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Editors:

Roy Hendroko Setyobudi, Hugo Scheer, Leenawaty Limantara, Yuzo Shioi, Leszek Fiedor, Tatas H.P. Brotosudarmo and Monika N.U. Prihastyanti

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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 Δt_R and t_R ratio for selected pigments and resolution for structural isomers, such as α- and β-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.

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014

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

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Nomenclature

Δt_R	retention ti
t_R	retention ti
t_R **Ratio**	ratio betwe
v/v	solvents vo

1. Introduction

Chromatography n
separation[1]. Since t
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Pigment analyses of lea

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HPLC/UFLC columns
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Procedia Chemistry

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ments; reverse phase.

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Nomenclature

Δt_R	retention time difference
t_R	retention time
t_R Ratio	ratio between retention time of two pigment peaks
v/v	solvents volume ratio

1. Introduction

Chromatography method has been introduced since 1905 as specialized technique for photosynthetic pigments separation[1]. Since then, several methods have been developed and commonly used, e.g., thin-layer chromatography[1,2], column chromatography[3,4], and high-performance liquid chromatography (HPLC)[5–7]. 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[8,9]. 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[10–12].

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[13]. Ability of these columns in separating pigments had reported for various samples and their improvements[5,14–16]. 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[17]. There are several reports on the monolithic column performance in separating photosynthetic pigments[16,18,19]. This column type is known for its advantages in providing good separation and short time analysis[20]. 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[5,6,14,15].

Recently, new types of column based on napthylethtyl bonded silica packing and cholesteryl bonded silica packing were invented. Cholester column is basically similar with conventional ODS column as their equivalent hydrophobicity. Nevertheless, cholester column has high sensitivity for hydrophobic compound due to their strong stereo-selectivity. Like cholester 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[21]. 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 cholester 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[21]. 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[5,6,22,23].

2.5. Data analysis

UFLC data were reve
polynomial regression was
data represent an average

3. Results and discussion

Six photosynthetic pigm
the properties of four colu
with comparison of absorp
zeaxanthin (2nd peak), chl
peak) (Table 1), as genera
after separation with mobi
per min and column temp
Moreover, particulate pac
(Fig. 1. A and C), despit
column types. This is prob
40 °C, retention time is al
were also obtained by C8 c

Table 1. Identification of the pigm

Peak No.	Pigment
1	Violaxanthin
2	Zeaxanthin
3	Chlorophyll *b*
4	Chlorophyll *a*
5	α-Carotene
6	β-Carotene

*Represent I-II-III bands for caro
**Mobile phase, 50 : 50 (System
***References: Hegazi[5]; Jeffrey[6]

Cholesteryl bonded sil
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hydrophobic compounds.
which have a broad spectr
column could be clearly s
carotene compared with C
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polar pigments (data not s
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a pigment source. Samples were
(S 7° 57' 21.4632", E 112° 35'
then frozen and stored at -20 °C

Germany), Shim-Pack XR-ODS,
mm (Shimadzu) were purchased
cosmosil π-NAP, 2 i.d. × 50 mm

f sodium ascorbate and calcium
) was added to prevent enzymatic
t weight) of *P. angustifolia* was
al bottom tube, by shaking with
ie pigments, the extractions and
e under ultra-high purity (99 %)
method was conducted less than
filter (0.2 μm, nylon, Whatman,

d with photodiode array detector
. In briefly, HPLC analysis was
C Grade, MERCK) and methanol
he following: 20 : 80 (System 1);
5). Column temperature used was
n. Injection was automated by an
d to analysis.

ra were obtained by UV-Visible
ents were measured in different
anol, while carotenoids group in
ose of reference spectra from the

Data analysis

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

Results and discussion

Six photosynthetic pigments were separated with the columns used, except for π-NAP column. In here, therefore, properties of four columns were mainly compared, excluding π-NAP column. The pigments were identified comparison of absorption spectra of isolated pigments in different solvents as follows: violaxanthin (1st peak), xanthin (2nd peak), chlorophyll *b* (3rd peak), chlorophyll *a* (4th peak), α-carotene (5th peak), and β-carotene (6th k) (Table 1), as generally found in most of the higher plants[23–26]. Fig. 1 shows representative chromatograms r separation with mobile phase of acetonitrile-methanol, 50 : 50 (v/v) (System 3) at a fixed flow rate of 0.5 mL min and column temperature at 30 °C and 40 °C. Rapid separation was observed in C18 than C8 column. reover, particulate packing column needed longer time analysis than monolithic column at both temperatures g. 1. A and C), despite the large column volume. High column temperature enhanced time analysis in both mn types. This is probably due to decrease in solvent density with increasing temperature. In both columns at °C, retention time is able to reduce about 0.7 times of 30 °C to accomplish all peak separation. Similar results re also obtained by C8 column, XR-C8 (Fig. 1. E and F).

ble 1. Identification of the pigments extracted from *P. angustifolia*

Peak No.	Pigment	λ_{max} (nm)*					Ref.***
		Acetone	n-Hexane	Diethyl ether	ethanol	eluent**	
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

epresent 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: Hegazi[5]; Jeffrey[6]; Britton[22]; Gross[23].

