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Separation of Photosynthetic Pigments by High-Performance Liquid Chromatography: Comparison of Column Performance, Mobile Phase, and Temperature

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Abstract

High-performance liquid chromatography (HPLC) has been commonly used as method of separating and identifying photosynthetic pigments such as chlorophylls and carotenoids because of such advantages as speed, high resolution and sensitivity. In this technique, high separation relies largely on the type of column material. This study compared the efficiency of five reverse-phase columns, C8, C18, C18 monolithic, π-NAP, and cholester, for separation of photosynthetic pigments at several fixed conditions of mobile phase and temperature. This investigation also analysed the parameters of $\Delta R$ and $R$ ratio for selected pigments and resolution for structural isomers, such as $\alpha$- and $\beta$-carotene. Among above columns tested, cholester column is suitable for separation of pigments not only for a broad range of polarity, but also for hydrophobic pigments in a simple mobile phase. This finding can help in the selection of column and HPLC parameters in separating photosynthetic pigments.

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Keyword: Cholesteryl bonded; HPLC column; monolithic packing; particulate packing; photosynthetic pigments; reverse phase.

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1. Introduction

Chromatography method has been introduced since 1905 as specialized technique for photosynthetic pigments separation. Since then, several methods have been developed and commonly used, e.g., thin-layer chromatography, column chromatography, and high-performance liquid chromatography (HPLC). Ultra-fast liquid chromatography (UFLC) was one of the newest generations of HPLC which provide special advantages in conducting researches with low time consuming and high resolution data. These advantages gave an opportunity in the development of a low cost and rapid analysis method. Generally, quality of UFLC separation is affected by four main parameters, i.e., mobile phase, flow rate, column temperature, and column type. Thus, an exploration of the best conditions for pigment separation had become a challenge for chromatography researchers in the world.

Pigment analyses of leaves of higher plants were reported using different UFLC/HPLC analytical methods. In main separation parameters, column material has been understood as an important part where pigment separation occurs during analysis. Other parameters are usually set depend on column type. Generally, HPLC/UFLC columns are distinguished as monolithic and particulate packing types. Ability of these columns in separating pigments had reported for various samples and their improvements. Monolithic column is consisted of small-sized skeletons and wide through-pores which can be achieved higher separation efficiency than the case with particulate packing columns at a similar pressure drop. There are several reports on the monolithic column performance in separating photosynthetic pigments. This column type is known for its advantages in providing good separation and short time analysis. Particulate packing columns have also been widely used for pigment separation. Two most well-used particulate packing column are octyl (C8) and octadecyl (C18) types based on silica. There are numbers of reports on these C8 and C18 which used to develop optimized method for analysis of photosynthetic pigments.

Recently, new types of column based on naphtylethyl bonded silica packing and cholesteryl bonded silica packing were invented. Cholesterol column is basically similar with conventional ODS column as their equivalent hydrophobicity. Nevertheless, cholesteryl column has high sensitivity for hydrophobic compound due to their strong stereo-selectivity. Like cholesteryl column, π-NAP column has unique specific selectivity in separation. This napthylethyl bonded silica packing column was built for π-π interactions for hydrophobic compound. These advantages, in case of photosynthetic pigments separation, provide better chance to provide good separation of carotenes group.

In previous investigation, two silica particulate packing columns (C18 and C8) were analysed as the standard in addition to C18 monolithic type column to understand the effect of carbon chain length and the difference between particulate and monolithic types on the pigment separation. The sample used here was pigments extracted from leaves of Pleomele angustifolia, an indigenous source of natural colorants as mentioned previously. It contains common six major pigments such as chlorophylls a and b, violaxanthin, zeaxanthin, α-carotene, and β-carotene. This investigation results showed that monolithic column provided better resolution and faster analysis, although each column had their characteristic features. In the present study, in addition to above three columns, an examination of two new type columns mentioned above, i.e., π-NAP and cholesteryl columns, were conducted. This investigation approaches would give basic information to develop simple and rapid HPLC separation method for photosynthetic pigments.
2. Materials and methods

2.1. Plant material

_Pleomele angustifolia_ Roxb. N. E. Brown was used throughout this study as a pigment source. Samples were collected from MRCPP Arboretum located in Malang, East Java, Indonesia (S 7° 57' 21.4632", E 112° 35' 24.7056°). Collected leaves were cleaned by rinsing with distilled water and were then frozen and stored at -20 °C for further analyses.

