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STUDY ON SEPARATION OF ω-HYDROXY AND N-FATTY ACIDS WITH AN HPLC TECHNIQUE

(*Mempelajari cara pemisahan ω-hidroksi dan n-asam lemak dengan menggunakan Kromatografi cair kinerja tinggi*)

By/*Oleh*:
Bambang Wiyono[1], P.J. Jordan[2] and Poedji Hastoeti[1]

Ringkasan

Penelitian ini bertujuan untuk mencari kondisi yang cocok untuk memisahkan ω- dan n-asam lemak tanpa derivitasi dengan menggunakan metode Kromatografi cairan kinerja tinggi. Dalam kromatografi cair kinerja tinggi digunakan kolom RP-18 Brownlee, fase bergerak campuran THF dan CH_3OCN, metode isokratik, dilakukan pada suhu kamar, kecepatan alir 1 ml/mn, menggunakan detektor UV dengan panjang gelombang 215 nm dan sensitivitasnya 0,5 AUFS. Hasil analisis menunjukkan bahwa peningkatan persentase air dalam fase bergerak meningkatkan waktu retensi n-asam lemak dan ω-hidroksi asam lemak. n-asam lemak dapat dipisahkan dengan baik bila menggunakan kondisi di atas dan menggunakan fase bergerak yang mengandung air sekitar 25-35 bagian. Sedangkan ω-hidroksi asam lemak dapat dipisahkan dengan baik dengan menggunakan fase bergerak yang mengandung air minimal 49 bagian berdasarkan volume.

Kata kunci: Kromatografi cair kinerja tinggi, ω-hidroksi asam lemak, n-asam lemak, pemisahan, fase bergerak

Summary

The objective of this study is to find out a suitable condition in separating ω-hydroxy and n-fatty acids into individual fatty acids without any derivatization with an HPLC technique. This separation was carried out on reversed phase column (Brownlee column RP-18, 5 μ particle size and 10 cm length) using isocratic method with a mixture of mobile phase (THF and CH_3OCN), flow rate at 1 ml/mn, sensitivity of 0.5 AUFS, integrator attenuation at 1, and at an ambient temperature with UV detector at 215 nm. The results indicated that the increase in water percentage in mobile phase tended to increase the retention time of n-fatty acids and ω-hydroxy fatty acids. With the above condition and mobile phase contained 25-35 part of water, n-fatty acids could be separated precisely, while ω-hydroxy fatty acids could be separated with mobile phase minimally containing 49 part of water by volume.

Key words: HPLC, n-fatty acids, ω-hydroxy fatty acids, separation, and mobile phase

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[1] Researcher at Forest Products Research Center

[2] Senior lecturer at Dep. of Chemical and Process Engineering, University of Canterbury New Zealand

I. INTRODUCTION

The most popular methods for the analysis of fatty acids and hydroxy fatty acids are gas liquid chromatography (GLC) and gas chromatography (GC). In GLC method, problems arise from forming the methyl ester derivative, including incomplete reaction, isomerization of unsaturated fatty acids and oxidation of hydrolysis (Bailie, et al. 1982). In GC method, the problem rises with respect to heat labile or short chain fatty acids (Hanis, et al. 1988). In order to overcome these problems, high performance liquid chromatography (HPLC) method has been introduced. This method offers several advantages. A major advantage is that the milder condition used in HPLC allows polar compounds of volatility or compound sensitivity to heat to be chromatographed. The detectors most often used in HPLC are nondestructive, including the visible ultra-violet, differential refractometer, infrared and fluorence spectrophotometer detectors. The eluate in HPLC is also convenient to be collected in separate fraction (Bailie, et al. 1982). Another advantage is that HPLC offers good resolution of important fatty acids (Hanis, et al. 1988).

