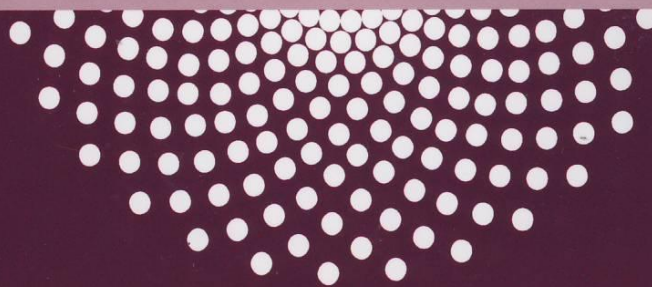


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2nd Humboldt Kolleg in Conjunction with International Conference on Natural Sciences 2014, HK-ICONS 2014

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.

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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 times
v/v	solvents volume

1. Introduction

Chromatography method for separation of photosynthetic pigments. Since the development of chromatography^{1,2}, column liquid chromatography conducting researches in the development of four main parameters, the best conditions for Pigment analyses of leaf

In main separation separation occurs during HPLC/UFLC columns separating pigments have of small-sized skeleton with particulate packing performance in separation good separation and slow separation. Two most silica. There are number photosynthetic pigment

Recently, new type packing were invented hydrophobicity. Nevertheless stereo-selectivity. Like naphthylethyl bonded silica advantages, in case of carotenes group.

In previous investigation addition to C18 monolithic particulate and monolithic leaves of *Pleomele* and common six major pigments investigation results show column had their characteristic two new type columns approaches would give pigments.

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 separation¹. 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 methods¹⁰⁻¹².

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 types¹³. 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 drop¹⁷. 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 analysis²⁰. 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 naphthylethyl 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 naphthylethyl 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 separation²¹. 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 cholesterol, 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 previously²¹. 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 by polynomial regression was used. The data represent an average of three runs.

3. Results and discussion

Six photosynthetic pigments were identified from the properties of four columns. The results were compared with the properties of four columns with comparison of absorption spectra. Zeaxanthin (2nd peak), chlorophyll *b* (3rd peak) (Table 1), as generated from the data after separation with mobile phase 50 : 50 (System 3) per min and column temperature 30 °C. Moreover, particulate pigments were also obtained (Fig. 1. A and C), despite of different column types. This is probably due to the high temperature 40 °C, retention time is also shorter. The results were also obtained by C8 column.

Table 1. Identification of the pigments

Peak No.	Pigment
1	Violaxanthin
2	Zeaxanthin
3	Chlorophyll <i>b</i>
4	Chlorophyll <i>a</i>
5	α -Carotene
6	β -Carotene

*Represent I-II-III bands for carotenoids

**Mobile phase, 50 : 50 (System 3)

***References: Hegazi⁵; Jeffrey⁶

Cholesteryl bonded silica (C8 and H) in terms of selectivity for hydrophobic compounds. The results showed that the C8 column could be clearly separated carotenoids compared with C18 column. It proved to be more suitable for the separation of carotenoids. The cholesterol column might be more suitable for the separation of polar pigments (data not shown). This column, however, might be more suitable for carotenoids and their isomers.

Data analysis

HPLC 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 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, the properties of four columns were mainly compared, excluding π -NAP column. The pigments were identified by 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 for 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. 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 <i>b</i>	455,592,649	-	455,595,641	463,590,645	465,595,648	6,22,23
4	Chlorophyll <i>a</i>	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, cholesterol 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 cholesterol 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.