Cholesteryl bonded silica packing column was superior for separation among all columns examined (Fig. 1.G d H) in terms of selectivity and resolution of hydrophobic pigments, as suggested by manufacturer for separating drophobic compounds. This investigation examined suitability for the separation of photosynthetic pigments hich have a broad spectrum of polarity. As shown in Fig. 1.G and H (see peaks 5 and 6) and also Fig. 4, cholester lumn could be clearly separated not only polar pigments, but also non-polar pigments, *trans* α-carotene and β-rotene compared with Chromolith and XR-ODS columns. On the other hand, as generally known, XR-C8 was wed to be more suitable for the separation of polar than hydrophobic pigments. These findings suggest that holester column might be good alternative from usual C18 columns. π-NAP column was unable to separate even in olar pigments (data not shown), suggesting that this column is unsuitable for separating photosynthetic pigments. his column, however, may have advantages and potential in separating isomeric compounds, especially for rotenoids and their isomer separation. Further investigation is needed for optimizing this column.

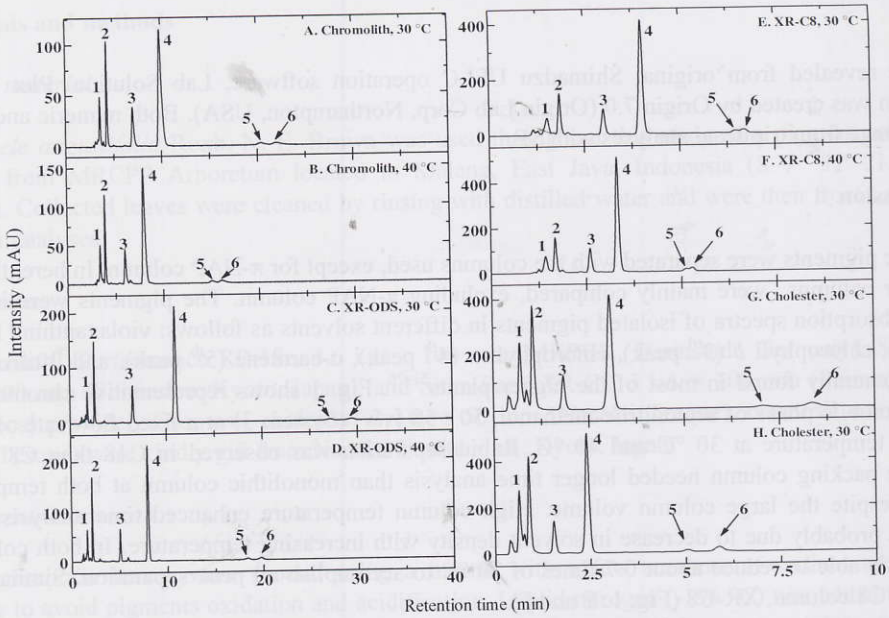


Fig. 1. UFPLC chromatograms of photosynthetic pigments from leaves of *P. angustifolia*. UFPLC was carried out an isocratic in System (50 : 50, v/v) and flow rate at 0.5 mL per min. Other conditions are described in the text.

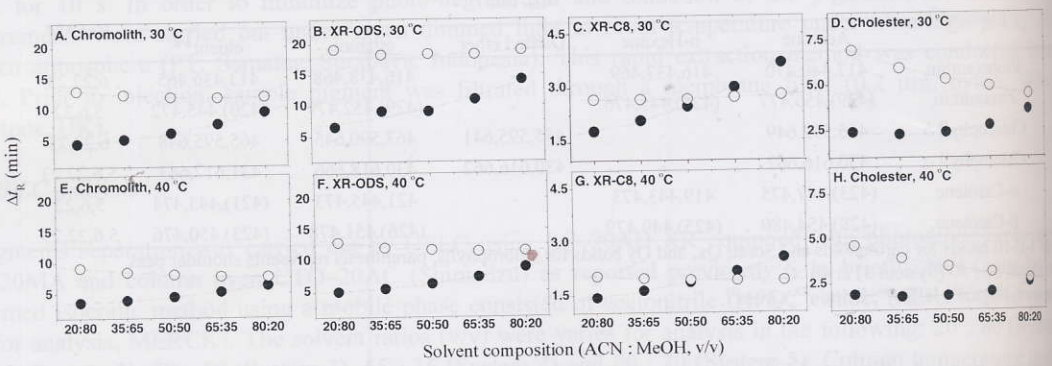


Fig. 2. $\Delta t_{Rchl\_a\text{-}viol}$ (solid circle) and $\Delta t_{Rb\text{-}car\text{-}chl\_a}$ (open circle) were calculated from the results of UFPLC separation of photosynthetic pigments extracted from leaves of *P. angustifolia*. Other conditions are the same as in Fig. 1. Data are average of 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 Δt_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 Δt_R. Generally 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, Δt_R of XR-C8 column was more conspicuously increased than any other columns. In contrast, Δt_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 pigments

separation. The results of provides useful information to

Table 2. $\Delta t_{R\ chl\_a\text{-}viol}$ and $\Delta t_{R\ b\text{-}car\text{-}ch}$

No	Column
1	Chromolith
2	XR-ODS
3	XR-C8
4	Cholester

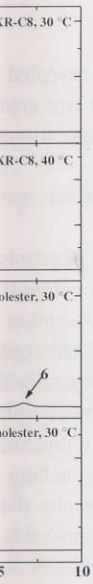
Peak retention time ratio and $t_{R\beta\text{-}car/Chl\_a}$, were also calc column temperatures on t_R ra XR-C8 columns, $t_{Rchl\_a/viol}$ w increasing solvent strength. columns were linearly decrea from analyzed samples are su