Most identification of fatty acids and hydroxy fatty acids in HPLC was done after their derivatization with hydroxyamic acids (Gutnikov and Streng. 1991), 2-nitrophenylhydrazides (Miwa, et al. 1990), methyl ester (Tagaki, et al. 1989), p-nitro benzyl (PNB) ester (Bandi and Reynold. 1985), 2,4,6-tritertier-butylphenol (Lockwood. Et al. 1983), 3,5-dinitrobenzoyl chloride (Bjorkvist and Toivonen. 1978), etc. These derivatizations cause difficulty if the desired component is needed in pure form as a raw material for producing other products, because the derivative agent should be taken out from the desired component. To surmount this difficulty, identification of fatty acids and ω-hydroxy fatty acids should be done without any derivatization. So far, HPLC identification of underivatized fatty acids has been done with the defferential refractometer as a detector (Bailie, et al. 1982). Mobile phase used in this analysis was a mixture of tetrahydrofuran (THF), acetonitril (CH_3CN) and water, with the composition of 25:35:45, repectively. In this work, it has been tried to identify fatty acids and ω-hydroxy fatty acids without any derivatization, with UV-visible detector. This stage was to find out the proper condition, especially the amount of water in mobile phase, which was suitable to separate n-fatty acids and ω-hydroxy fatty acids.

II. MATERIAL AND METHODE

A. Source of Chemical

The chemicals used for HPLC analysis of fatty acids and ω-hydroxy fatty acids were as follows:

- Tetrahydrofuran (THF), hipersolve grade from BDH chemical Ltd, Poole, England.
- Acetonitrile (CH_3CN), hipersolve grade from BDH chemical Ltd, Poole, England.

- n-fatty acids (C_{16} and C_{18}) standard from Department of Chemical and Process Engineering, University of Canterbury, Christchurch, New Zealand.
- ω-hydroxy palmitic acid and a mixture of ω-hydroxy fatty acids (C_{12} - C_{18}) from Dr. Franich, Forest Research Institute, Rotorua, New Zealand.
- ω-hydroxy palmitic acid standard from Dr. Franich, Forest Research Institute, Rotorua, New Zealand.
- Deonized water, from School of Medicine, Christchurch, New Zealand.

B. Sample Preparation

The procedure for the sample preparation of underivatized n-palmitic acid, n-stearic acid, a mixture of ω-hydroxy fatty acids and ω-hydroxy palmitic acid standard was adopted from Bailie's procedure (Bailie, et al. 1982) with some modification.

A certain amount of n-palmitic acid, n-stearic acid, a mixture of ω-hydroxy fatty acids and ω-hydroxy palmitic acid standard was weighed accurately into a phial. To dissolve the standard, tetrahydrofuran (THF) was added. When the standard did not dissolve properly, it was necessary to warm it up under hot water stream, then acetonitrile was added. Additional amount of THF was added when the solution was cloudy. The solution then was mixed and brought to concentration of 0.65 % by weight. These standard solutions were protected from the light by covering the phial with aluminium foil, and stored in a refrigerator at −15 °C when not in use.

C. HPLC Procedure

A modular high performance liquid chromatography (HPLC system used for fatty acids analysis is shown in Figure 1, along with details of the various components. As sample preparation, the procedure to analyze underivatized n-fatty acids and ω-hydroxy fatty acids was also adopted from Bailie's procedure (Bailie, et al. 1982).

Separation of n-fatty acids and ω-hydroxy fatty acids was carried out on a reversed phase column (Brownlee column, RP-18, 5 μ particle size and 10 cm length) using isocratic method, flow rate at 1 ml/mn, sensitivity of 0.5 AUFS, integrator attenuation at 1, and at an ambient temperature with UV detector at 215 nm. The solvent system to eluate n-fatty acids and ω-hydroxy fatty acids was a mixture of THF : CH_3CN : H_2O with 0.1 % by volume of acetic acid added to improve the peak symetry. The proportion of H_2O in the mixture of mobile phase varied from 35 to 49 parts by volume.

Using a defferential refractive index detector with THF : CH_3CN : H_2O (25:35:45) as a mobile phase, HPLC separation of the underivatized fatty acids has been done successfully (Bailie, et al. 1982). He also mentioned that the UV detector could be used to detect saturated and unsaturated fatty acids with the wavelength under 220 nm.