2.2. Columns

Chromolith® Performance RP-18e, 4.6 i.d. × 100 mm (MERCK, Darmstadt, Germany), Shim-Pack XR-ODS, 3 i.d. × 100 mm (Shimadzu, Kyoto, Japan), and Shim-Pack XR-C8, 3 i.d. × 100 mm (Shimadzu) were purchased from a local provider. Cosmosil cholester, 2 i.d. × 50 mm (Nacalai Tesque), cosmosil π-NAP, 2 i.d. × 50 mm (Nacalai Tesque) were kindly gift from Nacalai Tesque, Inc., Kyoto, Japan.

2.2. Pigments extraction

_P. angustifolia_ leaves were ground using a mortar with a few amounts of sodium ascorbate and calcium carbonate to avoid pigments oxidation and acidification. Liquid nitrogen (-196 °C) was added to prevent enzymatic reaction which can affect to the pigment stability. The homogenate (0.2 g wet weight) of _P. angustifolia_ was extracted with 3 mL of 100 % methanol (GR for analysis, MERCK) in a conical bottom tube, by shaking with vortex for 10 s. In order to minimize photo-degradation and oxidation of the pigments, the extractions and measurements were carried out under green dimmed light at room temperature under ultra-high purity (99 %) nitrogen atmosphere (PT. Samator, Surabaya, Indonesia). This rapid extraction method was conducted less than 1 min. Prior to injection, sample pigment was filtrated through a membrane filter (0.2 μm, nylon, Whatman, Maidstone, UK).

2.3. HPLC analysis

Pigments separation was carried out by UFLC using LC–20AD XR equipped with photodiode array detector SPD–20MA and column oven CTO–20AC (Shimadzu) as reported previously. In briefly, HPLC analysis was performed isocratic method using a mobile phase consisted of acetonitrile (HPLC Grade, MERCK) and methanol (GR for analysis, MERCK). The solvent ratios (v/v) were varies for analysis in the following: 20 : 80 (System 1); 35 : 65 (System 2), 50 : 50 (System 3), 65 : 35 (System 4) and 80 : 20 (System 5). Column temperature used was either 30 °C or 40 °C. Pigments were detected in the range of 190 nm to 800 nm. Injection was automated by an auto–sampler SIL–20AC XR (Shimadzu) and 20 μL pigment solution was subjected to analysis.

2.4. Pigment identification

All targeted peaks were isolated for identification. Visible absorption spectra were obtained by UV-Visible Spectrophotometer 1800 (Shimadzu) from 350 nm to 800 nm. Isolated pigments were measured in different solvents. Chlorophylls group was measured in acetone, diethyl ether, and ethanol, while carotenoids group in acetone, n-hexane, and ethanol. Spectral properties were then compared with those of reference spectra from the standard phytoplankton pigments.
2.5. Data analysis

UFLC data were revealed from original Shimadzu UFLC operation software, Lab Solution. Plot data and polynomial regression was created by Origin 7.0 (Origin Lab Corp, Northampton, USA). Both numeric and graphic data represent an average from triplicate analyses with SE.

3. Results and discussion

Six photosynthetic pigments were separated with the columns used, except for π-NAP column. In here, therefore, the properties of four columns were mainly compared, excluding π-NAP column. The pigments were identified with comparison of absorption spectra of isolated pigments in different solvents as follows: violaxanthin (1st peak), zeaxanthin (2nd peak), chlorophyll b (3rd peak), chlorophyll a (4th peak), α-carotene (5th peak), and β-carotene (6th peak) (Table 1), as generally found in most of the higher plants23–26. Fig. 1 shows representative chromatograms after separation with mobile phase of acetonitrile-methanol, 50 : 50 (v/v) (System 3) at a fixed flow rate of 0.5 mL per min and column temperature at 30 °C and 40 °C. Rapid separation was observed in C18 than C8 column. Moreover, particulate packing column needed longer time analysis than monolithic column at both temperatures (Fig. 1. A and C), despite the large column volume. High column temperature enhanced time analysis in both column types. This is probably due to decrease in solvent density with increasing temperature. In both columns at 40 °C, retention time is able to reduce about 0.7 times of 30 °C to accomplish all peak separation. Similar results were also obtained by C8 column, XR-C8 (Fig. 1. E and F).