Fig. 3. $t_{R\ chl\_a/viol}$ ratio (solid) and (diamond) employed at 30 °

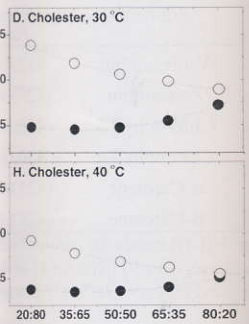
Table 3. t_R ratio polynomial r

No	Column
1	Chromolith
2	XR-ODS
3	XR-C8
4	Cholester

Δt_R and t_R ratio analysis pigments extracted from acceptable results in separa for separation of polar-sem β-carotene was not the case



s carried out an isocratic in System 3



separation of photosynthetic pigments
a are average of three experiments.

mes of Chl_*a* (Chlorophyll *a*),
in calculating Δt_R and t_R ratio.
a) pigments and between semi-
mpositions on Δt_R. Generally in
itrile concentrations (increasing
f polar and non-polar pigments
umn was more conspicuously
olester column decreased with
From these results, it is likely
for polar pigments, indicating
and, cholester column has high
uitable 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. $\Delta t_{R\ chl\_a\text{-}viol}$ and $\Delta t_{R\ b\text{-}car\text{-}chl\_a}$ polynomial regression from analyzed sample.

No	Column	Temperature	$\Delta t_{R\ chl\_a\text{-}viol}$		$\Delta t_{R\ b\text{-}car\text{-}chl\_a}$	
			Equation	R^2	Equation	R^2
1	Chromolith	30 °C	$Y = 4.55 - 0.06X + 0.21X^2$	0.99	$Y = 14.06 - 1.06X + 0.13X^2$	0.99
		40 °C	$Y = 3.54 + 0.01X + 0.10X^2$	1.00	$Y = 10.07 - 0.93X + 0.09X^2$	0.99
2	XR-ODS	30 °C	$Y = 6.66 + 0.28X + 0.23X^2$	0.94	$Y = 20.16 - 1.23X + 0.20X^2$	0.88
		40 °C	$Y = 4.98 - 0.07X + 0.17X^2$	0.99	$Y = 14.01 - 1.18X + 0.14X^2$	0.99
3	XR-C8	30 °C	$Y = 1.66 + 0.07X + 0.06X^2$	0.99	$Y = 2.726 - 0.04X + 0.01X^2$	0.98
		40 °C	$Y = 1.36 + 0.07X + 0.03X^2$	0.99	$Y = 2.14 - 0.09X + 0.01X^2$	0.99
4	Cholester	30 °C	$Y = 2.91 - 0.66X + 0.15X^2$	0.99	$Y = 7.94 - 1.16X + 0.09X^2$	0.99
		40 °C	$Y = 2.24 - 0.47X + 0.1X^2$	0.99	$Y = 5.37 - 0.82X + 0.06X^2$	0.99

Peak retention time ratio (t_R ratio) is also one of parameters to understand the peak separation. Ratios of $t_{RChl\_a/viol}$ and $t_{R\beta\text{-}car/Chl\_a}$, 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, $t_{Rchl\_a/viol}$ 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, $t_{R\beta\text{-}car/chl\_a}$ 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.

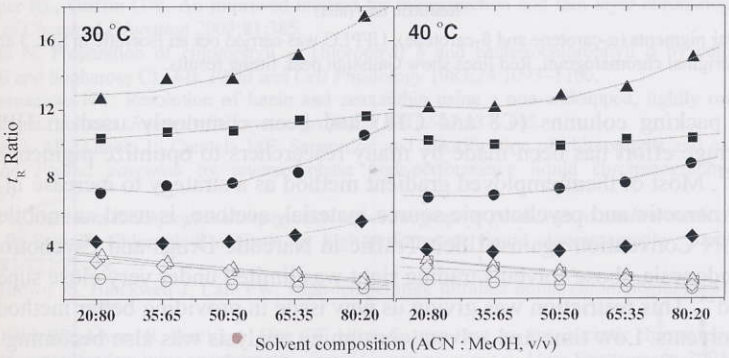


Fig. 3. $t_{R\ chl\_a/viol}$ ratio (solid) and $t_{R\ b\text{-}car/chl\_a}$ ratio (open), from Chromolith (Square), XR-ODS (triangle), XR-C8 (circle), and cholester column (diamond) employed at 30 °C and 40 °C column temperature.

Table 3. t_R ratio polynomial regression calculated from analyzed sample.

No	Column	Temperature	$t_{R\ chl\_a/viol}$		$t_{R\ b\text{-}car/chl\_a}$	
			Equation	R^2	Equation	R^2
1	Chromolith	30 °C	$Y = 10.14 + 0.20X + 0.02X^2$	0.96	$Y = 4.00 - 0.48X + 0.02X^2$	0.99
		40 °C	$Y = 10.54 - 0.41X + 0.07X^2$	0.72	$Y = 3.68 - 0.42X + 0.02X^2$	0.99
2	XR-ODS	30 °C	$Y = 13.05 - 0.40X + 0.24X^2$	0.96	$Y = 4.05 - 0.45X + 0.02X^2$	0.99
		40 °C	$Y = 12.68 - 0.68X + 0.23X^2$	0.98	$Y = 3.72 - 0.39X + 0.01X^2$	0.99
3	XR-C8	30 °C	$Y = 8.04 - 0.95X + 0.28X^2$	0.95	$Y = 2.47 - 0.20X + 0.01X^2$	0.99
		40 °C	$Y = 7.16 - 0.36X + 0.13X^2$	0.98	$Y = 2.39 - 0.20X + 0.01X^2$	0.99
4	Cholester	30 °C	$Y = 4.85 - 0.58X + 0.14X^2$	0.99	$Y = 3.40 - 0.14X - 0.02X^2$	0.99
		40 °C	$Y = 4.36 - 0.54X + 0.12X^2$	0.99	$Y = 3.00 - 0.13X - 0.02X^2$	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 cosmosil 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.