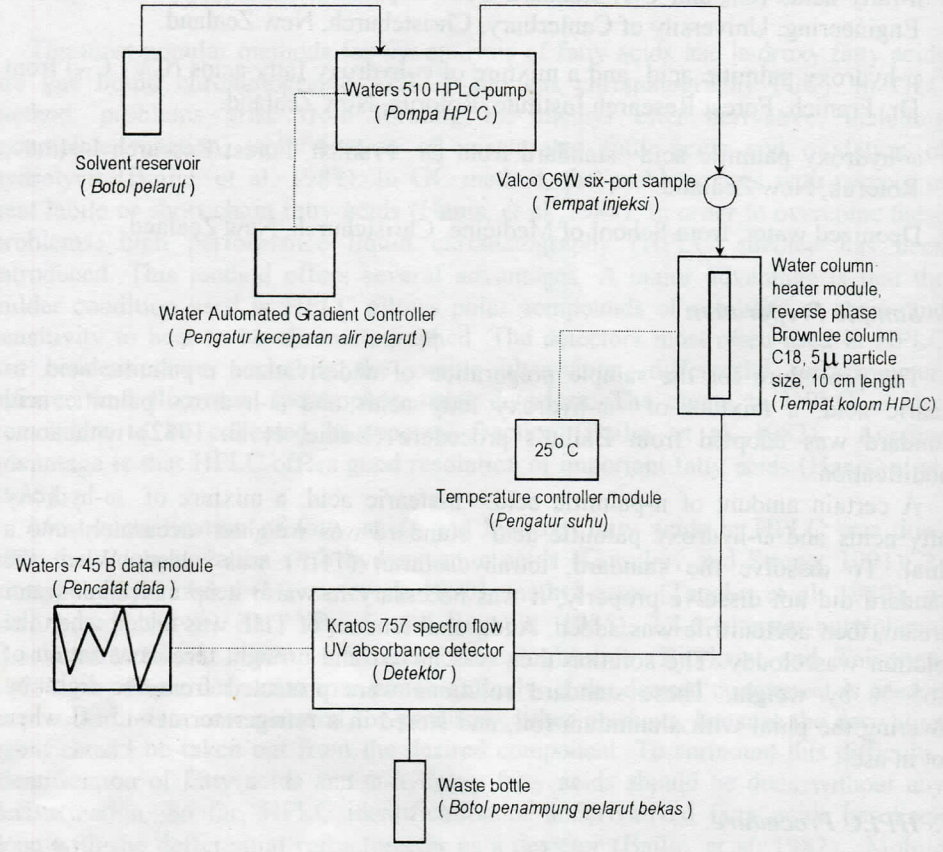

Figure 1. High performance liquid chromatographic system
Gambar 1. Sistem kromatografi cair kinerja tinggi

III. RESULTS AND DISCUSSION

During this experiment, the differential refractive index detector has been tried with similar condition as described by Bailie, *et al.* (1982), but the result was not satisfied because whenever the C_{16} or C_{18} sample was injected into HPLC, only did the peak of the solvent which was used to dissolve the sample appear in the chromatogram, did not injected sample. So, the UV detector was used throughout in this experiment with a wavelength of 215 nm. A mobile phase consisted of THF : CH_3CN : H_2O at proportion 25 : 35 : 45, respectively with the amount of H_2O in mobile phase varying from 35 to 49 parts by volume.

Table 1. Retention time of n-palmitic and n-stearic acid samples
Tabel 1. Waktu retensi n-asam palmitat dan stearat

Sample (*Contoh*)	Content of water in mobile phase (*Kandungan air dalam fase bergerak*) THF : CH_3CN : H_2O (25 : 35 : X) + 1 % Hac				
	Peak (*Puncak*)	X = 35		X = 45	
		Retention time (*Waktu retensi*)	Area (*Luas*)	Retention time (*Waktu retensi*)	Area (*Luas*)
n-palmitic acid (*Asam palmitat*)	C_{16}	32.13 29.96	26852 20139	+@	-
	C_{16}	31.94 29.93	24998 20040	+@@	-
n-stearic acid (*Asam stearat*)	C_{18}	44.23 39.69	20371 22328	-	-

Note: X part of water in mobile phase (*Bagian air yang terkandung dalam fase bergerak*)
* Injected at different date (*Diinjeksikan pada hari yang berbeda*)
@ Probably n-palmitic acid peak (*Mungkin puncak n-asam palmitat*)
@@ Probably n-stearic acid peak (*Mungkin puncak n-asam stearat*)

