and \(H_Q\)

---

\(^1\) Meta-protons of the aromatic ring have a coupling constant from 1 to 3Hz.
show triplet patterns with a coupling constant of 9Hz fit $H_{\text{III}}$. $H_R$ and $H_S$ are the protons of carbon IV.

- **$^{13}$C NMR**

  From C13 spectrum (Appendix2, 627), we can identify 25 carbon signals (without solvent signals):

  $\delta_{C_{01}}=169.544\text{ppm}$, $\delta_{C_{02}}=165.009\text{ppm}$, $\delta_{C_{03}}=157.264\text{ppm}$, $\delta_{C_{04}}=157.017\text{ppm}$, $\delta_{C_{05}}=147.819\text{ppm}$, $\delta_{C_{06}}=147.011\text{ppm}$, $\delta_{C_{07}}=145.97\text{ppm}$, $\delta_{C_{08}}=169.544\text{ppm}$, $\delta_{C_{09}}=120.047\text{ppm}$, $\delta_{C_{10}}=113.451\text{ppm}$, $\delta_{C_{11}}=113.184\text{ppm}$, $\delta_{C_{12}}=107.984\text{ppm}$, $\delta_{C_{13}}=104.043\text{ppm}$, $\delta_{C_{14}}=102.924\text{ppm}$, $\delta_{C_{15}}=97.523\text{ppm}$, $\delta_{C_{16}}=79.134\text{ppm}$, $\delta_{C_{17}}=78.853\text{ppm}$, $\delta_{C_{18}}=78.434\text{ppm}$, $\delta_{C_{19}}=77.869\text{ppm}$, $\delta_{C_{20}}=74.823\text{ppm}$, $\delta_{C_{21}}=74.626\text{ppm}$, $\delta_{C_{22}}=71.463\text{ppm}$, $\delta_{C_{23}}=71.300\text{ppm}$, $\delta_{C_{24}}=62.709\text{ppm}$, $\delta_{C_{25}}=62.532\text{ppm}$

- **HMQC**

  From HMQC spectrum (expanded spectrums in Appendix2), all the carbons that have directly attached protons are identified. They are: $\delta_{C_{08}}=169.544\text{ppm}=\delta_{C_{4}}$, 

  ![HMQC spectrum for 627 (delphinidin + 2 Hexoses)](image)

  Fig.3.15 HMQC spectrum for 627 (delphinidin + 2 Hexoses)

  From HMQC spectrum (expanded spectrums in Appendix2), all the carbons that have directly attached protons are identified. They are: $\delta_{C_{08}}=169.544\text{ppm}=\delta_{C_{4}}$, 

  ![HMQC spectrum for 627 (delphinidin + 2 Hexoses)](image)
\[ \delta C_{12} = 107.984 \text{ppm} = \delta C_6, \ \delta C_{15} = 97.523 \text{ppm} = \delta C_8, \ \delta C_{11} = 113.184 \text{ppm} = \delta C' = \delta C_6' \text{ and the hexose’s carbons.} \]

Anomeric protons: \( H_E--C_{13} \) (104.043ppm) and \( H_F--C_{14} \) (102.924ppm);
Protons on carbon II: \( H_K--C_{20} \) (74.823ppm) and \( H_M--C_{21} \) (74.626ppm);
Protons on carbon III: \( H_P--C_{18} \) (78.434ppm) and \( H_Q--C_{19} \) (77.869ppm);
Protons on carbon IV: \( H_R--C_{22} \) (71.463ppm) and \( H_S--C_{23} \) (71.300ppm);
Protons on carbon V: \( H_N--C_{16} \) (79.134ppm) and \( H_O--C_{17} \) (78.853ppm);
Protons on carbon VI: \( H_G \) and \( H_J--C_{24} \) (62.709ppm) and \( H_I \) and \( H_L--C_{25} \) (62.532ppm).

- **HMBC**

![HMBC Spectrum](image-url)
From the information in table 3.7 and the HMQC result, we can draw the following conclusions:

- H4, H6’, H2’ have long range couplings with C_{02} (165.009 ppm) and these protons belong to different rings (fig.3.12), so C_{02} must be C2, which connects ring B and has three-bond couplings to all the three protons;
- Other coupling partner of H2’ and H6’ are C_{05} (147.819 ppm), C_{07} (145.97 ppm), and C_{11} (113.184). C_{11} has already been confirmed to be C2’ and C6’. The other two carbon signals must represent the three carbons on ring B that have hydroxyl groups. Because of the symmetry, δC_{3’} and δC_{5’} are overlapped. It is difficult to distinguish C_{05} and C_{07} based on the information here. But in $^{13}$C, the intensity of C_{05} is much bigger than that of C_{07}. So C_{05} stands for C3’ and C5’ while C_{07} is C4’.
- H6, H8 and H4 all couple to C_{03} (157.264 ppm), C_{04} (157.017 ppm), while H6 and H8 both couple to C_{01} (169.544 ppm). There are three carbons (C5, C7 and C9) on ring A that connect to oxygen and can have higher chemical shifts. C7 is five-bond away from H4, so it is not easy to show the correlation based on the setting of this experiment. And H4 has three-bond couplings to C5 and C9. Thus, C_{03} and C_{04} must be C5 and C9. C_{01} must be C7. The other coupling partner of H6 and H8 is C_{10} (113.451 ppm), which should be C10 (three-bond coupling).

<table>
<thead>
<tr>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
<th>M</th>
<th>N</th>
<th>O</th>
<th>P</th>
<th>Q</th>
<th>R</th>
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<tr>
<td></td>
<td></td>
<td>C_{13}</td>
<td></td>
<td>C_{14}</td>
<td>C_{22}</td>
<td></td>
<td>C_{20}, C_{21}, C_{22}, C_{23}</td>
<td></td>
<td></td>
<td>C_{24}, C_{25}</td>
</tr>
<tr>
<td>H6’</td>
<td>H6’</td>
<td>H2’</td>
<td>H6’</td>
<td>H2’</td>
<td>H5’</td>
<td>H5’</td>
<td>H3’</td>
<td>H3’</td>
<td>H4’</td>
<td>H4’</td>
</tr>
</tbody>
</table>

Table 3.7 Data from HMBC and assignment of protons (the labels of sugar protons are the same as the one used in fig 3.17)
H4 also couples to C06 (147.011ppm). C3 is the only carbon left on ring A and C that has not been assigned. And the chemical shift 147.011 also fits. So C06 must be C3.

H1”” has a long range coupling with C04. As was discussed before, C04 and C03 are C5 and C9. But C9 can not connect to a glucose moiety. So C04 is C5. H1”” couples to C3. So the glycosidic linkage is from 3 and 5 positions of delphinidin.

Assignment of the glucose moieties is based on the correlation in Table 3.7. Finally, the structure of 627 (delphinidin-3,5-di-β–D-glucopyranose) is given in fig 3.17 with the assignment of all the carbons.

