Multi-Dimensional Fragmentation of Anthocyanins from Rose Petals by Ion Trap – Time-of-Flight (IT-TOF) Mass Spectrometry

Jeremy S. Barnes, Kevin A. Schug*
Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, TX
*corresponding author: 700 Planetarium Pl.; campus box 19065; Arlington, TX USA 76019-0065; (email) kschug@uta.edu; (phone) 817-272-3541; (fax) 817-272-3808

Abstract
A Shimadzu high performance liquid chromatography – ion trap – time of flight mass spectrometer (LCMS-IT-TOF) was used to aid in the identification of anthocyanin species in rose petals. The effects of varying the parameters which control the collision energy and the collision gas were observed on cyanidin-3,5-diglucoside and its related mono-glucoside and anthocyanidin fragment signals. Further variation in these parameters were used to optimize iterative multi-stage fragmentation (up to MS⁶) in order to elucidate the fragmentation pathway of cyanidin-3,5-diglucoside.

Keywords
Anthocyanins, flavonoids, rose, high mass accuracy, multi-dimensional fragmentation, electrospray, ESI-IT-TOF-MS

Introduction
Polyphenolic species are ubiquitous in nature, and are often found in complex mixtures. Anthocyanins are one of the many compound classes that fall under the polyphenolic flavonoid group, and are particularly well-suited for analysis by high performance liquid chromatography – mass spectrometry (LC-MS). This group of species is distinguished for their role in the bright red, blue and purple colors of berries and fruits and recently, is the focus of fervent health claims due to their antioxidant capacity. The core structure of an anthocyanin has a bi-phenolic structure, referred to as a flavylum cation, and may be described as a C6-C3-C6 skeleton. This skeleton has a phenolic ring fused to a pyran with an additional phenolic ring connected at the 2-position of the pyran. The flavylum cation is glycosylated, and the glycosides are often acylated, which allows for a multitude of potential structural and functional variants. The aglycone flavylum cation is referred to as an anthocyanidin. A positive charge is found on the oxygen of the pyran ring, which makes the anthocyanin (or anthocyanidin) well suited for positive-mode electrospray ionization (ESI) MS analysis. Specific detection of anthocyanins can also be achieved by virtue of their characteristic...
absorption at 520 nm. Separation of multiple species can be readily achieved through reverse phase liquid chromatography.

Analytical identification of these species can be extremely difficult without expensive and rare authentic standards and an extensive knowledge of the species common to the particular plant being analyzed. The petals of roses are well known for containing anthocyanins, which contribute to their eye catching colors. The anthocyanin content varies depending on which of the numerous rose species is being inspected. Often times, different anthocyanins are isobaric and can only be identified by retention time. Due to subtle changes in structure, it is difficult to confidently identify species without multiple identification tools. Thanks to the onset of technological innovation offered by the ion trap – time-of-flight mass spectrometer system provided by Shimadzu’s LCMS-IT-TOF, high mass accuracy and multi-dimensional higher order fragmentation can be used as a powerful tool to elucidate structural variants of anthocyanins and anthocyanidins based on fragmentation patterns.

To demonstrate these tools, the petals of a red hybrid tea rose (“Liberty”, *R. gallica*) was extracted and analyzed. Cyanidin-3,5-diglucoside is the major anthocyanin constituent found in the petal and was isolated by fraction collection to allow for further MS optimization studies. The collision energy and collision gas settings for the ion-trap can be systematically varied to enhance certain fragment signals. Higher order fragmentation may be limited to signal strength, especially in increasing mass stages. It was observed that little or no signal stops the ability to create additional orders of fragmentation. A higher order of MS^n fragmentation can be achieved by improving the signal strength. Supplementary high mass accurate fragment signals can lead to a better understanding of initial species identity, and more definitive identification of the compound. Determining the fragmentation pathways of individual species through optimized multi-dimensional fragmentation may also help provide a better means of identification of anthocyanin species in complex matrices when combined with chromatographic workflows.

**Materials and Methods**

*Sample Preparation*

Rose petals were ground to a powder in a coffee grinder, and then lyophilized. Powder (0.1 grams) was extracted in 70:30:0.1, methanol/water/trifluoroacetic acid (1 milliliter), then centrifuged and filtered to remove any residual solids. Cyanidin-3,5-diglucoside was isolated in a solution by eluent collection using a Foxy Jr Fraction Collector in combination with reversed phase liquid chromatography for use in ion-trap optimization experiments, via direct infusion.