At 35 parts of water in mobile phase, the injected n-palmitic acid and n-stearic acid produced peaks with the retention time of 29.96 minutes and 39.69 minutes. After comparing with the HPLC results of the Bailie's investigation, the peak with 29.96 minutes retention time was identified as n-palmitic acid and the peak with 39.69 minutes was identified as n-stearic acid. With this mobile phase composition, when these samples were injected into the HPLC at a different date, their retention time increased. This was probably due to the change of mobile phase composition, where the part of water in the mobile phase increased because of evaporating the solvent (see Table 1). At 45 part of water in the mobile phase, from the sample of

Table 2. Retention time of n-palmitic and n-stearic acid samples separated from their mixture
Tabel 2. Waktu retensi n-asam palmitat dan stearat yang dipisah dari campurannya

Sample (*Contoh*)	Content of water in mobile phase (*Kandungan air dalam fase bergerak*) THF : CH_3CN : H_2O (25 : 35 : X) + 1 % HAc				
	Peak (*Puncak*)	X = 35		X = 36	
		Retention time (*Waktu retensi*)	Area (*Luas*)	Retention time (*Waktu retensi*)	Area (*Luas*)
n-palmitic acid (*Asam palmitat*)	C_{16}	31.72	277301	34.02	32421
n-stearic acid (*Asam stearat*)	C_{18}	43.97	8969	+@	

Note: X part of water in mobile phase (*Bagian air yang terkandung dalam fase bergerak*)
@ Probably n-stearic acid peak (*Mungkin puncak n-asam stearat*)

n-palmitic and stearic acids, certain peak appeared in the chromatogram. When its retention time of both chromatogram was measured manually, it was 62.20 minutes and 64.90 minutes, respectively (see @ in Table 1). Probably this peak was n-palmitic acid because the pattern of this peak was similar to the pattern of the peak in the chromatogram at 35 part of water. At 35 part of water in mobile phase, when a mixture of n-palmitic and stearic acid samples was injected to the HPLC (see Table 2), both acids appeared in the chromatogram, the peak which came out after n-palmitic acid's peak was n-stearic acid, and their retention time was similar to their retention time in Table 1. Then when the part of water in mobile phase was increased to 36 part, only did n-palmitic acid appear in the chromatogram with the retention time longer than its retention time at 35 part of water. However, one peak without retention time came up after n-palmitic acid's peak. This probably was the peak of n-stearic acid, as shown in Table 2.

Table 3. Retention time of n-palmitic and ω-hydroxy palmitic acid
Tabel 3. Waktu retensi n-asam palmitat dan ω-hidroksi asam palmitat

Sample (*Contoh*)	Content of water in mobile phase (*Kandungan air dalam fase bergerak*) THF : CH_3CN : H_2O (25 : 35 : X) + 1 % HAc									
	X = 35		X = 36		X = 45		X = 47		X = 49	
	RT	Area (*Luas*)	RT	Area (*Luas*)	RT	Area (*Luas*)	RT	Area (*Luas*)	RT	Area (*Luas*)
ω-OH C_{16}	2.81	39566	3.00	38795	4.15	39597	5.0	39298	5.38 5.21@@	34601 22440
C_{16}	29.99	37507	33.94	40304	+	na	+@	na	+@	na

Note: X, part of water in mobile phase (*Bagian air yang terkandung dalam fase bergerak*)
RT Retention time (*Waktu retensi*)
@ Probably n-palmitic acid peak (*Mungkin puncak n-asam palmitat*)
@@ Using pure standard of ω-hydroxy palmitic acid (*Menggunakan standar ω-hidroksi asam palmitat*)