Table 1. Identification of the pigments extracted from P. angustifolia

| Peak No. | Pigment        | λ_{max} (nm)* | Acetone | n-Hexane | Diethyl ether | Ethanol | Eluent** | Ref.*** |
|---------|----------------|---------------|---------|----------|--------------|---------|----------|---------|
| 1       | Violaxanthin   | 417,440,470   | 416,437,469 | -        | 416,438,468 | 413,436,465 | 6,22,23  |
| 2       | Zeaxanthin     | (429),450,477 | (425),445,476 | -        | (429),452,479 | (420),445,472 | 5,6,22  |
| 3       | Chlorophyll b  | 455,592,649   | -       | 455,595,641 | 463,590,645 | 465,595,648 | 6,22,23  |
| 4       | Chlorophyll a  | 430,616,662   | -       | 430,616,662 | 430,618,666 | 431,617,663 | 5,6,22,23 |
| 5       | α-Carotene     | (423),447,475 | 419,443,473 | -        | 421,445,473 | (421),443,474 | 5,6,22  |
| 6       | β-Carotene     | (428),454,480 | (425),449,479 | -        | (426),451,478 | (423),450,476 | 5,6,22,23 |

*Represent I-II-III bands for carotenoids and Soret, Qx, and Qy bands for chlorophylls, parenthesis represents shoulder peak

**Mobile phase, 50 : 50 (System 3) at 40 °C

***References: Hegazi5; Jeffrey6; Britton22; Gross23.

Cholesteryl bonded silica packing column was superior for separation among all columns examined (Fig. 1.G and H) in terms of selectivity and resolution of hydrophobic pigments, as suggested by manufacturer for separating hydrophobic compounds. This investigation examined suitability for the separation of photosynthetic pigments which have a broad spectrum of polarity. As shown in Fig. 1.G and H (see peaks 5 and 6) and also Fig. 4, cholester column could be clearly separated not only polar pigments, but also non-polar pigments, trans α-carotene and β-carotene compared with Chromolith and XR-ODS columns. On the other hand, as generally known, XR-C8 was proved to be more suitable for the separation of polar than hydrophobic pigments. These findings suggest that cholester column might be good alternative from usual C18 columns. π-NAP column was unable to separate even in polar pigments (data not shown), suggesting that this column is unsuitable for separating photosynthetic pigments. This column, however, may have advantages and potential in separating isomeric compounds, especially for carotenoids and their isomer separation. Further investigation is needed for optimizing this column.
Fig. 1. UFPLC chromatograms of photosynthetic pigments from leaves of *P. angustifolia*. UFPLC was carried out an isocratic in System 3 (50 : 50, v/v) and flow rate at 0.5 mL per min. Other conditions are described in the text.

Fig. 2. $\Delta t_{Rchl_a-viol}$ (solid circle) and $\Delta t_{Rb-car-chl_a}$ (open circle) were calculated from the results of UFPLC separation of photosynthetic pigments extracted from leaves of *P. angustifolia*. Other conditions are the same as in Fig. 1. Data are average of three experiments. SE is less than ±0.5.