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**3.3.4. Cyanidin-3,5-di-β–D-glucopyranose (611)**

**1H-NMR (Appendix 2, 611)**

Table 3.7 is the data from the one dimensional 1H-NMR. The designations of 1H signals are based on letters in decreasing chemical shift order from A to U (skipping H to avoid confusion).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Chemical shift/ppm</th>
<th>Coupling constant/Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.129</td>
<td>s</td>
</tr>
<tr>
<td>B</td>
<td>8.335</td>
<td>dd 2.4/9</td>
</tr>
</tbody>
</table>

Fig.3.17 structure of delphinidin-3,5-di-β–D-glucopyranose (627) with the chemical shift of carbons (red labels)
The aglycone of 611 predicted in LC-MS was cyanidin. (fig.3.18). Six aglycone proton signals are identified: from A to F. According to the discussion about naphthalene in 3.3.3, H_D (7.067, d 2.4Hz) is H6 while H_E (7.066, dd 0.6/2.4Hz) is H8. And δ_H_D is a little higher than δ_H_E (in expanded 1H spectrum-Appendix 2, 611). H_A (9.129s) is H4; H6’ has a three-bond coupling with H5’ (3J_{ortho}=7-10Hz) and a four-bond coupling with H2’ (4J_{meta}=1-3Hz). So depending on the coupling constant, H_B (8.335, dd 2.4/9Hz) is H6’, H_C (8.057, d 2.4Hz) is H2’ and H_F (7.021, d 9Hz).

The rest protons from H_G to H_U are hexose protons. The type of the hexose was identified similarly like delphinidin-3,5-di-β-D-glucopyranose (627) in 3.3.3, based

<p>| | | |</p>
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<tr>
<td>C</td>
<td>8.057</td>
<td>d 2.4</td>
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<tr>
<td>D</td>
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<td>d 2.4</td>
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<td>dd 0.6/2.4</td>
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<tr>
<td>F</td>
<td>7.021</td>
<td>d 9</td>
</tr>
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<td>G</td>
<td>5.29</td>
<td>d 7.8</td>
</tr>
<tr>
<td>I</td>
<td>5.154</td>
<td>d 7.8</td>
</tr>
<tr>
<td>J</td>
<td>3.973</td>
<td>dd 1.8/12</td>
</tr>
<tr>
<td>K</td>
<td>3.958</td>
<td>dd 1.8/12</td>
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<tr>
<td>M</td>
<td>3.716</td>
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<td>dd 7.8/9</td>
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<td>O</td>
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<td>dd 7.8/9</td>
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</tr>
<tr>
<td>Q</td>
<td>3.582</td>
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<td>R</td>
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</tr>
<tr>
<td>S</td>
<td>3.538</td>
<td>t 9</td>
</tr>
<tr>
<td>T</td>
<td>3.459</td>
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</tr>
<tr>
<td>U</td>
<td>3.41</td>
<td>t 9.6</td>
</tr>
</tbody>
</table>

Table 3.8 1H-NMR data for 611 (Cyanidin + 2 Hexoses)

![Fig.3.18. structure of cyanidin](image)
on the coupling constant information.

Anomeric protons: $H_G$ (5.29, d 7.8Hz) and $H_I$ (5.154, d 7.8Hz);
Protons on carbon II: $H_N$ (3.698, dd 7.8/9Hz) and $H_O$ (3.681, dd 7.8/9Hz);
Protons on carbon III: $H_R$ (3.554, t 9Hz) and $H_S$ (3.538, t 9Hz);
Protons on carbon IV: $H_T$ (3.459, t 9.6Hz) and $H_U$ (3.41, t 9Hz);
Protons on carbon V: $H_F$ (3.643, m) and $H_Q$ (3.582, m);
Protons on carbon VI: $H_J$ (3.973, dd 1.8/12Hz), $H_K$ (3.958, dd 1.8/12Hz), $H_L$ (3.756, dd 6/12Hz) and $H_M$ (3.716, dd 6/12Hz).

The labels from I to VI are consistent with the ones in fig.3.14. The two hexoses in 611 are β-D-glucopyranose.

➢ ¹³C NMR

From C13 spectrum (Appendix2, 611), we can identify 27 carbon signals (without solvent signals):

\[
\begin{align*}
\delta C_{01} &= 169.740 \text{ppm}, & \delta C_{02} &= 165.176 \text{ppm}, & \delta C_{03} &= 157.362 \text{ppm}, & \delta C_{04} &= 157.065 \text{ppm}, \\
\delta C_{05} &= 156.797 \text{ppm}, & \delta C_{06} &= 147.779 \text{ppm}, & \delta C_{07} &= 146.851 \text{ppm}, & \delta C_{08} &= 136.268 \text{ppm}, \\
\delta C_{09} &= 129.285 \text{ppm}, & \delta C_{10} &= 121.268 \text{ppm}, & \delta C_{11} &= 118.727 \text{ppm}, & \delta C_{12} &= 117.723 \text{ppm}, \\
\delta C_{13} &= 113.959 \text{ppm}, & \delta C_{14} &= 105.981 \text{ppm}, & \delta C_{15} &= 104.203 \text{ppm}, & \delta C_{16} &= 102.91 \text{ppm}, \\
\delta C_{17} &= 97.545 \text{ppm}, & \delta C_{18} &= 79.138 \text{ppm}, & \delta C_{19} &= 78.842 \text{ppm}, & \delta C_{20} &= 78.507 \text{ppm}, \\
\delta C_{21} &= 77.841 \text{ppm}, & \delta C_{22} &= 74.847 \text{ppm}, & \delta C_{23} &= 74.605 \text{ppm}, & \delta C_{24} &= 71.502 \text{ppm}, \\
\delta C_{25} &= 71.290 \text{ppm}, & \delta C_{26} &= 62.759 \text{ppm}, & \delta C_{27} &= 62.520 \text{ppm}
\end{align*}
\]

➢ HMQC
From HMQC spectrum (expanded spectrums in Appendix2), all the carbons that have directly attached protons are identified. They are: δC₀₈=136.268ppm=δC₄, δC₀₉=129.285ppm=δC₆’, δC₁₁=118.727ppm=δC₂’, δC₁₂=117.723ppm=δC₅’, δC₁₄=105.981ppm=δC₆, δC₁₇=97.545ppm=δC₈ and the hexose’s carbons (the labels from II to VI are consistent with the ones in fig.3.14).

Anomeric protons: H₆—C₁₅ (104.203ppm) and H₁—C₁₆ (102.910ppm);
Protons on carbon II: H₂—C₂₂ (74.847ppm) and H₃—C₂₃ (74.605ppm);
Protons on carbon III: H₄—C₂₀ (78.507ppm) and H₅—C₂₁ (77.841ppm);
Protons on carbon IV: H₆—C₂₄ (71.502ppm) and H₇—C₂₅ (71.290ppm);
Protons on carbon V: H₆—C₁₈ (79.138ppm) and H₇—C₁₉ (78.842ppm);
Protons on carbon VI: H₁ and H₁—C₂₆ (62.759ppm) and H₂ and H₂—C₂₇ (62.520ppm).

