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IDENTIFICATION OF GLYCONE TYPES IN THE CROWN FLOWER OF BATU LOCAL ROSES USING LC-MS ANALYSIS

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ABSTRACT

Rose flower is symbol of love that has plenty of benefits. It can be used for preventing and treating various diseases. Rose is known to contain anthocyanin pigments in which a glycone compound is bonded to aglycone as its anthocyanidine. Anthocyanins of Batu local rose were characterized as cyanidin and malvidin-glycosides. This study aimed to identify the type of glycone contained in the crown of roses using LC-MS (Liquid Chromatography Mass Spectrometry) analysis. Research was conducted by observing changes in the content of anthocyanin pigment of roses stored for few days and by identifying the type of glycone contained in the crown of roses. Selected local roses from Punten, District of Sidomulyo, Batu City, Indonesia were used to improve the usability of these widely cultivated roses. Anthocyanin pigment isolates were obtained from C18 column chromatographic fractionation (Shepadex G25 as stationary phase, Shimadzu). Prior to isolation, anthocyanin pigments were extracted using aquadest and lactic acid (with ethanol, HCl, BAA, and BuOH-HCl as developer materials), then were analyzed using a TLC plate. LC-MS analyses in molecular weights indicated that the crown of roses contained six types of glycone namely maltose (180), glucose (162), rhamnose (146), acids coumaril (146), xylose (132), and rutinose (308) m/z.

Keywords: anthocyanins, glycone, roses of Batu, LC-MS.

1. INTRODUCTION

Some people prefer food with attractive colors; therefore food coloring/dye is an important factor in food processing. A safe and healthy food coloring has been chosen by consumer, which is commonly obtained from natural dyes/pigments. These dyes may also contribute as an antioxidant due to its content of polyphenols.

Natural dyes are dyes (pigments) obtained from natural materials such as plant, animal,or mineral sources. Natural dyes are preferable because they are not harmful if consumed. Natural pigments or dyes, however, generally have a lower level of color stability compared to that of the synthetic dyes. One of the natural dyes commonly foundis anthocyanin. Anthocyanins are natural pigments found in many plant (fruits and flowers) organs such as strawberries, dragon fruit, grape, pomegranate, purple sweet potato, rose, canna flower, turired,and hibiscus. Source of anthocyanin pigments could be easily found in Indonesia.Roses (*Rosa sp.)*, for example, are widely cultivated in the area of Batu, Pasuruan,and Nganjuk- East Java, Indonesia (Saati*et al.,* 2012).

Roses are flower symbol of love with lots of benefits.It can be used to prevent and treat various diseases. Types of roses commonly loved by Indonesians are hybrids and Batu local. Both of these varieties have more variations among others, such as white, pink, crimson,and yellow. The Batu local roses are known to contain anthocyanin pigments of manifold cyanidin and malvidin-glycosides (Saati*et al.,* 2012).It contains sugar components bound by the aglycone via a glycosidic-bond. Anthocyanin dye is composed of the aglycone form of anthocyanidins glycone esterified with sugar molecules. A heating process may result in the breaking down of anthocyanin pigments into anthocyanidins and sugar.The type of sugar commonly encountered in anthocyanin is glucose, galactose, arabinose, and xylose (Rein, 2005). This study intends to identify the type glicone contained in the crown of Batu local roses using LC-MS (Liquid Chromatography Mass Spectrometry) analysis method.

2. THEORETICAL BASIS

Anthocyanins are flavonoid pigments, found in large quantities of fruits and vegetables. The main structure of anthocyanin is characterized by the presence of two aromatic rings of benzene (C_6H_6) - three carbon atoms linked to form a ring (Talavera*et al*., 2003). In plants, they are in the form of glycosides which bind monosaccharides (glucose, galactose, rhamnose).

Anthocyanin dissolves easily in water or polar solvents,more stable in acidic conditions (Rein, 2005), and gives the appearance of red, orange, purple and blue colors (Nollet, 1996). It has a maximum wavelength of515-700nm (Zussiva and Laurent, 2012). Anthocyanins are believed to provide benefits to human health and is absorbed in the form of the molecule intact in the stomach(Passamonti*etal.*,2003).