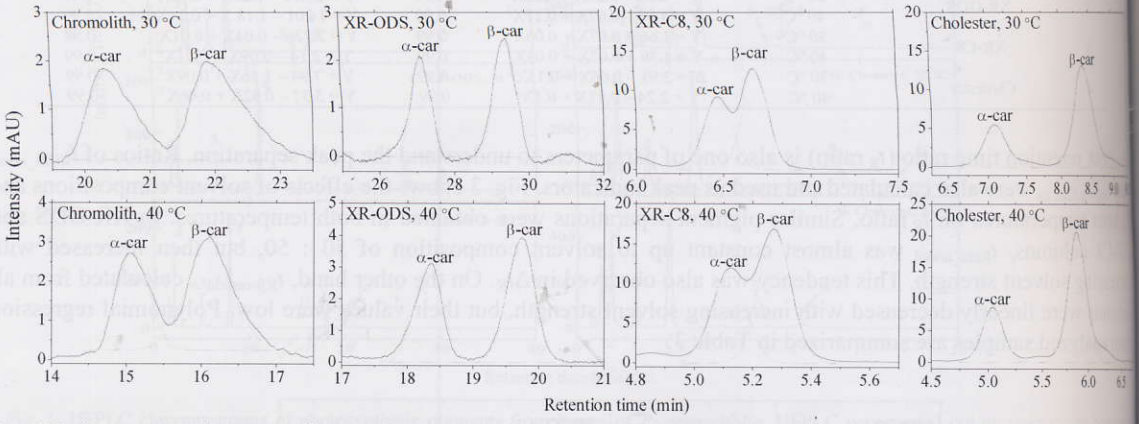


Fig. 4. Peak separation of non-polar pigments (α-carotene and β-carotene). UFPLC was carried out an isocratic in Sys. 3 and 0.5 mL per min flow rate. Black line represents original chromatogram. Red lines show Gaussian peak fitting results.

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[5,10,14,15,27]. 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[5,14,15]. Since the adoption of the 1988 UN Convention against Illicit Traffic 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[28]. 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[29,30]. This may cause in decreasing accuracy of the data.

In the previous study[21], 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[13,31]. 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 efficien
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References

1. Jeffrey S. Quantitative thin layer c
Bioenergetics 1968;162:271–285.
2. Quach HT, Steeper RL, Griffin C
spinach. *Journal of Chemical Educ*
3. Omata T, Murata N. Preparation
Sepharose CL–6B and Sepharose C
4. Gilmore AM, Yamamoto HY. Re
liquid chromatographic column. *Jo*
5. Hegazi MM, Ruzafa AP, Almela
Jania rubens and *Padina pavo*
1998;829:153–159.
6. Jeffrey S, Wright S, Mantoura R. *P*
7. Shioi Y, Fukae R, Sasa T. Chlor
Bioenergetics 1983;722:72–79.
8. Yan B, Zhao J, Brown JS, Blackw
1262.
9. Romanyshyn L, Tiller PR, Alvaro R
by liquid chromatography/tandem m
10. Canjura FL, Schwartz SJ. Separatio
Journal of Agricultural and Food Ch
11. Hart DJ, Scott KJ. Development an
carotenoid content of vegetables and
12. Shioi Y, Watanabe K, Takamiya
Chenopodium album. *Plant and Cell*
13. Unger KK, Skudas R, Schulte MM
comparison and critical appraisal. *Jou*
14. Wright S, Jeffrey SW, Mantoura
phytoplankton. *Marine Ecology Prog*
15. Zapata M, Rodriguez F, Garrido JL.
reversed phase C8 column and pyridin
16. Garrido JL, Rodriguez F, Campana
derivatives using a monolithic silica C
17. Nunez O, Nakanishi K, Tanaka N.
Chromatography A 2008;1191:231–25
18. Ruhle W, Paulsen H. Preparation of
2004;684:113–125.
19. Pol J, Hyotylainen T, Ranta-Aho O,
monolithic column for trapping and sep
20. Tanaka N, Kobayashi H, Ishizuka N
chromatography A 2002;965:35–49.

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.

