Research Journal of Chemical Sciences ISSN 2231-606X
Vol. **3(12),** 60-64, December **(2013)** *Res. J. Chem. Sci.*

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

Available online at: www.isca.in, www.isca.me
Received 8th December 2013, revised 12th December 2013, accepted 18th December 2013

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 preparativehplc. 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 becouse they have similar reaction characteristics[7].

Preparative liquid chromatographyis 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 operatedefficiently 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 parameterto 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-injectmixed 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 comparingwith the spectral data of published data[9], and confirming by the aid ofhigh-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, citricAcid, hydrocholric acid, formic acid were obtained from Merck , Germany. All other chemicals used in this study were analytical grade.

Instrumentation: A Shimadzu HPLC equiped 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 containing100 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 vacum 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 weredetermined based on the method of Huang *et al*[7], (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[10]. Anthocyanins are polar molecules and consequently more soluble in polar solvents, however extraction conditions are also key factors in their overall solubility[11,12]. 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 temperatureand incubation time are important for the stability and concentration of anthocyanins[12-17]. 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 is has less toxicity and can also recover anthocyanins with good quality characteristics[10]. The use of acid at the exctraction process is to stabilize anthocyanins in the flavylium cation form, which is red at low pH[18]. 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[19].

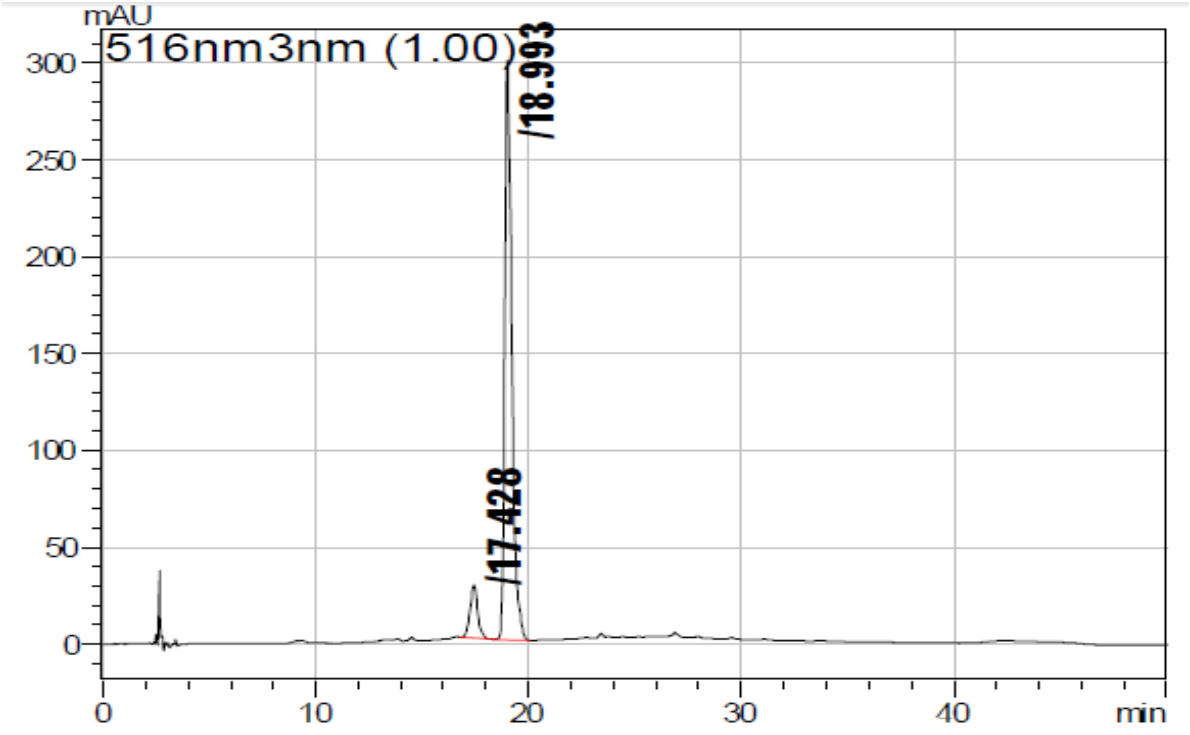
Test for anthocyanins: The red, purple and blue colors found in many plants are due to two classes of water soluble pigments: anthocyanins and betacyanins. The anthocyanins are flavonoids, a class of phenolic molecules that are synthesized through the Shikimic acid pathway and are widespread in the plant kingdom. Betalains, a group of pigments that includes the betacyanins are indole-derived alkaloids and contain nitrogen. The extracts in acidied 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.