The heating process can lead to the loss of the glycosyl anthocyanins with glycosidic bond hydrolysis, resulting inthe breaking of anthocyanin pigments into anthocyanidins and sugar (Francis, 1989).The diversity of anthocyanins may occur due to differences in the nature of sugar, sugar unit number, and the location of sugar bond. Sugar cluster in anthocyaninis very varied,but mostly is in the form of glucose, rhamnose, galactose, orarabinose. These sugar molecules can have an impact on the stability of anthocyanin molecules.Acylation of the third sugar molecule normally forms ferulic, p-coumaric, cafeice, malonic, oracetice acids (Francis, 2000).

3. MATERIALS AND METHODS

3.1 Materials and equipment

Roseswere obtained from flower farmers in Punten Batu and its surroundings.Samples are Batu local and hybrids Holand (Netherlands)rose varieties, with the age of 3 months-old after grafting. Chemicals used were of analytical grade such as methanol, ethanol, citric acid, malic acid, gallic acid, sulfuric acid, lactic acid, hydrochloric acid, hexane, acetone, petroleum ether, sodium sulfate, and potassium bromide. Whatman paper no. 2 and no. 41, silica gel (white powder), alumina, glasswool, kieselgel TLC plates GF 254, C18 (Shepadex G 25), were also used. Some equipments such as rotary vacuum evaporator (Heidolph VV 2000), C18 column (Shimadzu CTO-10 AS VP; SCL-10 A VP), UV-Vis spectrophotometer (Spectronic Thermo Genesys 20), HPLC (10AVPJapan), LCMS/Liquid chromatography-mass spectrometry (Mariner Biospectrometry), pH meter (CG 832 Gerale Scholl), Color Reader (CR 10 Konica Minolta), analytical balance (Pioneer), vacuum filter (2X-0.5 VWR Scientific) wereused throughout the research.

3.2 Material preparation

Anthocyanin pigments from crown of roses were extracted using aquades and citric acid as solvent with the ratio of 95 to 5. The obtained extracts were macerated while stored in a refrigerator for 30 minutes at temperature of 10ºC-12ºC, then centrifuged for 10 minutes at a speed of 4000 rpm. The filtrate was then filtered through a Whatman no. 41 filter paper and concentrated by using a rotary vacuum evaporator at temperature of 50°C. Isolates were then obtained after fractionation using C18 column (Shimadzu: with 100% acetonitrile and 4% formic acid)(Kim*et al.,* 2008).

3.3 Fractionation of pigments and glycone

Pigment component separation and fractionation of sugar as glycone and anthocyanins were performed gradually using fractionation column with silica gel as stationary phase, these include a white powder (5 cm), alumina (5 cm), adsorbent Na_2SO_4 (5 cm), and glass wool at the bottom. Fractionation of pigment were conducted using the developer phase of methanol-HCl 1% with hexane (ratio of 6 to 4) as many as 15 mL, then proceed with further development using a developer solution of methanol-HCl 1% and ethyl acetate (ratio of 6 to 4) as much as 5 mL, to elute 5 mL of concentrated pigments. To perform fractionation, 5 mL of concentrated Batu local rose pigments were put in a C-18 Shimadzu column, with a stationary phase of Shepadex G-25 and the mobile phase of solvent A: acetonitrile 100% and phosphoric acid 4%, solvent in water (AOAC, 1995; Fazeelat *etal.*, 2007).Flow rate of mobile phase used was 1.00 mL/min.

3.4 Observation and data analysis

Both fractionation methods gave three fractions, namely (1) fraction of translucent color components as much as 0.243 g glycone compounds (sugars), (2) fraction of aglycone or red pigments (carmine, took approximately 8.5 -10 hours) as much as 1.052 g, and (3) fraction of aglycone or red colored pigment (younger red, took approximately 10 to 13.5 hours), as much as 1.362 g.LC-MS (Liquid Chromatography Mass Spectrometry)analyses in molecular weight were conducted twice, namely to isolate the results of TLC before fractionation (A) and pure pigments as results of fractionation using a C18 column (B). This paper discusses the identification of sugar contained anthocyanin pigments as glycone.

