Preparative HPLC for the Purification of major Anthocyanins from Ficus padana burm.L

Syukri D.*, Darwis D. and Santoni A.
Department of Chemistry, Andalas University, West Sumatra, INDONESIA

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Abstract
Anthocyanin can be purified from raw extract of Ficus padana burm.L by applying preparative high performance liquid chromatography (HPLC). For every separation process, the chromatographic condition need to be optimized in order to gain the efficient purification process instantly. The current study shows that analytical scale chromatographic condition can be used as a condition for preparative HPLC. Two known acylated anthocyanins are isolated from the Ficus padana burm.L by Step gradient polarity preparative HPLC. The structures are set based on spectrometric analysis. The data from each obtained anthocyanin is being compared with both mass spectral data and published data. The first compound (peak 1) was identify as a pelargonidin 3-(6”-p-coumarylglucoside)-5-(4’’-Malonylglucoside) and the second compound was identify as a pelargonidin 3-(6’’-Malonylglucoside).

Keywords: preparative HPLC, anthocyanin, Ficus padana burm.L

Introduction
Color is an important factor determining fruit outer quality which has been used successfully in the characterization of fruits\(^1\). This property has an important effect on overall acceptability to the consumer\(^2\). Anthocyanins are responsible for most of the red, blue and purple colors of fruits, vegetables, flowers and other plant tissues or products\(^3\)\(^-\)\(^6\). The isolation and identification of anthocyanins are difficult as a result of their ability to undergo structural transformations and complex reactionary. Moreover, they are difficult to be analyzed independently from other flavonoids because they have similar reaction characteristics\(^7\).

Preparative liquid chromatography is widely accepted as an outstanding purification technique that developed at this time. The purification processes can be quickly gained very rapidly by developing of preparative liquid chromatography, so that it can be operated efficiently to produce pure compounds. The most important factors in preparative liquid chromatography such as the cycle time and the separation factor can be received from analytical-scale chromatographic data. Optimization of a preparative high-performance liquid chromatography (HPLC) separation is needed to achieve high purity result. This paper describes a preparative HPLC method procedure can be adopted from analytical-scale method where it can used to predict optimal preparative operation conditions for the separation.

For theseparation studied, the most critical parameter is to separate a specified amount of pure compounds is the production rate. Furthermore, the separation should obtain the very pure compounds and have a high product recovery as well, so that re-inject mixed of the fractions no longer needed. By following operating procedure will be useful to gain a little or no overlapping of the chromatographic bands\(^8\).

The structure of the anthocyanins were elucidated by comparing with the spectral data of published data\(^9\), and confirming by the aid of high-resolution electron spray-mass spectrometry (ESI-MS).

Material and Methods
Plant samples: The fruit samples were picked at Andalas University botanical garden in West Sumatera, and transported to the laboratory immediately.

Chemicals: HPLC-grade water, methanol, ethanol and acetonitrile, citric Acid, hydrocholric acid, formic acid were obtained from Merck, Germany. All other chemicals used in this study were analytical grade.

Instrumentation: A Shimadzu HPLC equipped with fraction collector (FRC-10A) were used for anthocyanin purification and an Agilent LC-MSD 6100 series, equipped with a DAD and ESI-MS detector were used for identification analysis.

Procedure: Extraction of anthocyanins: Acidified of ethanol pH 1.5 were prepared by mixed of ethanol with citric acid 35 %(3:7). 200 ml acidified ethanol was added into 1000 ml Erlenmeyer flask containing 100 g fruit. Anthocyanins were extracted at room temperatur for 6 hours in dark environment; this procedure was repeated three times to collect the extract solution. The extraction was concentrated under vacuum at room temperatur using a rotary evaporator until left 1/3. About 10 ml
of extracted solution was passed through a 0.45 μm millipore filter for analysis.

**Semi-preparative HPLC for purification of each fraction:** Preparative reversed-phase HPLC was performed Shimpack PRC-ODS (250 X 20 mm id, 5 μm, shimadzu). The mobile phase was determined based on the method of Huang et al.². (A) 2% formic acid solution, and (B) acetonitrile: water : formic acid (49 : 49 : 2). The gradient was start from 6 to 10% B for 4 min, from 10 to 25% B for 8 min, isocratic 25% B for 1 min, from 25 to 40% for 7 min, from 40 to 60% for 15 min, from 60 to 100% for 5 min, from 100 to 6% for 5 min, at a flow rate of 10.0 ml/min. Injection volumes were 5.0 mL, and the detection wavelength was 516 nm.

**HPLC-DAD-ESI-MS analysis:** Agilent Zorbax SB-C18 column was used. The condition of MS were as follows: ESI interface, positive ion model, 35 psi nebulizer pressure, 10 L/min dry gas flow rate, 350°C dry gas temperature and scans at m/z 150 to 1000. All analyses were duplicated.