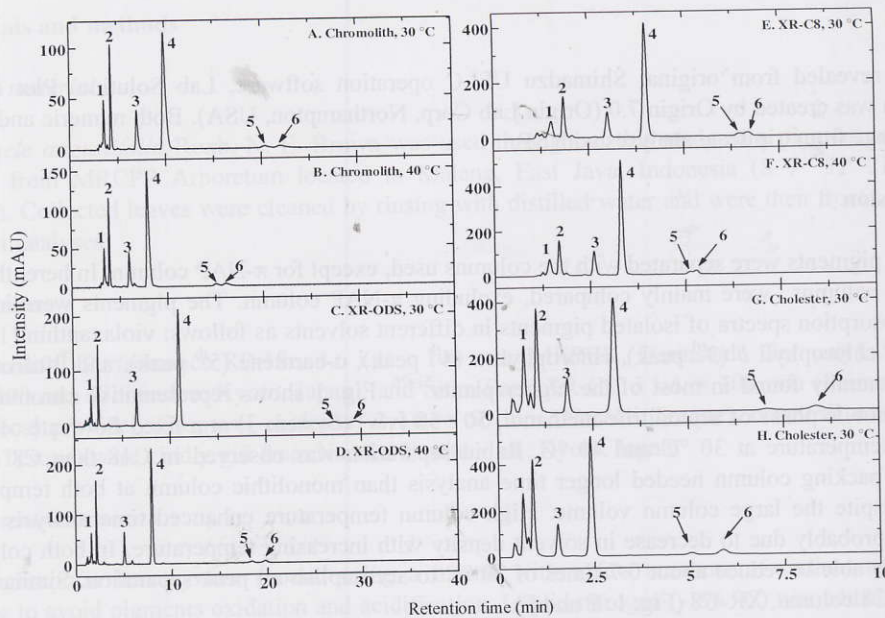


Fig. 1. UFPLC chromatograms of photosynthetic pigments from leaves of *P. angustifolia*. UFPLC was carried out an isocratic in System (50 : 50, v/v) and flow rate at 0.5 mL per min. Other conditions are described in the text.

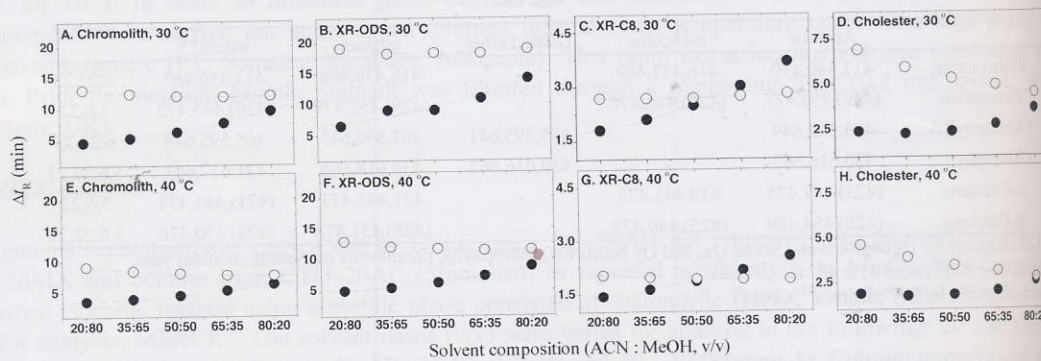


Fig. 2. $\Delta t_{Rchl_a/viol}$ (solid circle) and $\Delta t_{Rb-car-chl_a}$ (open circle) were calculated from the results of UFPLC separation of photosynthetic pigments extracted from leaves of *P. angustifolia*. Other conditions are the same as in Fig. 1. Data are average of three experiments. SE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

Table 2. $\Delta t_{Rchl_a/viol}$ and $\Delta t_{Rb-car-chl_a}$

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

Peak retention time ratio $t_{Rchl_a/viol}$ and t_{Rb-car/Chl_a} were also calculated at different column temperatures on t_R ratio. For XR-C8 columns, $t_{Rchl_a/viol}$ was increasing solvent strength. The t_R ratios of columns were linearly decreased from analyzed samples are shown