To analyze time distance between pigments with different polarities, retention times of Chl$_a$ (Chlorophyll $a$), viol (violaxanthin), and b-car (β-carotene) were selected as peak position indicators in calculating $\Delta t_R$ and $t_R$ ratio. These pigments peaks show time distance between polar (viol) to semi-polar (Chl$_a$) pigments and between semi-polar (Chl$_a$) to non-polar (b-car) pigments. Fig. 2 shows the effects of solvent compositions on $\Delta t_R$. Generally in reverse phase columns, separation time of pigments decreased with increasing acetonitrile concentrations (increasing ionic strength). This investigation can be conventionally compared the behaviour of polar and non-polar pigments against solvent compositions. In separation of polar pigments, $\Delta t_R$ of XR-C8 column was more conspicuously increased than any other columns. In contrast, $\Delta t_R$ of non-polar pigments in cholester column decreased with increasing acetonitrile concentrations, although other columns were almost constant. From these results, it is likely concluded that under used simple mobile phase, XR-C8 has high flexible retentivity for polar pigments, indicating that this column is suitable for the separation of non-polar pigments. On the other hand, cholester column has high flexibility for non-polar pigment than any other columns. Thus this column is suitable for non-polar pigment.
separation. The results of calculation by polynomial regression for columns used are shown in Table 2. This provides useful information to optimize chromatographic conditions in each column.

Table 2. Δ\text{R}_{\text{chl-a/viol}} and Δ\text{R}_{b\text{-car-chl-a}} polynomial regression from analyzed sample.

| No | Column       | Temperature | \(Δ\text{R}_{\text{chl-a/viol}}\) Equation | \(Δ\text{R}_{\text{chl-a/viol}}\) R² | \(Δ\text{R}_{b\text{-car-chl-a}}\) Equation | \(Δ\text{R}_{b\text{-car-chl-a}}\) R² |
|----|--------------|-------------|----------------------------------------|-----------------------------------|----------------------------------------|-----------------------------------|
| 1  | Chromolith   | 30 °C       | Y = 4.55 – 0.06X + 0.21X²               | 0.99                              | Y = 14.06 – 0.86X + 0.13X²             | 0.99                              |
| 2  | XR-ODS       | 30 °C       | Y = 6.66 – 0.28X + 0.23X²               | 0.94                              | Y = 20.16 – 1.21X + 0.20X²             | 0.88                              |
| 3  | XR-C8        | 30 °C       | Y = 4.98 – 0.07X + 0.17X²               | 0.99                              | Y = 14.01 – 1.18X + 0.14X²             | 0.98                              |
| 4  | Cholester    | 30 °C       | Y = 4.16 – 0.07X + 0.20X²               | 0.99                              | Y = 2.726 – 0.04X + 0.11X²             | 0.98                              |

Peak retention time ratio (\(t_R\) ratio) is also one of parameters to understand the peak separation. Ratios of \(t_{\text{Rchl-a/viol}}\) and \(t_{\text{R\beta-car/chl-a}}\) were also calculated and used as peak indicators. Fig. 3 shows the effects of solvent compositions and column temperatures on \(t_R\) ratio. Similar pigment separations were obtained in both temperatures. In XR-ODS and XR-C8 columns, \(t_{\text{Rchl-a/viol}}\) was almost constant up to solvent composition of 50 : 50, but then increased with increasing solvent strength. This tendency was also observed in \(Δt_R\). On the other hand, \(t_{\text{R\beta-car/chl-a}}\) calculated from all columns were linearly decreased with increasing solvent strength, but their values were low. Polynomial regression from analyzed samples are summarized in Table 3.

![Fig. 3. \(t_{\text{Rchl-a/viol}}\) ratio (solid) and \(t_{\text{R\beta-car/chl-a}}\) ratio (open), from Chromolith (Square), XR-ODS (triangle), XR-C8 (circle), and cholester column (diamond) employed at 30 °C and 40 °C column temperature.](image)

Table 3. \(t_R\) ratio polynomial regression calculated from analyzed sample.