- HMBC
Designation | Spin Coupling partners | assignment  
---|---|---  
A | C₀₂, C₀₃, C₀₄, C₀₇ | H₄  
B | C₀₂, C₀₃, C₁₁(C2’') | H₆’  
C | C₀₂, C₁₀₅, C₀₆, C₀₉(C₆’’') | H₂’  
D | C₀₃, C₀₄, C₀₁, C₁₃, [C₁₄ (C₆)-C₁₇(C₈)] | H₆  
E | | H₈  
F | C₀₅, C₀₆, C₁₀ | H₅’  
G | C₀₇ | H₁’’  
I | C₀₄ | H₁’’’  
J | | H₆”A  
K | | H₆”’A  
L | | H₆”B  
M | | H₆”’B  
N | C₁₅ | H₂”  
O | C₁₆ | H₂”  
P | C₂₄, C₂₅ | H₅”  
Q | | H₅”’  
R | C₂₂, C₂₃, C₂₄, C₂₅ | H₃”  
S | | H₃”’  
T | C₁₈, C₁₉, C₂₆, C₂₇ | H₄”  
U | | H₄”’

Table 3.9 Data from the HMBC spectrum and assignment of protons (the labels of sugar protons are the same as the one used in fig 3.21)
From the information in table 3.9 and the HMQC result, we can make such conclusions:

✧ H4, H6, H2’ have long range couplings with C02 (165.176ppm) and these three protons belong to different rings (fig.3.18), so C02 must be C2 which has three-bond couplings to all the three protons;

✧ H6, H8, H4 couple to C03 (157.362ppm) and C04 (157.065ppm). Depending on the high chemical shifts and their positions on cyanidin, C03 and C04 must be C5 and C9. But right now we can not tell exactly which one is which one;

✧ H6 and H8 also couple to C01 (169.740ppm) and C13 (113.959ppm). C01 must be C7 which can reach such a high chemical shift. By now, almost all the carbons on ring A and ring C have been assigned except C3 and C10. Based on the chemical shift, C13 must be C10;

✧ H4 couples to C07 (146.851ppm). The high chemical shift of C07 indicates its connection with oxygen. Among the carbons near C4 that connect to oxygen and have not yet been assigned, only C3 is left. So C07 must be C3;

✧ Both H2’ and H6’ have long range couplings with C02 (C2) and C05 (156.797ppm), while H2’ alone couples to C06 (147.779ppm). C3’ and C4’ of ring B connect to hydroxyl groups and can have higher chemical shifts. Thus C05 and C06 must represent C3’ and C4’. Besides H2’ and H6’ have three-bond couplings with C4’, while H2’ has two-bond coupling with C3’. It fits with the information shown in the spectrum. So C05 must be C4’ and C06 must be C3’.

✧ H5’ couples to C05 (C4’), C06 (C3’), and C10 (121.268ppm). C10 must be C1’.

✧ H1’’’ has long range coupling to C04. As was discussed before, C04 and C03 are C5 and C9. But C9 can not connect to a glucose moiety. So C04 is C5. H1’’ has long range coupling to C3. So the glycosidic linkage is from 3 and 5 positions of cyanidin.

So the structure of cyanidin-3,5-di-β-D-glucopyranose (611) is shown in fig 3.21.
with the assignment of all the carbons.

Fig.3.21 structure of cyanidin-3,5-di-β–D-glucopyranose (611) with the chemical shift of carbons (red labels)

### 3.3.5. Peonidin-3,5-di-β–D-glucopyranose (625)

1H-NMR (Appendix 2, 625)

Table 3.10 gives the data from 1D 1H-NMR spectra. The designations of 1H signals are based on letters in decreasing chemical shift order from A to V (skipping H to avoid confusion).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Chemical shift/ppm</th>
<th>Coupling constant/Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.18</td>
<td>s</td>
</tr>
<tr>
<td>B</td>
<td>8.358</td>
<td>dd 2.4/9</td>
</tr>
<tr>
<td>C</td>
<td>8.262</td>
<td>d 2.4</td>
</tr>
<tr>
<td>D</td>
<td>7.135</td>
<td>s</td>
</tr>
<tr>
<td>E</td>
<td>7.086</td>
<td>s</td>
</tr>
<tr>
<td>F</td>
<td>7.078</td>
<td>d 9</td>
</tr>
<tr>
<td>G</td>
<td>5.315</td>
<td>d 7.8</td>
</tr>
<tr>
<td>I</td>
<td>5.164</td>
<td>d 7.8</td>
</tr>
<tr>
<td>J</td>
<td>4.031</td>
<td>s</td>
</tr>
<tr>
<td>K</td>
<td>3.971</td>
<td>dd 1.8/12</td>
</tr>
<tr>
<td>L</td>
<td>3.96</td>
<td>dd 1.8/12</td>
</tr>
<tr>
<td>M</td>
<td>3.757</td>
<td>dd 5.4/12</td>
</tr>
</tbody>
</table>
The aglycone of 625 predicted in LC-MS is peonidin (fig.3.22), which has six proton signals from A to F. H_B, H_C and H_F are assigned to be protons of ring B, depending on their coupling constants. H_B (8.358, dd 2.4/9Hz) is H6’, which have a vicinal coupling to H5’ (3J_ortho=7-10Hz) and a four-bond coupling to H2’ (4J_meta=1-3Hz). Similarly H_C (8.262, d 2.4Hz) is H2’ and H_F (7.078, d 9Hz) is H5’. Then there are three aromatic proton signals left: H_A (9.18, brs), H_D (7.135, brs) and H_E (7.086, brs). From the recordings in literatures (Appendix 1) as well as the experience in assigning anthocyanins, δH4 is the biggest while δH8 is always bigger than δH6. So, H_A =H4, H_D =H8 and H_E =H6. The strong singlet H_J (4.031, s) stands for the methoxyl protons.

The rest protons from H_G to H_V are hexose protons. The type of the hexose is identified similarly like delphinidin-3,5-di-β-D-glucopyranose (627) in 3.3.3, based on the coupling constant information (labels from I to VI are consistent with the ones in fig.3.14).
Anomeric protons: $H_G$ (5.315, d 7.8Hz) and $H_I$ (5.164, d 7.8Hz);
Protons on carbon II: $H_O$ (3.687, dd 7.8/9Hz) and $H_P$ (3.664, dd 7.8/9Hz);
Protons on carbon III: $H_S$ (3.556, t 9Hz) and $H_T$ (3.537, t 9Hz);
Protons on carbon IV: $H_U$ (3.461, dd 9/ 9.6Hz) and $H_V$ (3.396, dd 9/9.6Hz);
Protons on carbon V: $H_Q$ (3.63, m) and $H_R$ (3.57, m);
Protons on carbon VI: $H_K$ (3.971, dd 1.8/12Hz), $H_L$ (3.96, dd 1.8/12Hz), $H_M$(3.757, dd 5.4/12Hz) and $H_N$ (3.721, dd 6.6/12Hz).

So the two hexoses in 625 are $\beta$–D-glucopyranose.

➢ ¹³C NMR

From C13 spectrum (Appendix2, 625), we can identify 28 carbon signals (without solvent signals):

$\delta C_{01}=169.980$ppm, $\delta C_{02}=165.122$ppm, $\delta C_{03}=163.480$ppm, $\delta C_{04}=157.572$ppm, $\delta C_{05}=157.117$ppm, $\delta C_{06}=149.877$ppm, $\delta C_{07}=146.791$ppm, $\delta C_{08}=136.744$ppm, $\delta C_{09}=129.898$ppm, $\delta C_{10}=121.150$ppm, $\delta C_{11}=118.086$ppm, $\delta C_{12}=115.589$ppm, $\delta C_{13}=113.781$ppm, $\delta C_{14}=106.051$ppm, $\delta C_{15}=104.378$ppm, $\delta C_{16}=102.932$ppm, $\delta C_{17}=97.689$ppm, $\delta C_{18}=79.211$ppm, $\delta C_{19}=78.868$ppm, $\delta C_{20}=78.605$ppm, $\delta C_{21}=77.853$ppm, $\delta C_{22}=74.991$ppm, $\delta C_{23}=74.612$ppm, $\delta C_{24}=71.549$ppm, $\delta C_{25}=71.294$ppm, $\delta C_{26}=62.761$ppm, $\delta C_{27}=62.527$ppm, $\delta C_{28}=57.103$ppm

➢ HMQC
From HMQC spectrum (expanded spectrums in Appendix2), the carbons that have directly attached protons are identified. They are: δC₀₈=136.744ppm=δC₄, δC₀₉=129.898ppm=δC₆’, δC₁₂=115.589ppm=δC₂’, δC₁₁=118.086ppm=δC₅’,
δC₁₄=106.051ppm=δC₆, δC₁₇=97.689ppm=δC₈, δC₂₈=57.103ppm=δC₅e and the hexose’s carbons (the labels from II to VI are consistent with the ones in fig.3.14).