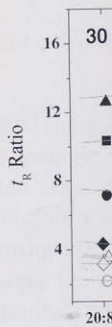
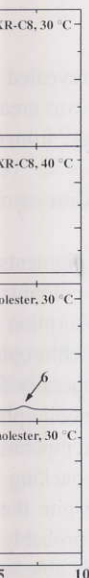


Fig. 3. $t_{Rchl_a/viol}$ ratio (solid) and t_{Rb-car/Chl_a} ratio (diamond) employed at 30 °C

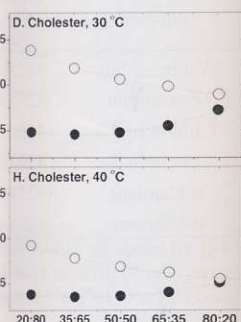
Table 3. t_R ratio polynomial regression

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

Δt_R and t_R ratio analysis of pigments extracted from *P. angustifolia* provides acceptable results in separation of polar-semi-polar pigments. The separation of β-carotene was not the case.



s carried out an isocratic in System 3



separation of photosynthetic pigments
are average of three experiments.

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

No	Column	Temperature	$\Delta t_{R \text{ chl } a/\text{viol}}$		$\Delta t_{R \text{ b-car/chl } a}$	
			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 $t_{R \text{ chl } a/\text{viol}}$ and $t_{R \text{ b-car/chl } a}$ 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, $t_{R \text{ chl } a/\text{viol}}$ 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, $t_{R \text{ b-car/chl } a}$ 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.

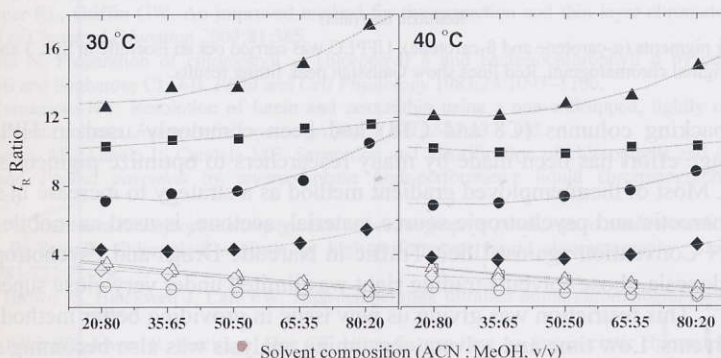


Fig. 3. $t_{R \text{ chl } a/\text{viol}}$ ratio (solid) and $t_{R \text{ b-car/chl } a}$ ratio (open), from Chromolith (Square), XR-ODS (triangle), XR-C8 (circle), and cholester column (diamond) employed at 30 °C and 40 °C column temperature.

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

No	Column	Temperature	$t_{R \text{ chl } a/\text{viol}}$		$t_{R \text{ b-car/chl } a}$	
			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 cholesterol column. Combined together with the previous results, cholesterol column is superior for the separation of non-polar pigments in terms of selectivity and resolution.