Acknowledgement

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References

1. Jeffrey S. Quantitative thin layer chromatography of chlorophylls and carotenoids from marine algae. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1968;162:271–285.
2. Quach HT, Steeper RL, Griffin GW. An improved method for the extraction and thin-layer chromatography of chlorophyll *a* and *b* from spinach. *Journal of Chemical Education* 2004;81:385.
3. Omata T, Murata N. Preparation of chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll *a* by column chromatography with DEAE–Sepharose CL–6B and Sepharose CL–6B. *Plant and Cell Physiology* 1983;24:1093–1100.
4. Gilmore AM, Yamamoto HY. Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C18 high-performance liquid chromatographic column. *Journal of Chromatography A* 1991;543:137–145.
5. Hegazi MM, Ruzafa AP, Almela L, Candela ME. Separation and identification of chlorophylls and carotenoids from *Caulerpa prolifera*, *Jania rubens* and *Padina pavonica* by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* 1998;829:153–159.
6. Jeffrey S, Wright S, Mantoura R. *Phytoplankton pigments in oceanography: guidelines to modern methods*. Paris: Unesco Pub; 1997.
7. Shioi Y, Fukae R, Sasa T. Chlorophyll analysis by high-performance liquid chromatography. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1983;722:72–79.
8. Yan B, Zhao J, Brown JS, Blackwell J, Carr PW. High-temperature ultrafast liquid chromatography. *Analytical Chemistry* 2000;72:1253–1262.
9. Romanyshyn L, Tiller PR, Alvaro R, Pereira A, Hop CE. Ultra–fast gradient vs. fast isocratic chromatography in bioanalytical quantification by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2001;15:313–319.
10. Canjura FL, Schwartz SJ. Separation of chlorophyll compounds and their polar derivatives by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry* 1991;39:1102–1105.
11. Hart DJ, Scott KJ. Development and evaluation of an HPLC method for the analysis of carotenoids in foods and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chemistry* 1995;54:101–111.
12. Shioi Y, Watanabe K, Takamiya K. Enzymatic conversion of pheophorbide a to the precursor of pyropheophorbide a in leaves of Chenopodium album. *Plant and Cell Physiology* 1996;37:1143–1149.
13. Unger KK, Skudas R, Schulte MM. Particle packed columns and monolithic columns in high-performance liquid chromatography-comparison and critical appraisal. *Journal of Chromatography A* 2008;1184:393–415.
14. Wright S, Jeffrey SW, Mantoura R, et al. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series* 1991;77:183–196.
15. Zapata M, Rodriguez F, Garrido JL. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. *Marine Ecology Progress Series* 2000;195:29–45.
16. Garrido JL, Rodriguez F, Campana E, Zapata M. Rapid separation of chlorophylls *a* and *b* and their demetallated and dephytylated derivatives using a monolithic silica C18 column and a pyridine-containing mobile phase. *Journal of Chromatography A* 2003;994:85–92.
17. Nunez O, Nakanishi K, Tanaka N. Preparation of monolithic silica columns for high-performance liquid chromatography. *Journal of Chromatography A* 2008;1191:231–252.
18. Ruhle W, Paulsen H. Preparation of native and recombinant light-harvesting chlorophyll-*a*/*b* complex. *Photosynthesis Research Protocols* 2004;684:113–125.
19. Pol J, Hyotylainen T, Ranta-Aho O, Riekkola ML. Determination of lycopene in food by on–line SFE coupled to HPLC using a single monolithic column for trapping and separation. *Journal of Chromatography A* 2004;1052:25–31.
20. Tanaka N, Kobayashi H, Ishizuka N, et al. Monolithic silica columns for high-efficiency chromatographic separations. *Journal of chromatography A* 2002;965:35–49.

21. Indriatmoko, Shioi Y, Brotosudarmo THP, Limantara L. *Comparison of column performance between monolithic and particulate packing for the separation of photosynthetic pigments*. Paper presented at: International Conference of Plant Physiology; August 26th-28th,2014; Bali, Indonesia.
22. Britton G, Liaaen–Jensen S, Pfander H. *Carotenoid volume 1A: isolation and analysis*. Basel: Brikhauser Verlag; 1995.
23. Gross J. *Pigments in vegetables: Chlorophylls and Carotenoids*. New York: Van Nostrand Reinhold; 1991.
24. Timperio AM, D'Amici GM, Barta C, Loreto F, Zolla L. Proteomics, pigment composition, and organization of thylakoid membranes in iron–deficient spinach leaves. *Journal of Experimental Botany* 2007;58:3695–3710.
25. Ottander C, Campbell D, Oquist G. Seasonal changes in photosystem II organisation and pigment composition in Pinus sylvestris. *Planta* 1995;197:176–183.
26. Schoefs B. Determination of pigments in vegetables. *Journal of chromatography A* 2004;1054:217–226.
27. Darko E, Schoefs B, Lemoine Y. Improved liquid chromatographic method for the analysis of photosynthetic pigments of higher plants. *Journal of Chromatography A* 2000;876:111–116.
28. Jayasuriya DC. The role of chemicals control in the fight against illicit drug production and trafficking. *Journal of Financial Crime* 1998;5:272–275.
29. Ballschmiter K, Truesdell K, Katz J. Aggregation of chlorophyll in nonpolar solvents from molecular weight measurements. *Biochimica et Biophysica Acta (BBA)-General Subjects* 1969;184:604–613.
30. Pepkowitz LP. The Stability of carotene in acetone and petroleum ether extracts of green vegetables. *Journal of Biological Chemistry* 1943;149:465–471.
31. Maruska A, Kornysova O. Application of monolithic (continuous bed) chromatographic columns in phytochemical analysis. *Journal of Chromatography A* 2006;1112:319–330.

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Abstract

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Nomenclature

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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 Δt_R and t_R ratio for selected pigments and resolution for structural isomers, such as α- and β-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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Nomenclature

Δt_R	retention time difference
t_R	retention time
t_R **Ratio**	ratio between retention time of two pigment peaks
v/v	solvents volume ratio

1. Introduction

Chromatography method has been introduced since 1905 as specialized technique for photosynthetic pigments separation[1]. Since then, several methods have been developed and commonly used, e.g., thin-layer chromatography[1,2], column chromatography[3,4], and high-performance liquid chromatography (HPLC)[5–7]. 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[8,9]. 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[10–12].

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[13]. Ability of these columns in separating pigments had reported for various samples and their improvements[5,14–16]. 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[17]. There are several reports on the monolithic column performance in separating photosynthetic pigments[16,18,19]. This column type is known for its advantages in providing good separation and short time analysis[20]. 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[5,6,14,15].

Recently, new types of column based on napthylethtyl bonded silica packing and cholesteryl bonded silica packing were invented. Cholester column is basically similar with conventional ODS column as their equivalent hydrophobicity. Nevertheless, cholester column has high sensitivity for hydrophobic compound due to their strong stereo-selectivity. Like cholester 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[21]. 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 cholester 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[21]. 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[5,6,22,23].