Anomeric protons: H₅—C₁₅ (104.378ppm) and H₁—C₁₆ (102.932ppm);
Protons on carbon II: H₂—C₂₂ (74.991ppm) and H₃—C₂₃ (74.612ppm);
Protons on carbon III: H₅—C₂₀ (78.605ppm) and H₇—C₂₁ (77.853ppm);
Protons on carbon IV: H₄—C₂₄ (71.549ppm) and H₆—C₂₅ (71.294ppm);
Protons on carbon V: H₃—C₁₈ (79.211ppm) and H₅—C₁₉ (78.868ppm);
Protons on carbon VI: H₇ and H₉—C₂₆ (62.761ppm) and H₈ and H₉—C₂₇ (62.527ppm).

➢ HMBC
Fig. 3.24 HMBC spectrum for 625 (Peonidin + 2 Hexoses) - the spectral width of the $^{13}$C dimension was too small so the signal folded automatically. But still there is useful information.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Spin Coupling partners</th>
<th>assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C02, C04, C05, C06, C07, C10, C13</td>
<td>H4</td>
</tr>
<tr>
<td>B</td>
<td>C04, C05, C02, C06</td>
<td>H6</td>
</tr>
<tr>
<td>C</td>
<td>C02, C03, C06, C10, C11</td>
<td>H2</td>
</tr>
<tr>
<td>D</td>
<td>C01, C04, C05, C13, C14</td>
<td>H8</td>
</tr>
<tr>
<td>E</td>
<td>C01, C17</td>
<td>H6</td>
</tr>
<tr>
<td>F</td>
<td>C02, C10, C12</td>
<td>H5</td>
</tr>
<tr>
<td>G</td>
<td>C07</td>
<td>H1</td>
</tr>
<tr>
<td>I</td>
<td>C05</td>
<td>H1</td>
</tr>
<tr>
<td>J</td>
<td>C06</td>
<td>OMe</td>
</tr>
<tr>
<td>K</td>
<td></td>
<td>H6</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td>H6</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td>H6</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>H6</td>
</tr>
<tr>
<td>O</td>
<td>C15</td>
<td>H2</td>
</tr>
<tr>
<td>P</td>
<td>C16</td>
<td>H2</td>
</tr>
<tr>
<td>Q</td>
<td></td>
<td>H5</td>
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<tr>
<td>R</td>
<td></td>
<td>H5</td>
</tr>
<tr>
<td>S</td>
<td>C22, C23, C24, C25</td>
<td>H3</td>
</tr>
<tr>
<td>T</td>
<td></td>
<td>H3</td>
</tr>
<tr>
<td>U</td>
<td>C26, C27</td>
<td>H4</td>
</tr>
</tbody>
</table>
From the information in table 3.11 and the HMQC result, we can get such information:

- Aromatic carbons of ring A and ring C are assigned similarly as the assignment in 3.3.3. And C07 (146.791 ppm) is C3, which has a three-bond coupling with H4. C04 (157.572 ppm) and C05 (157.117 ppm) are C5 and C9;
- From HMQC, C2′ (115.589 ppm), C5′ (118.086 ppm) and C6′ (129.898 ppm) are already confirmed. H2′ and H6′ both couple to C06 (149.877 ppm), which can be C3′ or C4′. H2′ also couples to C03 (163.480 ppm). So compared with the chemical shift and the position of H2′, C03 is C3′ and C06 is C4′. C06 is also the coupling partner of methoxyl protons (4J). C10 (121.150 ppm) was C1′;
- Carbons of glucose moieties were assigned based on the long range coupling information in table 3.11.

Finally, fig 3.25 is the structure of Peonidin-3,5-di-β-D-glucopyranose (625) with the assignment of all the carbons.

Fig.3.25 structure of Peonidin-3,5-di-β-D-glucopyranose (625) with the chemical shift of carbons (red labels)
3.3.6. Cyanidin-3-β–D-glucopyranose (449)

The assignment of 449 (cyanidin-3-β-D-glucopyranose) is quite similar to 611 (cyanidin-3,5-di-β-D-glucopyranose); they have the same aglycone- cyanidin and 449 has one glucose moiety less than 611.

1D $^1$H and $^{13}$C spectra were recorded successfully. But at the time of HMBC and HMQC experiment, the molecular weight analyzed on LC-MS was two amu larger than before. And the signals of H6 and H8 were even invisible. So it might due to deuterium exchange on ring A after long time of storage in CD$_3$OD-CF$_3$COOD (discussed later in 3.3.9 Summary).

- **1D $^1$H and 2D HMQC spectra**

Table 3.12 is a summary of signals from 1D $^1$H and 2D HMQC spectra (Appendix 2, 449), where the assignment of protons and their attached carbons is given. The labels are consistent with the ones in figure 3.26.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chemical shift/ppm</th>
<th>Coupling constant/Hz</th>
<th>carbon partners of the protons</th>
<th>Chemical shift/ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4</td>
<td>9.03</td>
<td>s</td>
<td>C4</td>
<td>137.148</td>
</tr>
<tr>
<td>H6'</td>
<td>8.266</td>
<td>dd 2.4/8.4</td>
<td>C6'</td>
<td>128.407</td>
</tr>
<tr>
<td>H2'</td>
<td>8.056</td>
<td>d 2.4</td>
<td>C2'</td>
<td>118.605</td>
</tr>
<tr>
<td>H5'</td>
<td>7.028</td>
<td>d 8.4</td>
<td>C5'</td>
<td>117.959</td>
</tr>
<tr>
<td>H8</td>
<td>6.901</td>
<td>dd 0.6/1.8</td>
<td>C8</td>
<td>95.249</td>
</tr>
<tr>
<td>H6</td>
<td>6.663</td>
<td>d 1.8</td>
<td>C6</td>
<td>103.494</td>
</tr>
<tr>
<td>H1&quot;</td>
<td>5.294</td>
<td>d 7.8</td>
<td>C1&quot;</td>
<td>103.896</td>
</tr>
<tr>
<td>H2&quot;</td>
<td>3.68</td>
<td>dd 7.8/9</td>
<td>C2&quot;</td>
<td>74.933</td>
</tr>
<tr>
<td>H3&quot;</td>
<td>3.541</td>
<td>t 9</td>
<td>C3&quot;</td>
<td>78.257</td>
</tr>
<tr>
<td>H4&quot;</td>
<td>3.446</td>
<td>dd 9/9.6Hz</td>
<td>C4&quot;</td>
<td>71.249</td>
</tr>
<tr>
<td>H5&quot;</td>
<td>3.56</td>
<td>m</td>
<td>C5&quot;</td>
<td>78.956</td>
</tr>
<tr>
<td>H6&quot;A</td>
<td>3.916</td>
<td>dd 2.4/12</td>
<td>C6&quot;</td>
<td>62.527</td>
</tr>
<tr>
<td>H6&quot;B</td>
<td>3.713</td>
<td>dd 6/12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.12 Data from 1 D $^1$H and 2D HMQC spectra (because of deuterium substitution of H6 and H8, the assignment of C6 and C8 was based on the comparison of the $^{13}$C signals with the known ones in Appendix 1)