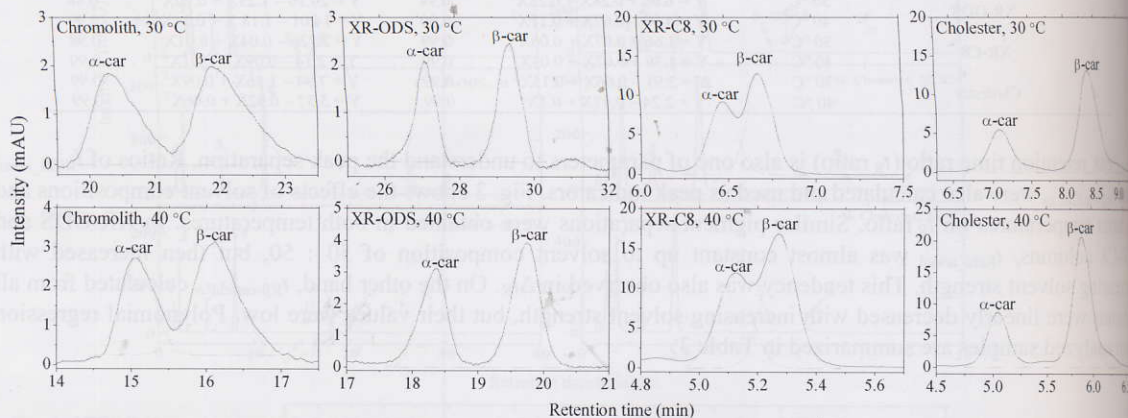


Fig. 4. Peak separation of non-polar pigments (α -carotene and β -carotene). UFPLC was carried out an isocratic in Sys. 3 and 0.5 mL per min flow rate. Black line represents original chromatogram. Red lines show Gaussian peak fitting results.

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 used²⁸. 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 study²¹, 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 cholesterol 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 cholesterol column.

4. Conclusion

In this study, the efficient separation of photosynthetic pigments was achieved. Among above columns tested, cholesterol column was especially for hydrophobic pigments. Cholesterol column is superior to resolution of structural parameters in separating photosynthetic pigments.

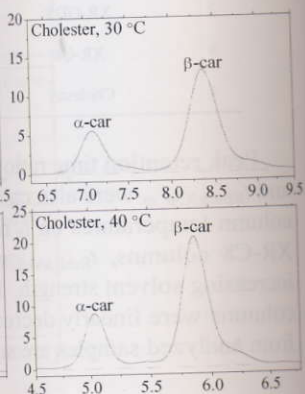
Acknowledgement

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References

1. Jeffrey S. Quantitative thin layer chromatography. *Bioenergetics* 1968;162:271–285.
2. Quach HT, Steeper RL, Griffin C. Separation of photosynthetic pigments in spinach. *Journal of Chemical Education* 1983;60:100–102.
3. Omata T, Murata N. Preparation of a reversed-phase Sepharose CL-6B and Sepharose C-6B column. *Journal of Chromatography* 1983;272:72–79.
4. Gilmore AM, Yamamoto HY. Reversed-phase liquid chromatographic column. *Journal of Chromatography* 1983;272:72–79.
5. Hegazi MM, Ruzafa AP, Almela J. Separation of *Jania rubens* and *Padina pavona* pigments. *Bioenergetics* 1998;829:153–159.
6. Jeffrey S, Wright S, Mantoura R. *Journal of Chromatography* 1983;272:72–79.
7. Shioi Y, Fukae R, Sasa T. Chlorophyll a and b in *Chlorella* sp. *Bioenergetics* 1983;113:15–21.
8. Yan B, Zhao J, Brown JS. *Blackwell Science* 1998;1262.
9. Romanyshyn L, Tiller PR, Alvaro R. Separation of photosynthetic pigments by liquid chromatography/tandem mass spectrometry. *Journal of Chromatography* 1998;829:153–159.
10. Canjura FL, Schwartz SJ. Separation of photosynthetic pigments. *Journal of Agricultural and Food Chemistry* 1998;46:1000–1004.
11. Hart DJ, Scott KJ. Development and application of a reversed-phase and carotenoid content of vegetables and fruits. *Bioenergetics* 1983;113:15–21.
12. Shioi Y, Watanabe K, Takamiya H. Separation of photosynthetic pigments in *Chenopodium album*. *Plant and Cell Physiology* 1983;24:1000–1004.
13. Unger KK, Skudas R, Schulte MM. Separation of photosynthetic pigments: a comparison and critical appraisal. *Journal of Chromatography* 1983;272:72–79.
14. Wright S, Jeffrey SW, Mantoura R. Separation of photosynthetic pigments in phytoplankton. *Marine Ecology Progress Series* 1983;11:1–10.
15. Zapata M, Rodriguez F, Garrido JL. Separation of photosynthetic pigments on a reversed phase C8 column and pyridine. *Journal of Chromatography* 1983;272:72–79.
16. Garrido JL, Rodriguez F, Campana M. Separation of photosynthetic pigments derivatives using a monolithic silica column. *Journal of Chromatography* 2008;1191:231–235.
17. Nunez O, Nakanishi K, Tanaka N. Separation of photosynthetic pigments. *Chromatography A* 2008;1191:231–235.
18. Ruhle W, Paulsen H. Preparation of a reversed-phase C8 column. *Journal of Chromatography* 2004;684:113–125.
19. Pol J, Hyotylainen T, Ranta-Aho O. Separation of photosynthetic pigments on a monolithic column for trapping and separation. *Journal of Chromatography* 2002;965:35–49.
20. Tanaka N, Kobayashi H, Ishizuka N. Separation of photosynthetic pigments. *Chromatography A* 2002;965:35–49.