4. RESULTS AND DISCUSSIONS

4.1 The chemical content of raw materials

The research was conducted by observing changes in the content of anthocyanin pigments stored for few days and identifying the glycone type contained in the crown of roses.Bat local roses were selected because it may improve the usability of roses widely cultivated in Punten, Sidomulyo, District of Batu, East Java; Indonesia.Prior to extraction, the chemical content of crown of red roses (local and hybrid varieties) was analyzed. The analyses include water content, total sugar content, vitamin C, essential oil content, total dissolved solids, and absorbance. The results can be seen in Table-1.

Water content contained in the crown of red roses, local and hybrid varieties are relatively the same, 83.32% and 83.51%, respectively. As comparison, the crown of roses (MBM) *Rosa chine sis* contains water ranging from 65.21 ± 0.66 (Ramamurthy*et al.*, 2010).Total sugar content of crown of red roses from two varieties is approximately 9.73 to 12.45%. This value is similar to the observations ofSaati (2007); total sugar content of crown of red roses Hybrid Netherlands was 9.71 to 12.50%.According to Saati and Musthofa (2008), total sugar content of crown of red roses Hybrid Netherlands was approximately 8.3 to 8.9%.The presence of sugar during observations showed indications of glycosidic bonds, which is one of the characters of the pigment anthocyanin, composed of aglycone (as anthocyanidins) and glycone (as sugar compounds) (Li, 2009; Hendry and Houghton, 1996).These results are relatively closed to *boysenberry* sugar content, ranged from 8.5 to 14.0%, which is also bound to the pigment anthocyanin (Oregon, 2007; Jettanaporn'sumran, 2009).Local rose varieties from Batu have total sugar content of 12.45 ± 0.63%, significantly higher than the total sugar content of crown of red *Canna coccinea* of 3.2% (Saati and Ragil, 2007), and crown of *Rosa chinensis*6.5 ± 0.22 (total sugars) (Ramamoorthy *et al.,* 2010). This indicates that the crown of Batu local roses has glycoside content and contributes sweetness more than other petals.

The content of vitamin C in the crown of roses was 17.23 mg for hybrid varieties and 15.69 mg/100 g for local varieties. This is in accordance with the observations of Blake (2004); the roses contain vitamin C of 0.5-2.0 mg/100 g). In some species, such as *Rosa canina* and *Rosa rugosa,* they contain very rich vitamin C (Anonymous, 2006)[a].

Observation on absorbance was conducted to determine the presence of anthocyanin pigments. The

mean absorbance of powdered pigment Dutch roses Hybrid was 0.123, higher than that of Batu local varieties (0.084 after 100 times dilution). This is influenced by the level of color of red roses crown, Hybrid has blackish-red (darker red) compared to Batu local roses. Differences in the absorbance values were attributed to differences in genetic factors of varieties of roses, as postulated by Hendry and Houghton (1996) and Anonymous (2006)[b].

Table-1. The mean values of water content, total sugar content, vitamin C, essential oil content, total dissolved solid, and absorbance of red roses crown.

Analysis of chemical constituents	Local varieties	Hybrid varieties
Water Content (%)	83.32 ±0.96	83.51 ±0.86
Total Sugar Content (%)	12.45 ±0.63	9.73 ± 0.66
Vitamin C (mg/100g)	15.69 ± 1.80	17.23 ± 4.18
EssentialOil (%)	0.802 ± 0.62	0.803 ± 0.62
Absorbance	0.86± 0.02	1.23± 0.03
Total Disolved Solid (°Brix)	8.33± 1.15	7.67± 0.58

Total dissolved solid analyses on rose petal pigment extracts of hybrid varieties gave smaller value (7.67° Brix) compared to the local varieties of roses (8.33°Brix), relatively closed to the total dissolved solids in *boysenberry* which ranges from 8.82 to 11.2°Brix (Oregon, 2007; Jettanapornsumran, 2009). This may be due to the differences in varieties and place to grow (Joy *et al.,* 1998; Tranggono, 1990), and also differences in total dissolved solid (Budiarto, 1991).Walyono (2007) found that the total sugar content of fresh roses hybrid varieties was of 9.71%, whilst the local rose was 10.99%.