| No | Column       | Temperature | \(t_R\) at acetic Equation | \(t_R\) at acetic R² | \(t_R\) at ethanol Equation | \(t_R\) at ethanol R² |
|----|--------------|-------------|---------------------------|---------------------|-----------------------------|---------------------|
| 1  | Chromolith   | 30 °C       | Y = 10.14 + 0.20X + 0.02X² | 0.96                | Y = 4.00 – 0.48X + 0.02X²   | 0.99                |
| 2  | XR-ODS       | 30 °C       | Y = 10.54 + 0.41X + 0.07X² | 0.92                | Y = 3.68 – 0.42X + 0.02X²   | 0.99                |
| 3  | XR-C8        | 30 °C       | Y = 13.05 + 0.40X + 0.24X² | 0.96                | Y = 4.05 – 0.45X + 0.02X²   | 0.99                |
| 4  | Cholester    | 30 °C       | Y = 12.68 – 0.68X + 0.23X² | 0.98                | Y = 3.72 – 0.30X + 0.01X²   | 0.99                |

\(Δt_R\) and \(t_R\) ratio analysis had provided clear description for the column performance in separating photosynthetic pigments extracted from \(P. angustifolia\). All investigated columns, except cosmostil π-NAP column, provide acceptable results in separating pigments from polar to non-polar species. Most of these columns had their abilities for separation of polar-semi polar pigments. However, separation of non-polar carotenoids such as α-carotene and β-carotene was not the case.
Subsequently, this investigation conducted Gaussian peak fitting analysis using Origin software to determine the resolution of columns. This analysis focused on the peaks of structurally similar pigments, α-carotene and β-carotene (Fig. 4). Under used conditions, poor pigment separation was observed in the XR-C8. Similarly Chromolith column gave low resolution probably due to peak broadening. XR-ODS provided good results of the separation, but much high resolution was obtained by cholester column. Combined together with the previous results, cholester column is superior for the separation of non-polar pigments in terms of selectivity and resolution.

Previously, particulate packing columns (C8 and C18) had been commonly used in HPLC for separating photosynthetic pigments. Huge effort has been made by many researchers to optimize pigment separation through these column types. Most of them employed gradient method as a strategy to increase in separation quality. In some HPLC methods, a narcotic and psychotropic source material, acetone, is used as mobile phase. Since the adoption of the 1988 UN Convention against Illicit Trafficking in Narcotic Drugs and Psychotropic Substances, in some countries including Indonesia, those solvents trading right was limited under very close supervision in order to minimize irresponsible used. This restriction was giving us new issue in providing better method for HPLC, which is not use of drug related solvents. Low time and solvent consuming analysis was also becoming strong demand for pigment separation analysis due to environmental problem and stability. Photosynthetic pigments were unstable against extreme uncontrolled environment. Long time HPLC analysis should be considered solvent-pigment interaction and column temperature which gives effect in pigment stability. This may cause in decreasing accuracy of the data.

In the previous study, the efficiency between particulate packing and monolithic columns were compared. Clearly different from particulate packing bed, monolith column composed by a continuous character of skeleton, which fulfills the separation chambers. Monolith contained a discrete bimodal pore size distribution. Chromolith column showed a typical characteristic of monolithic column in the separation of P. angustifolia pigments. It provided better resolution and faster analysis. Thus, high tolerates to flow rate system of this column provides us to optimize a rapid separation method.

Cosmosil cholester column is claimed as their abilities of enhanced selectivity over traditional C18 materials and greater performance in separating isomers or other closely related compounds. It is expected as an ideal column for method development and serves as an excellent alternative to traditional C18 columns. There was, however, limited information about this column performance relating to photosynthetic pigment separation. In this report, this column has shown its performance compared to other columns. This column has proved its advantages and specialized characteristic in separating hydrophobic pigment in such a rapid elution time. This is the first report on the separation of photosynthetic pigment by cosmosil cholester column.
4. Conclusion

In this study, the efficiency of five reverse-phase columns, C8, C18, C18 monolithic, π-NAP, and cholester, for separation of photosynthetic pigments at several fixed conditions of mobile phase and temperature were compared. Among above columns tested, cholester column is suitable for separation of pigments for a broad range of polarity, especially for hydrophobic pigments in rapid elution time and simple mobile phase. In addition, this column is also superior to resolution of structurally similar pigments. These findings can help in the selection of column and HPLC parameters in separating photosynthetic pigments by using simple mobile phase system.

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