Rapid Separation of Anthocyanins and Flavonol Glycosides Utilising Discovery® DSC-MCAX Solid Phase Extraction

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Introduction

Flavonols and anthocyanins are water-soluble vacuolar flavonoid compounds that are synthesized by organisms of the plant kingdom. Flavonoids are widely distributed in plants fulfilling many functions including pigmentation and protection from UV light and attack by microbes and insects [1].

The chemical structure of the anthocyanidins is based upon the flavonoid family of molecules that is in turn based on the C6-C3-C6 configuration in the flavan nucleus [2]. Figure 1 illustrates the structure of the flavlyum ion which makes up the backbone of the anthocyanidins. The chemical structure of the flavonols is also based upon the flavonoid family of molecules where flavonols use the 3-hydroxyflavone backbone. Anthocyanins are anthocyanidins linked with one or more sugars that are sometimes acylated. Flavonols and flavones are glycosylated and acylated similarly to anthocyanins [1].

Figure 1 Flavlyum ion backbone of anthocyanidins & anthocyanins

Since 1992 more than 277 anthocyanins have been reported and more recent literature estimates the total number of identified anthocyanins at 550 [1]. Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancers and cardiovascular disease [3,4]. The benefi
cial effects of fruit, vegetables, and tea or even red wine have been attributed to flavonoid compounds rather than to known nutrients and vitamins [5].

Both anthocyanins and flavonols are typically characterised through the utilisation of reversed-phase HPLC with UV-VIS detection coupled to electrospray ionisation mass spectrometry (ESI-MS). Due to the complexity of the flavonol and anthocyanin isomeric structures that may exist in plant tissues, the separation of these molecules by RP-HPLC can lead to co-eluting compounds. The separation of the flavonols and anthocyanins prior to analysis by LC-ESI-MS would greatly facilitate the identification.

The DSC-MCAX mixed-mode cation SPE cartridges were used in the separation of anthocyanin-O-glycosides (positively charged) and flavonol-O-glycosides (neutral) in red tulip flower petals.

**Extraction of Anthocyanins from Tulip**

Red tulip blooms (*Tulipa darwin hybrid ‘Apeldoorn’*) were treated with 50/50 methanol/water with 0.1 % formic acid, ground with a glass stirring rod and placed in sonicator for several minutes to extract the anthocyanins and flavonols. The mixture was filtered to remove large particles.

**SPE Fractionation of Flavonol Glycosides and Anthocyanins**

A DSC-MCAX SPE cartridge, 100 mg/3 mL (52783-U), was conditioned with 1.5 mL of methanol and equilibrated with 1.5 mL of water containing 0.1 % formic acid. A small aliquot of tulip extract was diluted with an equal volume of water with 0.1 % formic acid and loaded onto the cartridge. The cartridge was rinsed with 1.5 mL water with 0.1 % formic acid solution (0.5 mL x 3). The flavonol glycosides were eluted with 1.5 mL methanol (0.5 mL x 3). The anthocyanins were eluted with 1.5 mL (0.5 mL x 3) of a 50/50 solution of potassium phosphate buffer pH 6.0 and methanol.

**Instrumentation – HPLC/DAD/ESI-MS/MS Analyses**

LC/ESI-MS/MS experiments were performed on an Agilent MSD XCT ion trap mass
spectrometer (Palo Alto, CA) equipped with an electrospray ionisation (ESI) interface, 1100 HPLC, a DAD detector, and Chemstation software. The column used was a 150 x 0.5 mm i.d., C18 phase with 5 µm particle size. Solvents were (A) 0.1 % formic acid/ 99.9 % water (v/v) and (B) 0.1 % formic acid/ 99.9 % acetonitrile (v/v). Solvent gradient was 0–20 min, 10–50 % B; 20–31 min, 50 % B; and 31–35 min, 50–10 % B. Flow rate was 0.6 mL/min, injection volume was 0.5 µL, and column temperature was 25 °C. The ion trap mass spectrometer was operated in positive ion mode scanning from m/z 100 to m/z 2200 at a scan resolution of 13000 amu/s. Representative chromatograms, PDA UV Spectrums, and MS results are depicted in Figures 3–4. Using this data, identities were proposed for the flavonol glycoside and anthocyanin extracts (Table 1).

![Figure 3](Image1)

**Figure 3** HPLC Trace of Tulip Extract Before and After SPE Fractionation

![Figure 4](Image2)

**Figure 4** Mass spectrum of anthocyanin & flavonol glycoside
Table 1 Proposed identities of flavonol & anthocyanin constituents

<table>
<thead>
<tr>
<th>RT</th>
<th>Proposed Identity</th>
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<tbody>
<tr>
<td>A</td>
<td>14.6 Unknown flavonol</td>
</tr>
<tr>
<td>B</td>
<td>16.5 Apigenin-genistein acetyl-rhamnosyl glucoside</td>
</tr>
<tr>
<td>C</td>
<td>17.0 Quercetin di-glucoside</td>
</tr>
<tr>
<td>D</td>
<td>17.7 Apigenin-genistein acetyl-rhamnosyl glucoside (isomer)</td>
</tr>
<tr>
<td>E</td>
<td>18.1 Kampferol rhamnosyl glucoside xyloside</td>
</tr>
<tr>
<td>F</td>
<td>18.6 Unknown isorhamnetin derivative</td>
</tr>
<tr>
<td>G</td>
<td>19.2 Luteolin rutinoside</td>
</tr>
<tr>
<td>H</td>
<td>20.1 Luteolin rutinoside (isomer)</td>
</tr>
<tr>
<td>I</td>
<td>13.9 Cyanidin rutinoside</td>
</tr>
<tr>
<td>J</td>
<td>15.2 Pelargonidin rutinoside</td>
</tr>
<tr>
<td>K</td>
<td>16.1 Pelargonidin acetyl-rhamnosyl glucoside</td>
</tr>
<tr>
<td>L</td>
<td>17.3 Cyanidin acetyl-rhamnosyl glucoside</td>
</tr>
</tbody>
</table>

Conclusion

Flavonol glycosides and anthocyanins are compounds that are found in plants, flowers and fruits. These compounds are involved in the protection of the plant from UV light and aid in pollination by producing brilliant colours to attract insects and animals. Because the structures of the flavonols and anthocyanins are very similar, it is extremely difficult to successfully separate and identify the compounds in plant extracts. The method described can be used as a simple and rapid separation of the flavonol glycosides and anthocyanins. By exploiting the different ionisation characteristics between the two compound classes, DSC-MCAX (mixed-mode cation exchange) was used as a fractionation tool prior to analysis. Once separated, the characterisation of both classes of compounds is made much easier and their potential uses can be more effectively evaluated.

References