4.2 Absorbance of pigment's filtrat

Observation on the quality of anthocyanin pigment was performed by observing the absorbance value, namely the emergence of the maximum peak at a specific waveleng thre presented anthocyanin pigments character. Important character of anthocyanin pigments have absorbance peak at two cups as glycone (tape I) and a glycone as anthocyanidins (tape II).Glycone compounds generally represent the sugar component (glycone: mono, di or tri-saccharide) which is tied to the pigment and often appeared at λ = 230-280 nm (Markham, 1988), whereas anthocyanidins compound presented as aglycone is often known at λ = 490-535 nm (Hendry and Houghton, 1996; Harborne, 1987), 494-510 nm (Wrolstad*et al.*, 2005), and 520-546 nm in methanol-HCl (Barczak, 2005).Peak distribution of glycone and aglycone is shown in Table-2 and Figure-1.

Table-2. Peak absorbance bands I and II of Bat local roses pigment filtrates at various solvents.

Solvent	Tape I on λ (nm)	Glycone absorbance (dilution100x)	Tape II onλ (nm)	Anthocyanidins absorbance (dilution10x)	Total dissolved solid(°Brix)	Levels (%)
Aq-citrate	235	3.263	514	0.408	5.03 c	7.1253
Aq-lactate	240	2.737	513.5	0.679	5.83 d	14.0293
MetHCl	244	2.458	518	0.157	0.22 a	1.0179
Aq-H_2SO_4	239.5	0.240	517.5	0.087	0.47 b	0.6439

Note: Tape I = glycone, Tape II = aglycone / anthocyanidins

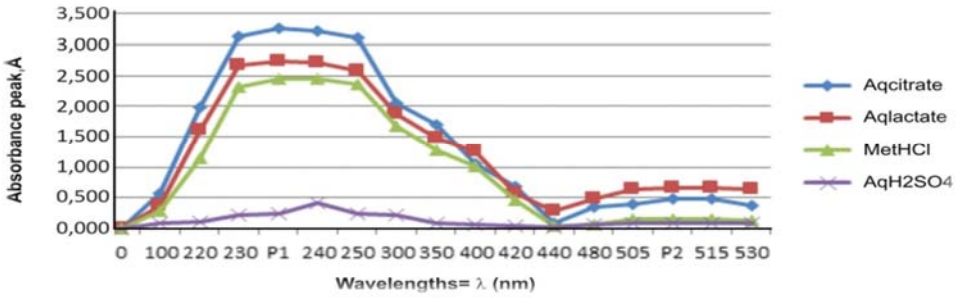

Figure-1. Peak distribution of glycone absorbance value (P1) and a glycone (P2)of Batu local roses at various solvent extractions.

The result of peak distribution shows that the four extraction solvents used to produce anthocyanin pigments were in accordance with the character of tape I and II, respectively, asglycone and aglycone (Li, 2009).The maximum absorbance peak in the second tape shows that both of these bands correspond to the presence of anthocyanin pigment character at defining point, which has a maximum absorbance in the range of 235-244 nm and aglycone / anthocyanidins in the range of 513.5 to 518 nm, as mentioned by Markham (1988) and defined as glycone flavonoids and aglycone (Macheix*et al*., 1990).

Solvent containing distilled water and lactic acid generates anthocyanin pigments peak (tape II)with the high of 0.679 A, whereas solvent containing distilled water and citric acid produces anthocyanin pigments at the highest peak (tape I, 3.263 A).This indicates that pigments with solvent extraction of distilled water and citric acid have more glycone content (glucoside).On the other hand, pigments containing anthocyanidins (aglycone) were generated using solvent made from distilled water and lactic acid. This result is consistent with several studies showing that a more stable anthocyanin pigment could be obtained from extraction using solution containing polar solvents and acidic (Markakis, 1982; Nollet, 1996; Li, 2009).Such treatment also resulted in the highest value of pigment levels for Bat local roses, *i.e.* 14.0293 mg/100 ml, in accordance with the research of Gizir*et al.* (2007). Their research has shown that the use of lactic acid in the extraction of black carrots provides significantly higher extraction efficiency (up to temperature of 100°C) when compared to water acidified with sulfuric acid and citric acid.