- **$^{13}$C spectrum**

The left carbon signals from $^{13}$C spectrum are designated based on letters in
decreasing chemical shift order from A to I (skipping C to avoid confusion):
\[ \delta_{C_A}=170.674 \text{ppm}, \quad \delta_{C_B}=164.562 \text{ppm}, \quad \delta_{C_D}=157.919 \text{ppm}, \quad \delta_{C_E}=157.875 \text{ppm}, \]
\[ \delta_{C_F}=155.919 \text{ppm}, \quad \delta_{C_G}=147.581 \text{ppm}, \quad \delta_{C_H}=145.810 \text{ppm}, \quad \delta_{C_I}=121.437 \text{ppm}, \]
\[ \delta_{C_J}=113.555 \text{ppm}, \]

**HMBC**

<table>
<thead>
<tr>
<th>protons</th>
<th>Spin Coupling partners</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4</td>
<td>C_B, C_E, C_D, C_H, C_I(very wake), C_J</td>
</tr>
<tr>
<td>H6'</td>
<td>C_F, C_B, C_2', C_5'</td>
</tr>
<tr>
<td>H2'</td>
<td>C_B, C_F, C_I, C_G, C_6'</td>
</tr>
<tr>
<td>H5'</td>
<td>C_I, C_F, C_G</td>
</tr>
<tr>
<td>H8</td>
<td></td>
</tr>
<tr>
<td>H6</td>
<td></td>
</tr>
<tr>
<td>H1''</td>
<td>C_H</td>
</tr>
</tbody>
</table>

Table 3.13 Data from the HMBC

A brief discussion of the result:

- H4, H2’, H6’ have a same coupling partner C_B (164.562ppm), which is C2 that connects ring B and ring C and has three-bond couplings to these three protons.
- Ring B: Information about C2’ C5’ C6’ can be found in table 3.12. the rest C3’ and C4’ should have higher chemical shifts because of the hydroxyl group. From the coupling partners of H5’- C_I (121.437ppm), C_F (155.919ppm) and C_G (147.581ppm), we can confirm C_I is C1’, while C_F and C_G represent C3’ and C4’. Besides, H2’ couples to C_G while H6’ doesn’t. S C_G is C3’ that is four-bond away from H6’, and C_F is C4’.
- Because of the deuteration, information of ring A can only be obtained from H4. From its coupling partners C_E (157.875ppm), C_D (157.919ppm), C_H (145.810ppm), C_J (113.555ppm), we can confirm C_J is C10 which has a smaller chemical shift than the other three. Depending on the results from other researchers (Appendix 1) and my assignment of 611 (cyanidin-3,5-di-β-D-glucopyranose), C_H=C3, C_E=5 and C_D=9.
- Glucoside was confirmed to be bonded with the 3-hydroxyl of cyanidin
through a glucosidic bond because of the cross peak from H1’’ (δ5.294) to Cβ (145.810ppm).

As a result, the structure of Cyanidin-3-β-D-glucopyranose (449) is given in fig.3.26 with assignment of carbons.

![Fig.3.26. structure of Cyanidin-3-β-D-glucopyranose (449) with the chemical shift of carbons (red labels)](image)

### 3.3.7. Peonidin-3-β-D-glucopyranose

Only 1H-NMR spectrum of 463 (Peonidin+ Hexose) was recorded because of its small amount (Appendix 2).

Table 3.14 shows the data from the 1D 1H-NMR spectrum. The 1H signals are designated by letters in decreasing chemical shift order from A to O (skipping H to avoid confusion).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Chemical Shift δ/ppm</th>
<th>Coupling Constant J/Hz</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.07</td>
<td>8</td>
<td>Aglycone</td>
</tr>
<tr>
<td>B</td>
<td>6.674</td>
<td>1.8(d)</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>6.944</td>
<td>0.6/1.8(dd)</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>8.249</td>
<td>1.8(d)</td>
<td>2’</td>
</tr>
<tr>
<td>E</td>
<td>7.071</td>
<td>8.4(d)</td>
<td>5’</td>
</tr>
<tr>
<td>F</td>
<td>8.276</td>
<td>1.8/8.4(dd)</td>
<td>6’</td>
</tr>
</tbody>
</table>
Based on the structure of peonidin as well as the principles of meta- and ortho-coupling constants (3.3.3), six aromatic protons (From A to F) are assigned as shown in table 3.14. The anemic proton was assigned at δ5.318 (d, J=7.8Hz). According to the vicinal coupling constants (7.8~12Hz) of the hexose (from I to O), it should be β-D-glucopyranose. Protons of the methoxyl group were H_G (4.024 ppm, s).

463 had the same aglycone as 625 (Peonidin-3,5-di-β-D-glucopyranose). Comparing the chemical shifts of the aromatic protons (table 3.15 in 3.3.9 Summary), the major difference was in H6 and H8, which had much smaller chemical shifts than the ones in 625. The same pattern was observed in 449 (Cyanidin-3-β-D-glucopyranose) and 611 (Cyanidin-3,5-di-β-D-glucopyranose). 449 differed from 611 in the absence of the glucose moiety on C5. Glucoside of 463 was probably bonded with the 3-hydroxyl of peonidin. Besides, the chemical shifts we recorded were almost the same as Peonidin-3-β-D-glucopyranose (Appendix 1/Fossen et al., 2002). So 463 probably was Peonidin-3-β-D-glucopyranose.
3.3.8. Assignment of impure anthocyanins

As was introduced before, two mixtures were also collected from semi-preparative HPLC; one had 641 (Petunidin+ 2Hexose) as a major component while the other has a dominant component 655 (malvidin+ 2Hexose).

Only $^1$H NMR spectra were recorded for these two mixtures.

- **641 (Petunidin+ 2Hexose)**

![Fig.3.28 spectra from HPLC-UV/Vis-MS of the mixture sample (641-Petunidin+ 2Hexose, 625-Peonidin-3,5-di-$\beta$–D-glucopyranose and 611-Cyanindin-3,5-di-$\beta$–D-glucopyranose): A is the mass spectrum showing the intensity of these three components; B is the UV/Vis spectrum.]

Based on the HPLC-UV/Vis-MS chromatogram (figure 3.28), the impure analyte used for NMR was a mixture of 641, 625 (Peonidin-3,5-di-$\beta$–D-glucopyranose) and 611 (Cyanindin-3,5-di-$\beta$–D-glucopyranose). But the amount of 611 was very small. So the assignment was based on comparison with 625. And there are also references in the literatures (Appendix 1).