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used in HPLC for separating the pigment separation through increase in separation quality. used as mobile phase^{5,14,15}. Since and Psychotropic Substances, in very close supervision in order to better method for HPLC, which is becoming strong demand for synthetic pigments were unstable and considered solvent-pigment. This may cause in decreasing

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over traditional C18 materials and expected as an ideal column for separation. There was, however, limited resolution. In this report, this column is its advantages and specialized. This is the first report on the

4. Conclusion

In this study, the efficiency of five reverse-phase columns, C8, C18, C18 monolithic, π -NAP, and cholesterol, for separation of photosynthetic pigments at several fixed conditions of mobile phase and temperature were compared. Among above columns tested, cholesterol 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

- Jeffrey S. Quantitative thin layer chromatography of chlorophylls and carotenoids from marine algae. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1968;162:271-285.
- 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.
- 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.
- Gilmore AM, Yamamoto HY. Resolution of lutein and zeaxanthin using a non-encapped, lightly carbon-loaded C18 high-performance liquid chromatographic column. *Journal of Chromatography A* 1991;543:137-145.
- 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.
- Jeffrey S, Wright S, Mantoura R. *Phytoplankton pigments in oceanography: guidelines to modern methods*. Paris: Unesco Pub; 1997.
- Shioi Y, Fukae R, Sasa T. Chlorophyll analysis by high-performance liquid chromatography. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1983;722:72-79.
- Yan B, Zhao J, Brown JS, Blackwell J, Carr PW. High-temperature ultrafast liquid chromatography. *Analytical Chemistry* 2000;72:1253-1262.
- Romanishyn 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Nunez O, Nakanishi K, Tanaka N. Preparation of monolithic silica columns for high-performance liquid chromatography. *Journal of Chromatography A* 2008;1191:231-252.
- Ruhle W, Paulsen H. Preparation of native and recombinant light-harvesting chlorophyll-*a/b* complex. *Photosynthesis Research Protocols* 2004;684:113-125.
- 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.
- 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: Birkhauser 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. Ballschmitter 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

Spirulina is one of the Bioactive compound in *Spi* problem in the world. The microalgae was conducted antihyperglycemic activity phycoecyanin. Blood glucos administration of biomass a blood glucose level.

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Keywords: Antihyperglycemic;

Nomenclature

wk	week
d	day

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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 separation¹. 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 methods¹⁰⁻¹².

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 types¹³. 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 drop¹⁷. 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 analysis²⁰. 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 naphthylethyl 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 naphthylethyl 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 separation²¹. 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 μ m, 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 previously²¹. 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 μ L 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 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)*					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 <i>b</i>	455,592,649	-	455,595,641	463,590,645	465,595,648	6,22,23
4	Chlorophyll <i>a</i>	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, cholesterol 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 cholesterol 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.