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 plants[23–26]. 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)*					Ref.***
		Acetone	n-Hexane	Diethyl ether	ethanol	eluent**	
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: Hegazi[5]; Jeffrey[6]; Britton[22]; Gross[23].

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.

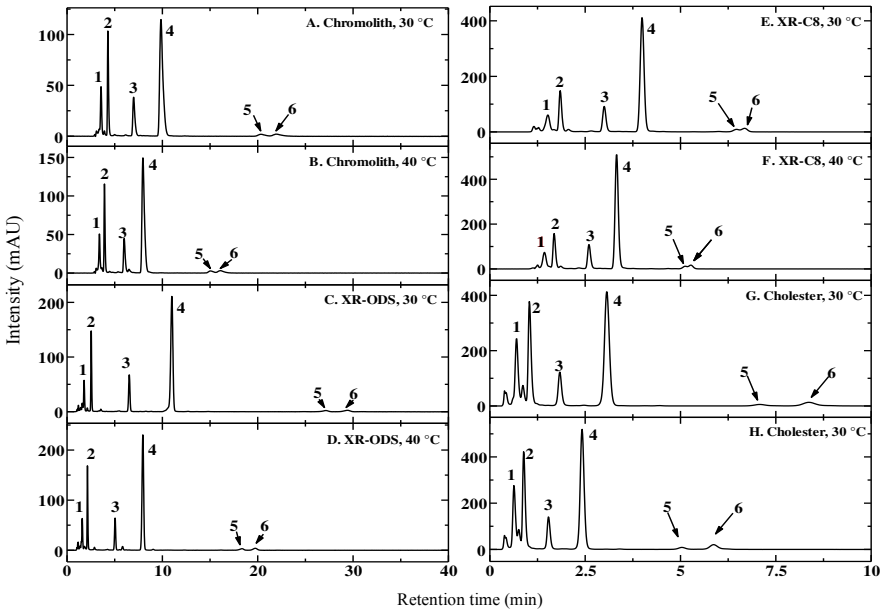

Fig. 1. UFPLC chromatograms of photosynthetic pigments from leaves of *P. angustifolia*. UFPLC was carried out an isocratic in System 3 (50 : 50, v/v) and flow rate at 0.5 mL per min. Other conditions are described in the text.

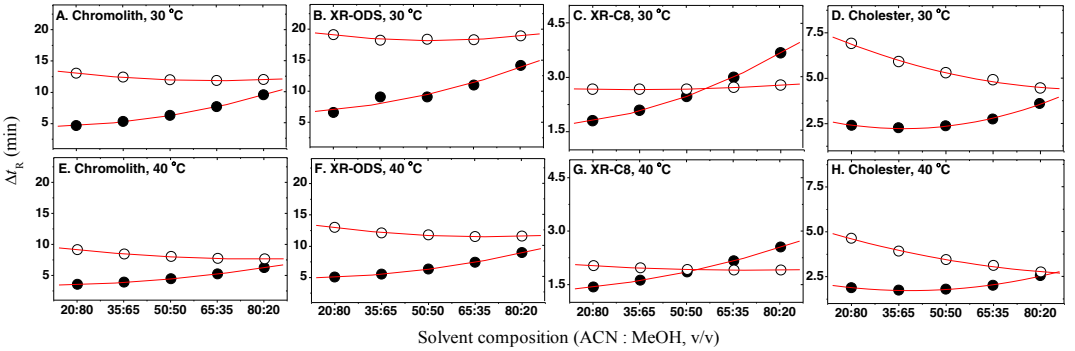

Fig. 2. $\Delta t_{Rchl\_a\text{-}viol}$ (solid circle) and $\Delta t_{Rb\text{-}car\text{-}chl\_a}$ (open circle) were calculated from the results of UFPLC separation of photosynthetic pigments extracted from leaves of *P. angustifolia*. Other conditions are the same as in Fig. 1. Data are average of 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 Δt_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 Δt_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, Δt_R of XR-C8 column was more conspicuously increased than any other columns. In contrast, Δt_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. $\Delta t_{R\ chl\_a\text{-}viol}$ and $\Delta t_{R\ b\text{-}car\text{-}chl\_a}$, polynomial regression from analyzed sample.

No	Column	Temperature	$\Delta t_{R\ chl\ a\text{-}viol}$		$\Delta t_{R\ b\text{-}car\text{-}chl\ a}$	
			Equation	R^2	Equation	R^2
1	Chromolith	30 °C	$Y = 4.55 - 0.06X + 0.21X^2$	0.99	$Y = 14.06 - 1.06X + 0.13X^2$	0.99
		40 °C	$Y = 3.54 + 0.01X + 0.10X^2$	1.00	$Y = 10.07 - 0.93X + 0.09X^2$	0.99
2	XR-ODS	30 °C	$Y = 6.66 + 0.28X + 0.23X^2$	0.94	$Y = 20.16 - 1.23X + 0.20X^2$	0.88
		40 °C	$Y = 4.98 - 0.07X + 0.17X^2$	0.99	$Y = 14.01 - 1.18\ X + 0.14X^2$	0.99
3	XR-C8	30 °C	$Y = 1.66 + 0.07X + 0.06X^2$	0.99	$Y = 2.726 - 0.04X + 0.01X^2$	0.98
		40 °C	$Y = 1.36 + 0.07X + 0.03X^2$	0.99	$Y = 2.14 - 0.09X + 0.01X^2$	0.99
4	Cholester	30 °C	$Y = 2.91 - 0.66X + 0.15X^2$	0.99	$Y = 7.94 - 1.16X + 0.09X^2$	0.99
		40 °C	$Y = 2.24 - 0.47X + 0.1X^2$	0.99	$Y = 5.37 - 0.82X + 0.06X^2$	0.99

Peak retention time ratio (t_R ratio) is also one of parameters to understand the peak separation. Ratios of $t_{RChl\_a/viol}$ and $t_{R\beta\text{-}car/Chl\_a}$, 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, $t_{Rchl\_a/viol}$ 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, $t_{R\beta\text{-}car/chl\_a}$ 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.