![Fig.3.29 structure of petunidin]

The signals that can be identified in the spectrum were as follows:
$\delta H4=9.139\text{ppm}, \delta H6=7.081\text{ppm (d, 1.8Hz)}, \delta H8=7.116\text{ppm (dd, 0.6/1.8Hz)},$
$\delta H2'=8.045\text{ppm (d, 2.4Hz)}, \delta H6'=7.864\text{ppm (d, 2.4Hz)}, \delta H_{OMe}=4.018\text{ppm},$
$\delta H1''=5.336\text{ppm (d, 7.8Hz)}, \delta H1'''=5.163\text{ppm (d, 7.8Hz)}$

The coupling constant information was very useful in assigning the aromatic protons. Five aromatic protons were assigned to be protons of Petunidin. Two anomeric protons were assigned at $\delta 5.336$ (d, $J=7.8\text{Hz}$) and $\delta 5.163$ (d, $J=7.8\text{Hz}$), so the hexoses were in β form. The chemical shift of 641 was more similar to petunidin-3,5-di-β-D-glucopyranose (appendix 1, Norbaek et al., 1999) instead of petunidin-3,7-di-β-D-glucopyranose (appendix 1, Norbaek et al., 1998). The major difference between C7 and C5 substitutions lay in the chemical shift difference of H6 and H8: $\delta H6$ in petunidin-3,7-di-β-D-glucopyranose was smaller than $\delta H8$ smaller while in petunidin-3,5-di-β-D-glucopyranose they are similar. But further approval needed to be done.

655 (Malvidin+2Hexose)

Based on the HPLC-UV/Vis-MS chromatogram (figure 3.30), the impure analyte was a mixture of 625-Peonidin-3,5-di-β-D-glucopyranose and 655-Malvidin+2Hexose. But 655 was the major component. The following assignment was based on comparison with 625. And there is also one reference
malvidin-3,5-di-β–D-glucopyranose (appendix 1, Mas et al., 2000).

![Structure of malvidin](image)

The signals that can be identified in 1D $^1$H spectrum were as follows:

- $\delta$H4=9.177ppm, $\delta$H6=7.096ppm (d, 2.4Hz), $\delta$H8=7.181ppm (dd, 0.6/1.8Hz), $\delta$H6’=$\delta$H2’=8.068ppm (brs), $\delta$HOMe=4.026ppm, $\delta$H1”=5.424ppm (d, 7.8Hz), $\delta$H1’’’=5.169ppm (d, 7.8Hz)

The aromatic protons were assigned depending on the coupling constant information. Four aromatic proton signals fitted malvidin. Two anomeric protons were assigned at $\delta$5.424 (d, J=7.8Hz) and $\delta$5.169 (d, J=7.8Hz), which indicated the hexoses were in β form. The chemical shifts here were very similar to those in malvidin-3,5-di-β–D-glucopyranose (appendix 1, Mas et al., 2000). But further approval needed to be done.

### 3.3.9. Summary

#### 3.3.9.1 Summary of spectra assignment
Table 3.15 Assignment of Proton in different anthocyanins

<table>
<thead>
<tr>
<th>Hexose</th>
<th>6''B</th>
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<td>s</td>
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<td>7.071</td>
<td>d</td>
<td>8.4 Hz</td>
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3.3.9.2 Deuteration

We have observed strange phenomena that the mass of the anthocyanins isolated increased by 2 amu after a long time of storage in the NMR solvent (CF3COOD-CD3OD). And the signals for H6 and H8 disappeared in 1H NMR spectra. It was probably due to deuteration.

Hydroxyl groups exchange with each other rapidly in the solution.

\[ \text{R-O-H}_a + \text{R'-O-H}_b \leftrightarrow \text{R-O-H}_b + \text{R'-O-H}_a \]

So the deuteration of H6 and H8 probably results from the proton exchange of the hydroxyl groups of C7. When the hydroxyl group -OH changes to -OD, the D atom goes to 6 or 8 position because of the equilibrium between ketone and enol.
3.4. Electrochemical Detection

3.4.1. Theories

3.4.1.1. Electrochemistry of Anthocyanins

Anthocyanins have been well-known for their antioxidant properties. It is the phenolic hydroxyl groups that confer such property (e.g. Fig.3.33). The catechol groups in ring B and the resorcinol groups in ring A can be oxidized electrochemically (example in Fig.3.33).

It was reported that the meta-hydroxyl groups (e.g. meta-OH on C5 and C7, Fig.3.33) was more difficult to be oxidized than the hydroxyl groups in the ortho- or para-positions (e.g. ortho-OH on C3’ and C4’, Fig.3.33) (Brenna et al., 1998/ Kożmiński & Brett, 2006). As a result, anthocyanins will be oxidized in several steps at
different potentials depending on the oxidation order of different OH groups.

Electrochemical detection of anthocyanins has been used by several research groups (e.g. Brenna et al., 1998/ Koźmiński & Brett, 2006/ Castro-Gamboa et al., 2003); Compared with UV detection, electrochemical detection is more sensitive and selective, which is good for detection of the anthocyanins with a smaller quantity.

3.4.1.2. Mechanism

Substances that are either reducible or oxidizable undergo a redox reaction at the surface of the electrode, which causes a current change and is detected by electrochemical detector. Fig.3.34 (a) is a simple illustration about the mechanism of an electrochemical cell: a pair of inert electrodes are placed in a cell and a potential is placed across the electrodes. If the potential is great enough, a reaction will occur and current will flow.

The electrolysis I is recorded as a function of time, and the peak area has the dimension “time * ampere” which corresponds to the total charge Q exchanged between the electrode and the electroactive analyte. The peak area is related to Q by eq. (1):

\[ \text{Peak area} = Q = \int I \, dt \]  \hspace{1cm} (1)

Faraday’s electrolysis law link Q to the electroactive analyte concentrations through equ. (2):

\[ Q = z * F * n_{\text{inj}} = z * [A] * V_{\text{inj}} * F \]  \hspace{1cm} (2)

Where \( z \) is the number of electrons transferred in the electrode process, i.e 2 in Fig. 3.33, \( n_{\text{inj}} \) is the number of injected mole analyte, \([A]\) is the concentration of the analyte, \( V_{\text{inj}} \) is the injection volume and \( F \) is Faraday’s constant = 96500 C/mol.

If the number of electrons \( z \) is known for the electroactive compound, it is possible to obtain the concentration of the analyte directly from the peak area in the HPLC-ECD chromatogram.
Fig3.34 (a) Simple illustration of the reaction in an electrochemical cell (two electrodes are placed in a solution containing an electroactive compound); (b) a real electrochemical cell—the auxiliary electrode (A) is held at a fixed potential by the first amplifier (Amp.1), the voltage being selected by the potentiometer (P) that is connected to a regulated power supply. The current flowing through the working electrode is processed by the second amplifier (Amp.2) and the output fed to the recorder or data acquisition system; (c) a symbol of the coulometric electrode system employing porous graphitic carbon electrodes—each electrode unit has a central porous carbon electrode, on either side of which is situated a reference electrode and a auxiliary electrode. As the pressure drop across the porous electrode is relatively small, these electrode units can be connected in series forming an array.

A basic electrochemical cell needs three electrodes (fig.3.34b & c): the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any changes in the background conductivity of the mobile phase). The electrochemical cell used was an ESA model 5011 high sensitivity analytical cell with a porous graphite working electrode with a large surface area which ensures 100% reduction or oxidation of the electroactive analyte. The current was measured with an output in “mV” (U=IR, U=voltage/mV, I= current/mA, R=resistor/Ω).