Pre**parative HPLC:** The term preparative HPLC is usually associated withlarge columns and high flow rates. However, it is not thesize of the instrumentation or the amount of mobile phasepumped through the system that determines a preparativeHPLC experiment, but rather the objective of the separation.The objective of an analytical HPLC run is the qualitativeand quantitative determination of a compound. Fora preparative HPLC run it is the isolation and purificationof a valuable product. Since preparative HPLC isa rather expensive technique, compared to traditionalpurification methods such as distillation, crystallization orextraction, it had been used only for rare or expensiveproducts. With increasing demand for production of highlypure compounds in varying amounts for activity, toxicologyand pharmaceutical screenings the field of operation forpreparative HPLC is changing.

Preparative HPLC is used for the isolation and purification of valuable products in the chemical and pharmaceutical industry as well as in biotechnology and biochemistry. Depending on the working area the amount of compound to isolate or purify differs dramatically. For identification and structure elucidation of unknown compounds in synthesis or natural product chemistry it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams[20].

In this study, separated anthocyanin'spreparative HPLC peak fraction of thecrude of sample were analyzed by analytical HPLC under the optimum conditions. Analytical HPLCconditions 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 equiped 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).



Name	Ret Time	Peak start	Peak End	Area	% Area
A	17.428	16.833	18.200	209099	8.592546
B	18.993	18.333	20.033	2224394	91.40745

Figure-1
HPLC chromatograms of extract of *Ficus padana burm.L* fruits (DAD, 516 nm)

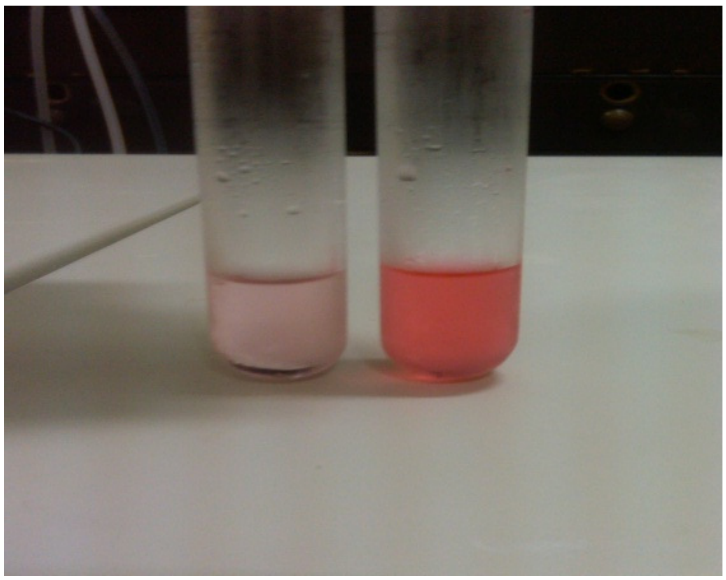


Figure-2
HPLC preparative fraction of anthocyanin compound of extract of *Ficus padana burm.L* fruits