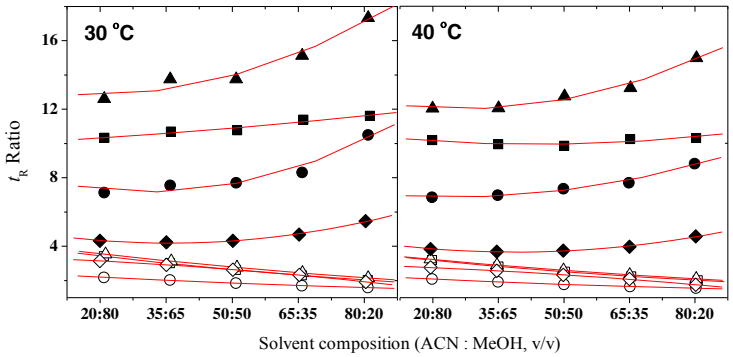

Fig. 3. $t_{R\ chl\_a/viol}$ ratio (solid) and $t_{R\ b\text{-}car/chl\_a}$ ratio (open), from Chromolith (Square), XR-ODS (triangle), XR-C8 (circle), and cholester column (diamond) employed at 30 °C and 40 °C column temperature.

Table 3. t_R ratio polynomial regression calculated from analyzed sample.

No	Column	Temperature	$t_{R\ chl\ a/viol}$		$t_{R\ b\text{-}car/chl\ a}$	
			Equation	R^2	Equation	R^2
1	Chromolith	30 °C	$Y = 10.14 + 0.20X + 0.02X^2$	0.96	$Y = 4.00 - 0.48X + 0.02X^2$	0.99
		40 °C	$Y = 10.54 - 0.41X + 0.07X^2$	0.72	$Y = 3.68 - 0.42X + 0.02X^2$	0.99
2	XR-ODS	30 °C	$Y = 13.05 - 0.40X + 0.24X^2$	0.96	$Y = 4.05 - 0.45X + 0.02X^2$	0.99
		40 °C	$Y = 12.68 - 0.68X + 0.23X^2$	0.98	$Y = 3.72 - 0.39X + 0.01X^2$	0.99
3	XR-C8	30 °C	$Y = 8.04 - 0.95X + 0.28X^2$	0.95	$Y = 2.47 - 0.20X + 0.01X^2$	0.99
		40 °C	$Y = 7.16 - 0.36X + 0.13X^2$	0.98	$Y = 2.39 - 0.20X + 0.01X^2$	0.99
4	Cholester	30 °C	$Y = 4.85 - 0.58X + 0.14X^2$	0.99	$Y = 3.40 - 0.14X - 0.02X^2$	0.99
		40 °C	$Y = 4.36 - 0.54X + 0.12X^2$	0.99	$Y = 3.00 - 0.13X - 0.02X^2$	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 cosmosil 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.

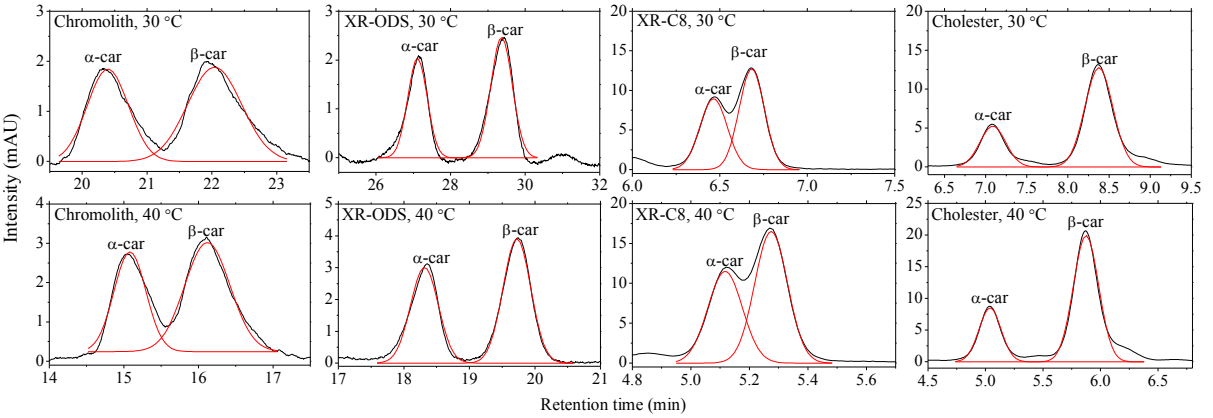

Fig. 4. Peak separation of non-polar pigments (α-carotene and β-carotene). UFPLC was carried out an isocratic in Sys. 3 and 0.5 mL per min flow rate. Black line represents original chromatogram. Red lines show Gaussian peak fitting results.

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[5,10,14,15,27]. 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[5,14,15]. Since the adoption of the 1988 UN Convention against Illicit Traffic 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[28]. 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[29,30]. This may cause in decreasing accuracy of the data.