1 http://www.chromatography-online.org/HPLC-Detectors/Electrical-Conductivity
3.4.1.3 External Standard-Ferrocene

The well-known electroactive compound-ferrocene was used as an external standard. Ferrocene undergoes a one-electron oxidation at a low potential, around 0.5 V vs. a saturated calomel electrode (SCE). Oxidation of ferrocene gives a stable cation called ferrocenium, which can be used as an oxidizing agent. In the oxidation process of Fig. 3.35 the number of exchanged electrons per ferrocene molecule is equal to z = 1.

3.4.2. Materials and Methods

Materials

Acetonitrile and HCOOH were HPLC grade. All the water used was MilliQ water.
Eluent A: 2.5% HCOOH in H$_2$O with 2mM KCl;
Eluent B: 2.5% HCOOH in Acetonitrile
Ferrocene (Sigma-Aldrich, CAS No. 102-54-5) was dissolved in Methanol with a concentration 10mg/L.
The NMR samples were used directly in HPLC-ECD.

Methods

HPLC was a DIONEX spectra system and equipped with an ASI-100 auto sampler, P680 gradient pump, the analytical column was a 250mm Luna 5U C18 column

1 http://en.wikipedia.org/wiki/Ferrocene
from Phenomenex with an i.d. of 4.60mm. The Model of the UV/Vis detector was PDA-100; electrochemical detector (ECD) was ESA CoulochemII detector equipped with an ESA Model 5011 analytical cell.

Table 3.17 gave the gradient program for elution. Four different scanning wavelengths were set on UV/Vis detector: 252nm, 320nm, 365nm and 520nm. The electron potentials for ECD were set in a series of experiment from 50mV to 1000mV. The sensitivity was set to 10μA or 100μA according to different test, but the sensitivity for a given series of test was the same. For example the sensitivity for testing 611 (cyanidin-3,5-di-β-D-glucopyranose) was 100μA throughout the testing.

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<th>B%</th>
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Table 3.17 gradient program for HPLC-UV/Vis-ECD

### 3.4.3. Results & Discussion

Both the extract (pre-purified by HLB-MCX-HLB) and fractions from semi-preparative HPLC were analyzed from HPLC-UV/Vis-ECD. But in the spectrum of the total anthocyanin extract, it was difficult to tell from the overlapped peaks. So, it was better to use the purified fractions in analysis.

**Comparison with UV detector about the sensitivity:**

A comparison of the spectrum from UV (black, at 520nm) and ECD (blue) was shown in fig.3.36.
The sample used in analysis here was the same as the one used in HPLC-UV/Vis-MS (fig.3.9), so each peak was labeled by comparison with the spectrum of HPLC-UV/Vis-MS.

The ECD detection showed a much higher sensitivity than UV detection (fig.3.36). The intensity of peaks in ECD spectrum was a lot bigger than in UV spectrum. Besides, the overlapped peaks (625/655, 611/641) in the UV/Vis spectrum were distinguished in the ECD because of different redox properties.

Both UV/Vis detection and ECD detection can proportion to the concentration of the analyte (3.2.1 & 3.4.1). However, the analyte without redox properties could not be detected on ECD. But it was apparent in fig.3.36 that ECD has a higher sensitivity than UV/Vis detector.
Redox properties of different anthocyanins in *Nesaea Crassicaulis*

Fig. 3.37 A: Standard curve of ferrocene (black-trendline); B: Comparison of the oxidation potential of five different anthocyanins: 611-Cyanidin-3,5-di-β-D-glucopyranose, 627-delphinidin-3,5-di-β-D-glucopyranose, 625-Peonidin-3,5-di-β-D-glucopyranose, 449-Cyanidin-3-β-D-glucopyranose, 463-deduced to be Peonidin-3-β-D-glucopyranose

From fig 3.37 A, the redox potential of ferrocene was 300mV. This value was used as a reference potential in the following discussion. For example, the oxidation potential observed at 700mV was represent as 400mV vs ferrocene, which meant that analyte was more resistant to oxidation than ferrocene by 400mV.
Fractions Cyanidin-3,5-di-β-D-glucopyranose (611) and Cyanidin-3-β-D-glucopyranose (449) were derived from the same aglycone: cyanidin (fig.3.38), which has ortho-hydroxyl groups (C3'-OH and C4'-OH) and meta-hydroxyl groups (C5-OH and C7-OH). Depending on the rules that the meta-hydroxyl groups were more difficult to oxidize than the hydroxyl groups in the ortho- or para-positions (Brenna et al., 1998/ Koźmiński & Brett, 2006), the ortho-hydroxyl groups on the B ring should be first oxidized. The first oxidation potential of them was almost similar about at 450mV (150mV vs ferrocene). But Cyanidin-3,5-di-β-D-glucopyranose (611) which had one more O-glucose substitution seemed to be more resistant to oxidation. And the second oxidation potential of 611 was more than 950mV, while 449 underwent gradually oxidation and had no significant secondary oxidation potential.

The aglycone of delphinidin-3,5-di-β–D-glucopyranose (627) was delphinidin, which has one pair more ortho-hydroxyl groups than cyaniding (3’, 4’ 5’ of ring B). The area change between 200mV and 450mV was almost linear, indicating the oxidation of the three hydroxyl groups on the B ring. However, the curve reached plateau at 450mV (150mV vs ferrocene), same as the first oxidation potential of Cyanidin-3,5-di-β–D-glucopyranose (611) and Cyanidin-3-β–D-glucopyranose (449). And the second oxidation potential was almost similar to 611 at 950mV (650mV vs ferrocene).

Peonidin-3,5-di-β–D-glucopyranose (625) and 463 (deduced to be Peonidin-3-β–D-glucopyranose), which had peonidin as the aglycone seemed to be more resistant to oxidation. The first oxidation potential observed in 463 was 700mV (400mV vs ferrocene). And like Cyanidin-3-β–D-glucopyranose (449), 463
underwent gradual oxidation after the first oxidation potential. Peonidin differs from cyanidin in the methoxyl group of C3’ (fig.3.38), but the oxidation potential differs very much. So methoxyl substitution might make anthocyanins more resistant to oxidation and thus more stable in oxidizing agents.

Generally speaking, ortho-hydroxyl groups on the B ring (in the anthocyanins tested here) were oxidized at 450mV (150mV vs ferrocene). Methoxyl groups and glucose substitution might make anthocyanins resistant to oxidation.
4. Conclusion

Seven anthocyanins were isolated and identified from *Nesaea Crassicaulis*. Five of them were obtained from semi-preparative HPLC as very pure compounds, while four of them were abundant enough to acquire 1D $^1$H, $^{13}$C and 2D HMQC and HMBC spectra. So the molecular structures of these four anthocyanins were confirmed to be Delphinidin-3,5-di-β-D-glucopyranose (M.W.=627), Cyanidin-3,5-di-β-D-glucopyranose (M.W.=611), Peonidin-3,5-di-β-D-glucopyranose (M.W.=625) and Cyanidin-3-β-D-glucopyranose (M.W.=449). The other three of them were deduced to be Peonidin-3-β-D-glucopyranose (M.W.=463), Petunidin-3,5-di-β-D-glucopyranose (M.W.=641) and Malvidin-3,5-di-β-D-glucopyranose (M.W.=655). (463) was pure but not abundant enough to obtain all the NMR spectra, however very nice $^1$H spectrum was recorded. And based on the information in $^1$H spectrum and comparison with the assignment in literatures, it probably was Peonidin-3-β-D-glucopyranose (Fossen et al., 2002). (641) and (655) not only had a very small amount but also overlapped with the other anthocyanins, so it was too difficult to get pure compounds for NMR.