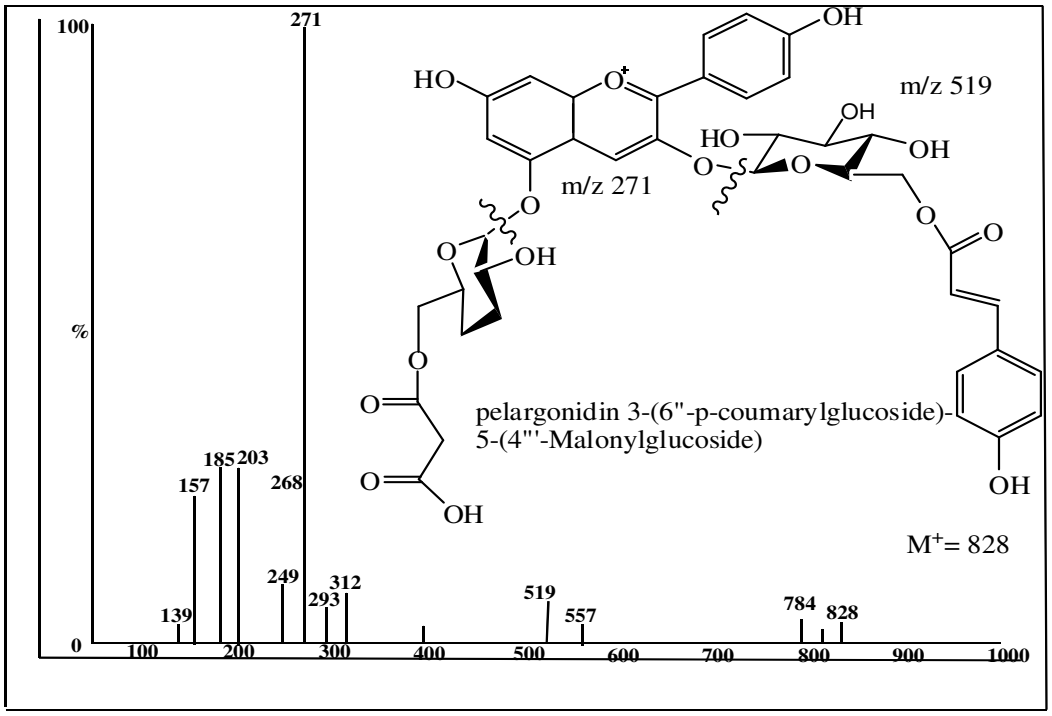


Figure-3
Structure and MS data of anthocyanin peak 1

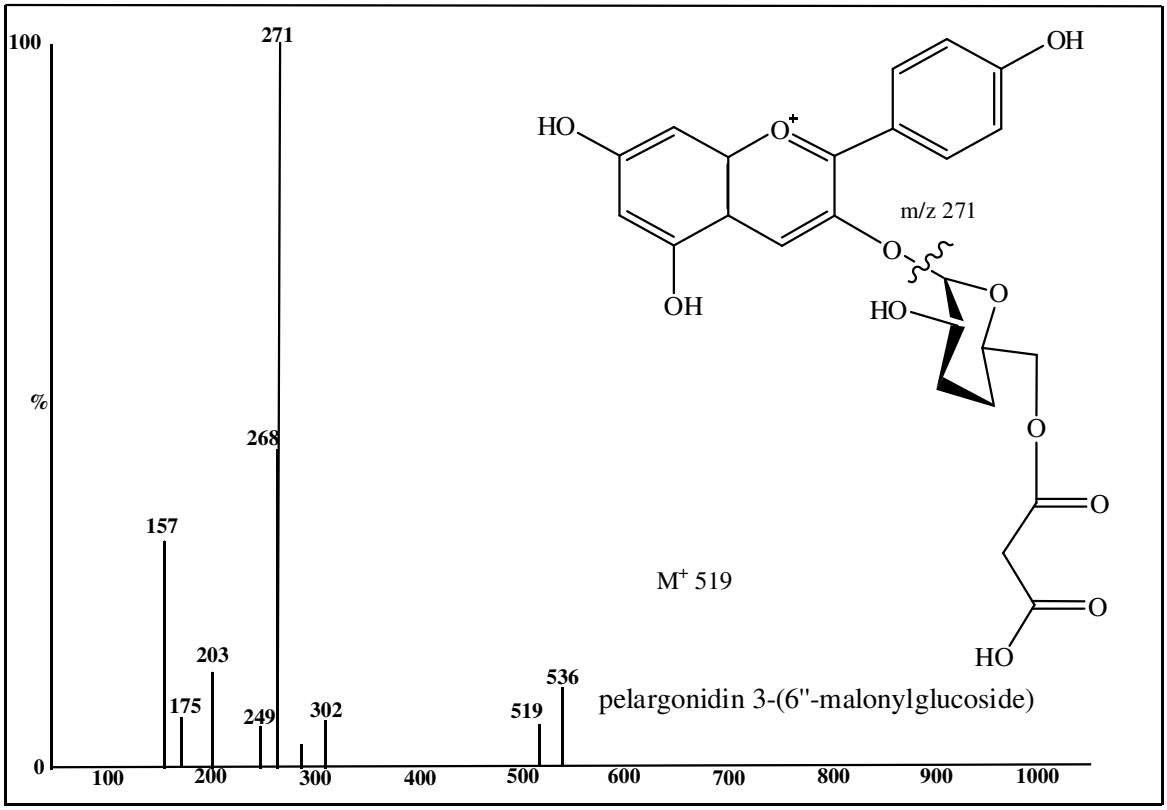


Figure-4
Structure and MS data of anthocyanin peak 2

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 anthocyanidin were tentatively identified in *Ficus padana burm.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.

Acknowledgement

The authors would like to thank Professor Fauzan Azima for giving granted to use the laboratory instrumental and Mr. Ario Betha Juanssilfero,M.Sc CPE for helpfull discussion on laboratory research.