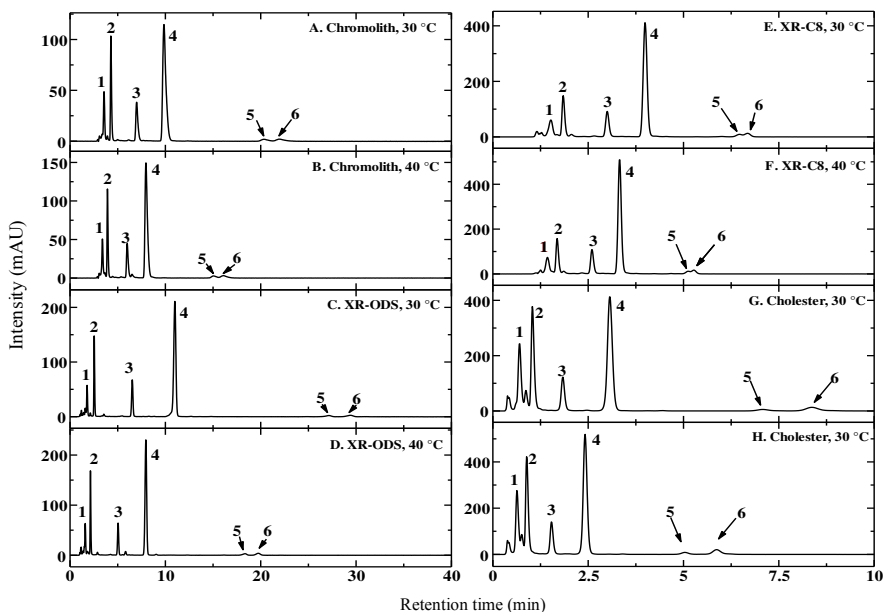


Fig. 1. UFPLC chromatograms of photosynthetic pigments from leaves of *P. angustifolia*. UFPLC was carried out an isocratic in System 3 (50 : 50, v/v) and flow rate at 0.5 mL per min. Other conditions are described in the text.

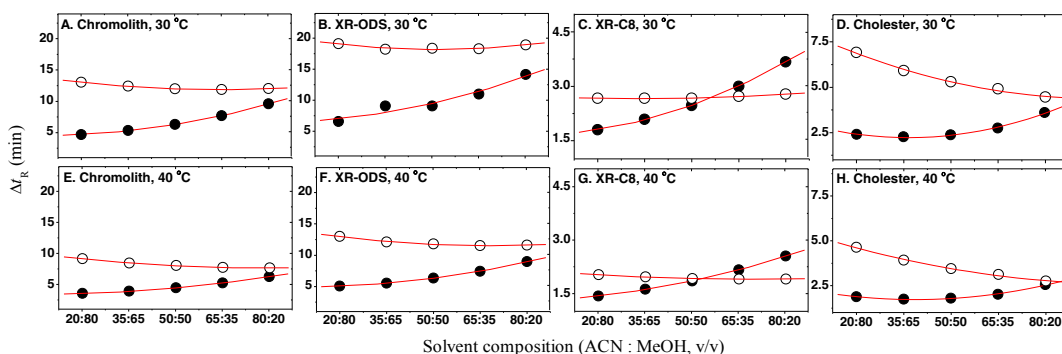


Fig. 2. Δt_{Rchl_a-viol} (solid circle) and $\Delta t_{Rb-car-chl_a}$ (open circle) were calculated from the results of UFPLC separation of photosynthetic pigments extracted from leaves of *P. angustifolia*. Other conditions are the same as in Fig. 1. Data are average of three experiments. SE is less 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 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. $\Delta t_{R \text{ chl}_a/\text{viol}}$ and $\Delta t_{R \text{ b-car-chl}_a}$ polynomial regression from analyzed sample.

No	Column	Temperature	$\Delta t_{R \text{ chl}_a/\text{viol}}$		$\Delta t_{R \text{ b-car-chl}_a}$	
			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 $t_{R \text{ chl}_a/\text{viol}}$ and $t_{R \text{ b-car}/\text{chl}_a}$, 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, $t_{R \text{ chl}_a/\text{viol}}$ 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, $t_{R \text{ b-car}/\text{chl}_a}$ 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.