In the previous study[21], 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[13,31]. 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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References

1. Jeffrey S. Quantitative thin layer chromatography of chlorophylls and carotenoids from marine algae. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1968;162:271–285.
2. Quach HT, Steeper RL, Griffin GW. An improved method for the extraction and thin-layer chromatography of chlorophyll *a* and *b* from spinach. *Journal of Chemical Education* 2004;81:385.
3. Omata T, Murata N. Preparation of chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll *a* by column chromatography with DEAE–Sepharose CL–6B and Sepharose CL–6B. *Plant and Cell Physiology* 1983;24:1093–1100.
4. Gilmore AM, Yamamoto HY. Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C18 high-performance liquid chromatographic column. *Journal of Chromatography A* 1991;543:137–145.
5. Hegazi MM, Ruzafa AP, Almela L, Candela ME. Separation and identification of chlorophylls and carotenoids from *Caulerpa prolifera*, *Jania rubens* and *Padina pavonica* by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* 1998;829:153–159.
6. Jeffrey S, Wright S, Mantoura R. *Phytoplankton pigments in oceanography: guidelines to modern methods*. Paris: Unesco Pub; 1997.
7. Shioi Y, Fukae R, Sasa T. Chlorophyll analysis by high-performance liquid chromatography. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1983;722:72–79.
8. Yan B, Zhao J, Brown JS, Blackwell J, Carr PW. High-temperature ultrafast liquid chromatography. *Analytical Chemistry* 2000;72:1253–1262.
9. Romanyshyn L, Tiller PR, Alvaro R, Pereira A, Hop CE. Ultra–fast gradient vs. fast isocratic chromatography in bioanalytical quantification by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2001;15:313–319.
10. Canjura FL, Schwartz SJ. Separation of chlorophyll compounds and their polar derivatives by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry* 1991;39:1102–1105.
11. Hart DJ, Scott KJ. Development and evaluation of an HPLC method for the analysis of carotenoids in foods and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chemistry* 1995;54:101–111.
12. Shioi Y, Watanabe K, Takamiya K. Enzymatic conversion of pheophorbide a to the precursor of pyropheophorbide a in leaves of Chenopodium album. *Plant and Cell Physiology* 1996;37:1143–1149.
13. Unger KK, Skudas R, Schulte MM. Particle packed columns and monolithic columns in high-performance liquid chromatography-comparison and critical appraisal. *Journal of Chromatography A* 2008;1184:393–415.
14. Wright S, Jeffrey SW, Mantoura R, et al. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series* 1991;77:183–196.
15. Zapata M, Rodriguez F, Garrido JL. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. *Marine Ecology Progress Series* 2000;195:29–45.
16. Garrido JL, Rodriguez F, Campana E, Zapata M. Rapid separation of chlorophylls *a* and *b* and their demetallated and dephytylated derivatives using a monolithic silica C18 column and a pyridine-containing mobile phase. *Journal of Chromatography A* 2003;994:85–92.
17. Nunez O, Nakanishi K, Tanaka N. Preparation of monolithic silica columns for high-performance liquid chromatography. *Journal of Chromatography A* 2008;1191:231–252.
18. Ruhle W, Paulsen H. Preparation of native and recombinant light-harvesting chlorophyll-*a*/*b* complex. *Photosynthesis Research Protocols* 2004;684:113–125.
19. Pol J, Hyotylainen T, Ranta-Aho O, Riekkola ML. Determination of lycopene in food by on–line SFE coupled to HPLC using a single monolithic column for trapping and separation. *Journal of Chromatography A* 2004;1052:25–31.
20. Tanaka N, Kobayashi H, Ishizuka N, et al. Monolithic silica columns for high-efficiency chromatographic separations. *Journal of chromatography A* 2002;965:35–49.

21. Indriatmoko, Shioi Y, Brotosudarmo THP, Limantara L. *Comparison of column performance between monolithic and particulate packing for the separation of photosynthetic pigments*. Paper presented at: International Conference of Plant Physiology; August 26th-28th,2014; Bali, Indonesia.
22. Britton G, Liaaen–Jensen S, Pfander H. *Carotenoid volume 1A: isolation and analysis*. Basel: Brikhauser Verlag; 1995.
23. Gross J. *Pigments in vegetables: Chlorophylls and Carotenoids*. New York: Van Nostrand Reinhold; 1991.
24. Timperio AM, D'Amici GM, Barta C, Loreto F, Zolla L. Proteomics, pigment composition, and organization of thylakoid membranes in iron–deficient spinach leaves. *Journal of Experimental Botany* 2007;58:3695–3710.
25. Ottander C, Campbell D, Oquist G. Seasonal changes in photosystem II organisation and pigment composition in Pinus sylvestris. *Planta* 1995;197:176–183.
26. Schoefs B. Determination of pigments in vegetables. *Journal of chromatography A* 2004;1054:217–226.
27. Darko E, Schoefs B, Lemoine Y. Improved liquid chromatographic method for the analysis of photosynthetic pigments of higher plants. *Journal of Chromatography A* 2000;876:111–116.
28. Jayasuriya DC. The role of chemicals control in the fight against illicit drug production and trafficking. *Journal of Financial Crime* 1998;5:272–275.
29. Ballschmiter K, Truesdell K, Katz J. Aggregation of chlorophyll in nonpolar solvents from molecular weight measurements. *Biochimica et Biophysica Acta (BBA)-General Subjects* 1969;184:604–613.
30. Pepkowitz LP. The Stability of carotene in acetone and petroleum ether extracts of green vegetables. *Journal of Biological Chemistry* 1943;149:465–471.
31. Maruska A, Kornysova O. Application of monolithic (continuous bed) chromatographic columns in phytochemical analysis. *Journal of Chromatography A* 2006;1112:319–330.