*Instrumentation*

Anthocyanin identification was performed on a LCMS-IT-TOF (Shimadzu Scientific Instruments, Kyoto, Japan), equipped with a surveyor HPLC system (LC-20AD pumping system, a SIL-20AHT autosampler, SPD-M20A diode array detector). Shimadzu’s *LCMS Solution* software was employed for data analysis. The Formula Predictor function of *LCMS Solution* was utilized in identification and confirmation of unknown signals. Ionization was performed using a conventional ESI source, in the positive ionization mode. The instrument was calibrated to < 5 ppm error in mass accuracy with an external standard of sodium TFA solution.

For HPLC-ESI-MS, a Gemini C18 column (2.0 mm x 100 mm, 3µm; Phenomenex, Torrance, CA, USA) and a Gemini C18 guard column (4.0 x 2.0 mm, 3µm; Phenomenex, Torrance, CA, USA) were used to chromatographically separate anthocyanin species using 1.0% formic acid as solvent A and acetonitrile as solvent B. The elution scheme was: linear gradient from 5% B to 10% B, 0-5 min;
isocratic elution 10% B, 5-30 min; linear gradient from 10% B to 13.5% B, 30-75 min; linear gradient from 13.5% B to 25% B, 75-90 min. The heat block and curved desolvation line (CDL) were maintained at 250 °C. Nitrogen was used as the nebulizing gas and drying gas, set at 1.5 L/min and 10 L/min, respectively. The ESI source voltage was set at 4.5kV and the detector voltage was set at 1.62 V.

Ion-Trap Optimization
The ion accumulation time was set at 50 msec. Precursor ion isolation parameters were set at a width of 3.000 amu and 20 msec. The energy and collision gas CID parameters were varied as demonstrated in the results, while keeping the activation time and frequency (q) constant at 30 msec and 0.251, respectively.

Results
Variation in the collision energy and collision gas parameters of the ion trap provided improved signal strength; the intensities of the higher order stage fragments were optimized by selecting the most efficient fragmentation settings. In the first CID (MS$^2$), the signal for the neutral loss of a single glucoside can be maximized using a 50% collision gas and 25% collision energy (Figure 1). Additional CID (MS$^3$) of cyanidin-glucoside results in further neutral loss of a glucoside, taking only a relatively low amount energy.

![Figure 1: Optimization of the major fragment ions of cyanidin-3,5-diglucoside by varying the collision gas and collision energy parameters. Colors of boxed species match data plots and chart outlines. The default setting of collision gas at 50% appears to be the optimal setting for this parameter.](image)
CID of the anthocyanidin signal can provide further identification of the species based on two observed fragmentation classes, retro-diels alder fragments and small neutral molecule losses (Figure 2). Adjustment of the collision energy maximizes the desired fragment signals (Figure 3). Continued multi-stage fragmentation can be pursued (in our case down to MS_6, Figure 4), providing adequate signal to trace multiple fragmentation pathway (Figure 5), which may be elucidated with the help of high mass accuracy and the Formula Predictor option in the LCMS Solution software.

Figure 2: Anthocyanidin fragments can be divided into two categories, Retro-Diels Alder (RDA) fragments and small molecule loss fragments. Anthocyanidins vary mainly by substitutions on their "B" ring (labeled R), which allows for identification of anthocyanidin types based on the tracking of these fragments in particular.

Figure 3: The ratio of observed RDA fragmentation of cyanidin changes based on the amount of collision energy used. Values represent fragmentation at the MS stage, with collision gas set at 50%.
Figure 4: Fragmentation pathway of cyanidin-3,5-diglucoside up to MS\textsuperscript{6}, using optimized CID parameters.
Figure 5: Proposed fragmentation pathway of cyanidin, determined from multi-stage CID using high mass accuracy and Formula Predictor.

Figure 6: Plot of mass accuracy of cyanidin-3,5-diglucoside and associated proposed fragment signals with energy at 25% and collision gas at 50%. As the event stage increases, mass accuracy remains consistent. Some deterioration in mass accuracy may occur when the signal intensity of the tracked ions becomes very low.
Conclusion
The LC-MS-IT-TOF from Shimadzu is a powerful tool for identification of naturally occurring polyphenolic compounds. This instrument provides the capability for higher order fragmentation and high mass accuracy measurement which were used to identify the anthocyanin species found in extracted rose petals. Signals up to MS^5 were used to elucidate the fragmentation pathways of cyanidin, the aglycone of the major anthocyanin constituent found in the rose petal. These experiments are fundamental to the design of an on-line method for rapid speciation of anthocyanins when IT-TOF mass spectrometry is combined with high efficiency chromatographic separations.

References
J.S. Barnes et al., J. Chromatogr. A, 2009, 1216, 4728-4735