References

1. Gautier-Hion A., Duplantier J.M., Quris R., Feer, F., Sourd C., Decoux J.P., Dubost G., Emmons L., Erard C., Hecketsweiler P., Fruit characters as a basis of fruit choice and seed dispersal in a tropical forest vertebrate community, *Oecologia*, **65**, 324-337 **(1985)**

2. Gamble J., Jaeger S.R. and Harker F.R., Preferences in pear appearance and response to novelty among Australian and New Zealand consumers, *Postharvest Biol Technol*, **41**, 38-47 **(2006)**

3. Gould K.S., McKelvie J. and Markham K., Do anthocyanins function as antioxidants in leaves? Imaging of H2O2 in red and green leaves after mechanical injury, *Plant Cell Environ*, **25**, 1261-1269 **(2002)**

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

5. Steyn W.J., Wand S.J.E., Holcroft D.M. and Jacobs G., Anthocyanins in vegetative tissues: a proposed unified function in photoprotection, *New Phytol*, **155**, 349-361 **(2002)**

6. Stintzing F.C. and Carle R., Functional properties of anthocyanins and betalains in plants, food, and in human nutrition, *Trends Food Sci. Tech*, **15**, 19-38 **(2004)**

7. Huang W., Shao-ling Zhang., Gai-hua Qin., Le Wenquan., Jun Wu., Isolation and determination of mayor anthocyanin pigments in the pericap of P.Communis L. Cv.'Red Du Comines' and their assoviation with antioxidant activity, *African Journal of Agricultural Research*, **7(26)**, 3772-3780 **(2012)**

8. Liu Y., Mukarami N Wang L., Si Zhang., Preparative Hing-Perfoemance Liquid Chromatography.,Preparative High-Performance Liquid Chromatography for the Purification of Natural Acylated Abthocyanins from Red Radish (*Raphanus sativus L.)., Journal of Chromatography Science,*46.743-746(2008)

9. www.lipidmaps.org,.*http://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=ProcessClassSearch&LMID=LMPK12*,.Retrieved on: November 10 **(2013)**

10. Kong J., Chia L., Goh N., Chia T., Brouillard R., Analysis and biological activities of anthocyanins. *Phytochemistry* 64. 923–933 **(2003)**

11. Delgado-Vargas, F., Paredes-Lopez, O., Natural Colorants for Food andNutraceutical Uses. *CRC Press, Boca Raton, FL*, p. 326 **(2003)**

12. Oki, T., Masuda, M., Furuta, S., Nishiba, Y., Terahara, N., Suda, I., Involvement of anthocyanins and other phenolic compounds in radical-scavengingactivity of purple fleshed sweet potato cultivars*, J. Food Sci*. 67 (5).1752–1756 (2002)

13. Pascual-Teresa, S., Santos-Buelga, C., Rivas-Gonzalo, J.C., LC–MS analysis of anthocyanins from purple corn cob*, J. Sci. Food Agric*, 82.1003–1006 **(2002)**

14. Lapornik B., Prosek M. and Golc Wondra A., Comparison of extracts prepared from plant by-products using different solvents and extraction time, *J. Food Eng*, **71(2)**, 214–222 **(2005)**

15. Jing P. and Giusti M., Effects of extraction conditions on improving the yield and quality of an anthocyanin-rich purple corn (Zea mays L.) color extract, *J. Food Sci*, **72(7)**, C363–C368 **(2007)**

16. Fan G., Han Y., Gu Z. and Chen D., Optimizing conditions for anthocyanins extraction from purple sweet potato using response surface methodology (RSM), *LWT Food Sci Technol*, **41**, 155–160 **(2008)**

17. Steed L.E. and Truong V.D., Anthocyanin content, antioxidant activity, and selected physical properties of flowable purple-fleshed sweetpotato purees, *J.Food Sci*, **73(5)**, S215–S221 **(2008)**

18. Rivas-Gonzalo J., Analysis of polyphenols. In Methods in Polyphenols Analysis; Santos-Buelga, C., Williamson, G., Eds.;Royal Society of Chemistry (Athenaeum Press, Ltd.), Cambridge,U.K. 95-98, 338-358 **(2003)**

19. Strack D. and Wray V., Anthocyanins. In Methods in Plants Biochemistry; Dey, P. M., Harbone, J. B., Eds.; Academic Press: San Diego, CA, Vol. 1*: Plant Phenolics,* 325- 359 **(1989)**

20. Huber U. and Majors R.E., Principle in preparative HPLC., *Agilent Technologies 5989-66EN* **(2007)**