**Results and Discussion**

**Extraction of anthocyanin:** Solvent extraction processes is the first step for isolation of anthocyanin pigment from plants. Anthocyanins are polar molecules and consequently more soluble in polar solvents, however extraction conditions are also key factors in their overall solubility. Alcoholic extraction is suitable for extracting anthocyanins from fruits and vegetables it shown in the previous research in study of anthocyanin from purple-fleshed sweetpotato powder, purple corn, red and black currants, and grapes. In the extraction process of anthocyanin from particular corps in plants solvent type, solvent concentration, solid–liquid ratio (solid loading), incubation temperature and incubation time are important for the stability and concentration of anthocyanins. Methanol is the most suitable solvent for the anthocyanin extraction process, but it has more toxic and hazardous to handle comparing with the other alcohols. Ethanol is a good option for replacing methanol because it has less toxicity and can also recover anthocyanins with good quality characteristics. The use of acid at the extraction process is to stabilize anthocyanins in the flavylium cation form, which is red at low pH. Hydrochloric acid is commonly used for solvent acidified but it may hydrolyze acylated anthocyanins, to avoid or at least minimize the breakdown of acylated anthocyanins, organic acids such as acetic, citric or tartaric acids, which are easier to eliminate during anthocyanin concentration, have been preferred.

**Test for anthocyanins:** The red, purple and blue colors found in many plants are due to two classes of water soluble pigments: anthocyanins and betacyanins. The anthocyanins are flavonoids, a class of phenolic molecules that are synthesized through the Shikimic acid pathway and are widespread in the plant kingdom. Betalains, a group of pigments that includes the betacyanins are indole-derived alkaloids and contain nitrogen. The extracts in acidiified ethanol were tested for the presence of anthocyanins by observing pigment color under acidic conditions by adding HCl. 3 ml of extract and 3 ml HCl were mixed in a test-tube and then placed in vessel with boiling water for 5 min. The mixture was stable and did not lose color when boiled indicated the presence of anthocyanins in the extracts.

**Preparative HPLC:** The term preparative HPLC is usually associated with large columns and high flow rates. However, it is not the size of the instrumentation or the amount of mobile phase pumped through the system that determines a preparative HPLC experiment, but rather the objective of the separation. The objective of an analytical HPLC run is the qualitative and quantitative determination of a compound. Fora preparative HPLC run it is the isolation and purification of a valuable product. Since preparative HPLC is rather expensive technique, compared to traditional purification methods such as distillation, crystallization or extraction, it had been used only for rare or expensive products. With increasing demand for production of high quality compounds in varying amounts for activity, toxicology and pharmaceutical screenings the field of operation for preparative HPLC is changing.

In this study, separated anthocyanin’s preparative HPLC peak fraction of the crude of sample were analyzed by analytical HPLC under the optimum conditions. Analytical conditions was used initially for the separation of anthocyanins. The non polar stationary phase and step gradient polarity system of mobile phase are selected to separated polar molecules of anthocyanins. The change the solvent strength of the mobile phase has be done because anthocyanins compounds will have different polarity depend of their structure. Figure-1 showed the anthocyanin profile of the extract using the preparative HPLC equipped with DAD chromatograms at 516 nm. A total of two anthocyanin compounds were identified by their elution order. The anthocyanins give two different colour that depend on their characteristic and concentration in the extract (figure-2).

By comparing the m/z of each anthocyanin molecule and its fragmentation to prepared database. Peaks 1 and 2 showed identical molecular ions at m/z 828 and 519. The first compound (peak 1) was identify as a pelargonidin 3-(6”-p-coumarylglucoside)-5-(4””-Malonylglucoside) (figure-3) and the second compound was identify as a pelargonidin 3-(6””-Malonylglucoside) (figure-4).
**Figure-1**
HPLC chromatograms of extract of *Ficus padana burm.* L. fruits (DAD, 516 nm)

<table>
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<tr>
<th>Name</th>
<th>Ret Time</th>
<th>Peak start</th>
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**Figure-2**
HPLC preparative fraction of anthocyanin compound of extract of *Ficus padana burm.* L. fruits
pelargonidin 3-(6"-p-coumarylglucoside)-5-(4"'-Malonylglucoside)

**Figure-3**
Structure and MS data of anthocyanin peak 1

pelargonidin 3-(6"-malonylglucoside)

**Figure-4**
Structure and MS data of anthocyanin peak 2
Conclusion

From the chromatogram as seen in figure 1 showed that analytical chromatographic experiment alone are useful for the prediction of scale-up conditions of preparative HPLC separations. There is two major anthocyanin that separated by preparative HPLC. By comparing their elution orders and mass spectrometric characteristics with published data in the literature one anthocyanadin were tentatively identified in Ficus padana burn.L samples. The anthocyanins showed pelargonidin aglycon and pelargonidin 3-(6’’-Malonylglucoside) was the major anthocyanin pigment that accounting for 94.1% of total anthocyanin.

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References

4. Manetas Y., Why some leaves are anthocyanic and why most anthocyanic leaves are red? _Flora_, 201, 163-177 (2006)