The four anthocyanins, whose molecular structures were confirmed (627, 611, 625, 449) and (463), were analyzed by electrochemical detector. (611), (627) and (449) had the same first oxidation potential at 450mV (150mV vs ferrocene). (449) and (463) underwent gradual oxidation and had no significant second oxidation potential after the first oxidation. Both of them had one glucose moiety less than the others, so the substitution of sugar moieties raised the antioxidant ability of anthocyanins. The oxidation of (625) and (463) was more gradual than all the other three and the first oxidation potential of (463) was 700mV (400mV vs ferrocene). So the methoxyl group on the B ring might also help to raise the antioxidant property.
5. Acknowledgement

I wish to thank my dear supervisor- Poul Erik Hansen, who provides me careful and outstanding instructions in almost every step of our project, and continuing encouragement and engagement during this study.

I am very grateful to and Professor Torben Lund, who give me a lot of suggestions and instructions in anthocyanins isolation and primary identification.

Thanks to Professor Søren Laurentius Nielsen who helps me in plant growth.

Thanks to Rita Buch and Annette Christensen, who help me ran all the NMR experiment. I quite appreciate the help from Jacob Krake, who has accompanied me in the whole experiment and helped me deal with all the instrument problems during the experiment.
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Wang H. et al., 2003. Characterization of Anthocyanins in Grape Juices by Ion Trap Liquid


### 7.1. NMR assignments for relevant anthocyanins

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**Aglycone**

| 4             | 9.24 d, 0.9Hz | 9.04 s | 9.26 d 0.9Hz | 9.07 | 8.97 br s |
| 6             | 7.16 d, 1.9Hz | 6.75 s | 6.78 d 1.9Hz | 6.72 | 6.85 d 1.8Hz |
| 8             | 7.18 dd, 0.9/1.9Hz | 6.98 s | 7.04 dd 0.9/1.9Hz | 6.98 | 7.28 d 1.8Hz |
| 2'            | 8.17 d, 2.3Hz | 8.11 brs | 8.16 d 2.2Hz | 8.24 | 8.00 br s |
| 5'            | 7.13 d, 8.8Hz | 7.09 d 8.2Hz | 7.50 d 8.8Hz | 7.10 |
| 6'            | 8.44 dd, 2.3/8.8Hz | 8.32 d br 7.9Hz | 8.35 dd 2.2/8.7Hz | 8.25 | 7.86 d (2.4) |
| OMe           | 4.08 | 3.98 brs |

**Ref**

- Fossen et al., 2003
- Torskangerpoll et al., 2005
- Fossen et al., 2002
- Fossen et al., 2003
- Norbaek et al., 1999

**solvent**

- TFA:CD$_3$OD(5.95)
- TFA:CD$_3$OD(5.95)
- TFA:CD$_3$OD(5.95)
- TFA:CD$_3$OD(5.95)
- TFA:CD$_3$OD(10/90)
### Table 1: Spectroscopic Data of Anthocyanins from *Nesaea Crassicaulis*

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**Aglycone**

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**Ref**

Norbaek et al., 1998  Norbaek et al., 1998  Mas et al., 2000  Norbaek et al., 1998  Norbaek et al., 1999

**Solvent**

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**Ref**

- Fossen et al., 2003
- Fossen et al., 2003
- Torskangerpoll et al., 2005
- Fossen et al., 2003
- Fossen et al., 2002

**solvent**

- TFA:CD3OD(5:95)
- TFA:CD3OD(5:95)
- TFA:CD3OD(5:95)
- TFA:CD3OD(5:95)
- TFA:CD3OD(5:95)
7.2. NMR spectra

- Proton NMR

![Proton NMR spectrum](image1)

- Carbon-13 NMR

![Carbon-13 NMR spectrum](image2)

- HMQC

![HMQC spectrum](image3)
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*
Supervisor: Poul Erik Hansen     Student: Jing Wang

HMBC
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*
Supervisor: Poul Erik Hansen     Student: Jing Wang
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

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$^1$H-NMR
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen  
Student: Jing Wang

$^{13}$C-NMR

HMQC
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

HMBC
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

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\(^1\)H-NMR
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen   Student: Jing Wang

$^{13}$C-NMR

HMQC
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

**HMBC**
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

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¹H-NMR
**Project:** Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

**Supervisor:** Poul Erik Hansen  
**Student:** Jing Wang

**13C-NMR**

**HMQC**
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*
Supervisor: Poul Erik Hansen     Student: Jing Wang

HMBC
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

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**HMBC**

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Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*
Supervisor: Poul Erik Hansen    Student: Jing Wang

![Graph 1](image1)

![Graph 2](image2)

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Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang
7.3. Data from HPLC-UV/Vis-MS

- **UV chromatogram (wavelength range from 520nm-540nm)**

![UV Chromatogram](image)

- **MS chromatogram**

![MS Chromatogram](image)

- **627**
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen  
Student: Jing Wang

**Figure 1:** UHPLC-MS/MS profiles of Anthocyanins from *Nesaea Crassicaulis*

- **RT:** 14.33-15.97 min  
- **AV:** 99 microA U
- **NL:** 7.85E4 microA U

- **Figure 2:** Spectral characteristics of Anthocyanins from *Nesaea Crassicaulis*

- **RT:** 14.74-16.06 min  
- **AV:** 27 microA U
- **NL:** 5.59E6 microA U

- **Figure 3:** ESI-HRMS of Anthocyanins from *Nesaea Crassicaulis*

- **RT:** 16.95-17.93 min  
- **AV:** 60 microA U
- **NL:** 8.52E5 microA U
Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

Supervisor: Poul Erik Hansen     Student: Jing Wang

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Project: Isolation and Identification of Anthocyanins from *Nesaea Crassicaulis*

**Supervisor:** Poul Erik Hansen  
**Student:** Jing Wang

- **RT:** 21.29-21.90  
  **AV:** 12  
  **NL:** 2.70E6

- **F:** + p ESI Full ms [120.00-1200.00]

- **wavelength (nm):**
  - 212.67
  - 217.66
  - 122.26
  - 655.02
  - 203.63
  - 331.29
  - 258.38
  - 337.29
  - 692.89
  - 166.70
  - 580.90
  - 189.65
  - 492.89
  - 580.90
  - 137.70
  - 463.08
  - 1107.87

- **m/z:**
  - 449.05
  - 287.32
  - 217.66
  - 451.24
  - 189.65
  - 896.58
  - 218.73
  - 766.38
  - 501.94
  - 730.53
  - 674.64
  - 331.23
  - 920.73
  - 550.50
  - 446.78
  - 654.75
  - 610.98
  - 802.77
  - 396.82
  - 127.12
  - 985.66
  - 1058.31
  - 827.32
  - 1193.96
  - 1169.34

- **449**

- **RT:** 21.79-22.35  
  **AV:** 37  
  **NL:** 3.27E5

- **F:** + p ESI Full ms [120.00-1200.00]

- **m/z:**
  - 463.00
  - 662.00

- **463** (because of longtime of storage, two Protons (H6 and H8) were deuterited—465)
7.4. Data from ECD

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