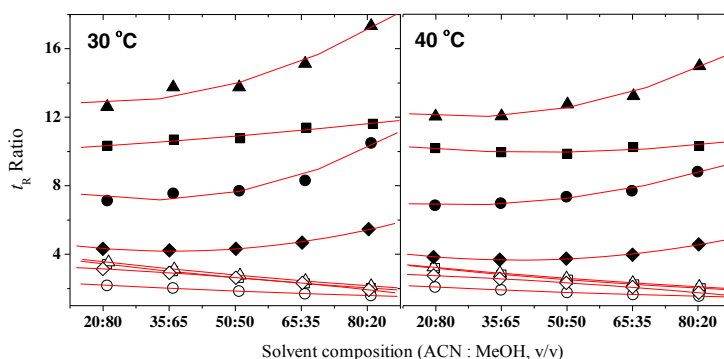


Fig. 3. $t_{R \text{ chl}_a/\text{viol}}$ ratio (solid) and $t_{R \text{ b-car}/\text{chl}_a}$ ratio (open), from Chromolith (Square), XR-ODS (triangle), XR-C8 (circle), and cholester column (diamond) employed at 30 °C and 40 °C column temperature.

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

No	Column	Temperature	$t_{R \text{ chl}_a/\text{viol}}$		$t_{R \text{ b-car}/\text{chl}_a}$	
			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 cholesterol column. Combined together with the previous results, cholesterol column is superior for the separation of non-polar pigments in terms of selectivity and resolution.

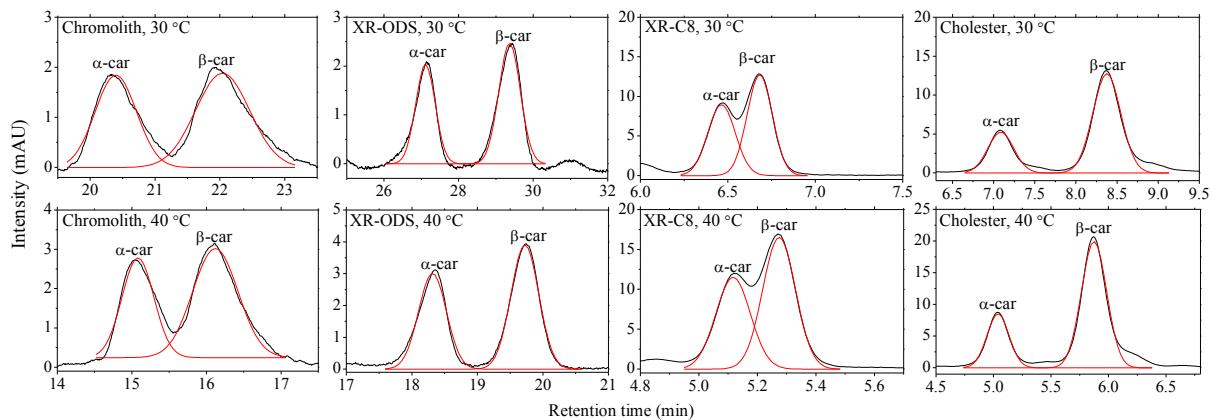


Fig. 4. Peak separation of non-polar pigments (α -carotene and β -carotene). UFPLC was carried out an isocratic in Sys. 3 and 0.5 mL per min flow rate. Black line represents original chromatogram. Red lines show Gaussian peak fitting results.

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 used²⁸. 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 study²¹, 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 cholesterol 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 cholesterol column.

4. Conclusion

In this study, the efficiency of five reverse-phase columns, C8, C18, C18 monolithic, π -NAP, and cholesterol, for separation of photosynthetic pigments at several fixed conditions of mobile phase and temperature were compared. Among above columns tested, cholesterol 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–Sephacel 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-encapped, 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 demethylated 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: Birkhauser 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.