4.3 LC-MS analyses

The results of LC-MS *(Liquid Chromatography - Mass Spectrometry)* analysescan be seen in Figure-2. Figure 2A shows the retention time of isolates fractionated using a TLC *(Thin Layer Chromatograph,* silica gel F 254*),* whilst Figure-2B shows the pure anthocyanin pigments as the results of fractionation using a C18 column. From the analyses, it was known that the retention times of anthocyanin pigments were at 3.4 and 3.9 minutes, each type of pigment showing the presence of cyanidin 3-glucoside (6"-malonylglucoside) and cyanidin-3-O-(2"O-glucosyl) rutinoside.

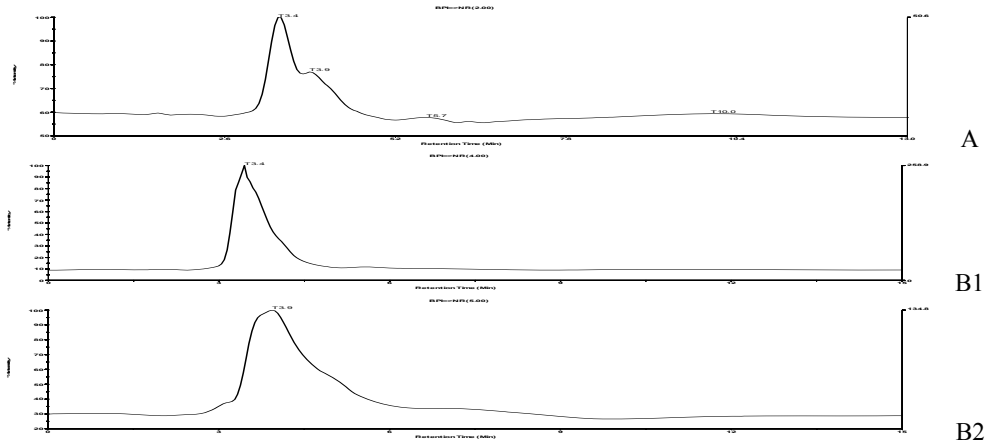

Figure-2. Time mooring of anthocyanin pigments (3.4 to 10.0 minutes): fraction I / B1 (3.4 minutes)and fraction II / B2 (3.9 min).

A type of sugar orglycone bound pigments was noticed. Several types of sugar were contained in a crown of Batu local rose pigments including glucose (162 m/z), maltose (180 m/z), rhamnose and acids coumaril (146 m/z), xylose (132 m/z), and rutinose (308 m/z). This is according to Wu and Prior (2005), as shown in Figure-3.

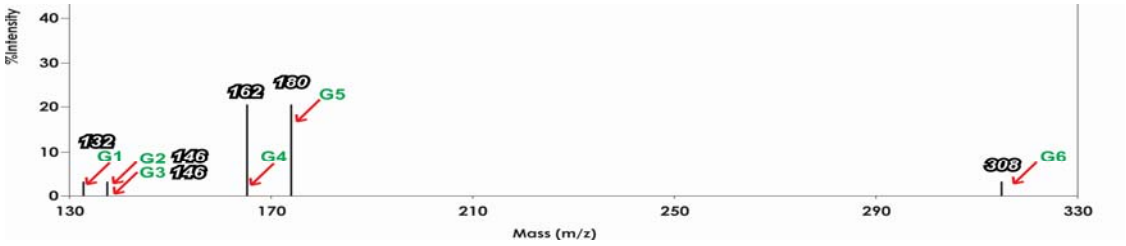

Figure-3. Distribution of type of sugar expected from LC-MS isolates bound in the crown of Batu local roses (G1 = xylose, 132 m/z, G2 = rhamnosa, 146 m/z, G3 =coumaric acid, 146 m/z, G4= glucose, 162 m/z, G5 = maltose,180 m/z, and G6 = rutinose, 308 m/z).

5. CONCLUSIONS

Isolation process of anthocyanin pigment extracts using a solvent of distilled water and lactic acid, with the developer material of ethanol, HCl, BAA, and BuOH-HCl, produced pigments with the best quality and levels, *i.e.* 14.0293%.LC-MS analysis results indicated that the crown of roses contained six types of glyconeor sugar, namely maltose (180m/z), glucose (162m/z), rhamnose (146m/z), acids coumaril (146 m/z), xylose (132 m/z), and rutinose (308 m/z).

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