The identification of ω-hydroxy palmitic acid was conducted based on the main peak which appeared in the chromatogram, and by comparing among its retention time which was chromatographed at different parts of water in the mobile phase. Initially ω-hydroxy palmitic acid sample separation of the hydrolysis product of solvent extracted wax was injected into the HPLC at 35 parts of water in the mobile phase. The peak with retention time of 2.81 minutes was identified as ω-hydroxy palmitic acid, and the retention time of 29.99 minutes was n-palmitic acid (Tabel 3). The reason why the peak with the retention time of 2.81 minutes was ω-hydroxy palmitic acid because the other peaks were always appeared in the chromatogram whatever the sample was injected into the HPLC. The peak with the retention time of 29.99 minutes was n-palmitic acid because its retention time with the same date of injection was similar to its retention time as shown in Table 1. When the part of water in mobile phase was increased to 36 parts, the retention time of ω-hydroxy palmitic acid and n-palmitic acid increased. Moreover, when the part

of water was increased to 45 parts, 47 parts and 49 parts, the retention time of ω-hydroxy palmitic acid always increased and became longer (Table 3). However, n-palmitic acid's peak appeared without its retention time, but if this peak was measured manually with a ruler from the beginning of elution, the increase in the part of water in the mobile phase caused the increased in its retention time.

Table 4. Retention time of ω-hydroxy lauric acid, ω-hydroxy and n-palmitic acid
Tabel 4. Waktu retensi ω-hidroksi asam laurat, ω-hidroksi dan n-asam palmitat

Sample (*Contoh*)	Content of water in mobile phase (*Kandungan air dalam fase bergerak*) THF : CH_3CN : H_2O (25 : 35 : X) + 1 % HAc							
	X = 35		X = 45		X = 47		X = 49	
	RT	Area (*Luas*)	RT	Area (*Luas*)	RT	Area (*Luas*)	RT	Area (*Luas*)
ω-OH C_{12}	1.96@	14192	2.59@	6375	2.95@	17948	5.0	39298
ω-OH C_{16}	2.86	9202	4.21	39597	5.0	39298	+@	na
C_{16}	29.99	4420	-	-	-	-		

Note: X, part of water in mobile phase (*Bagian air yang terkandung dalam fase bergerak*)
RT Retention time (*Waktu retensi*)
@ Probably ω-hydroxy lauric acid (*Mungkin ini ω-hidroksi asam laurat*)

When pure standard of ω-hydroxy palmitic acid was injected (Tabel 3), many peaks still appeared in the chromatogram as occurred to the ω-hydroxy palmitic acid sample from the separation of the hydrolysis product of solvent extracted wax. The retention time of this standard was around 5.16 and 5.21 minutes. The little difference of this retention time was probably due to using a new mobile phase (the same composition as mobile phase before). When a mixture of ω-hydroxy lauric, myristic and palmitic acid sample from the separation of the hydrolysis product of solvent extracted wax was injected into the HPLC, at 35 parts of water in mobile phase, only could the ω-hydroxy palmitic acid be identified; however, the peak with the retention time of 1.96 minutes was probably ω-hydroxy lauric acid. In this sample, n-palmitic acid also appeared in the chromatogram with the retention time 29.99 minutes. The increase in part of water in the mobile phase always led to the increase in the retention time of both ω-hydroxy lauric and palmitic acid (Tabel 4).

The detection of underivatized fatty acids was neither sensitive nor selective because these compounds generally do not contain suitable chromophores (chemical bonds or group which are responsible for UV-visible absorption). The use of the UV detector near 200 nm could not be recommended because the absorption of underivatized fatty acids was adversely affected by the impurities of organic solvent (Hanis, et al. 1988). Probably the properties and impurities of the solvent in the mobile caused many peaks appeared in the chromatogram when a single component such as n-palmitic acid was injected in the HPLC (se chromatograms in Appendix 1 to 9) This led to the difficulty to identify the desired component.

IV. CONCLUSION

During HPLC analysis of underivatized n-palmitic and stearic acids, and ω-hydroxy lauric, myristic and palmitic acid, increasing part of water in mobile phase gave a different effect. The increase in part of water in mobile phase led to lengthen the retention time of n-palmitic and stearic acids, ω-hydroxy lauric, myristic and palmitic acid. Every time a single component was injected into the HPLC, lot of interfering peaks appeared in the chromatogram along with it. The cause of interfering peaks should be work out to make easy in identifying the interest component. In the identifying of n-palmitic and stearic acids, less than 35 parts of water could be used in mobile phase to reduce their retention time; however, in the identifying of ω-hydroxy lauric, myristic and palmitic acid, the part of water in mobile phase was 49 parts or more to get better separation.

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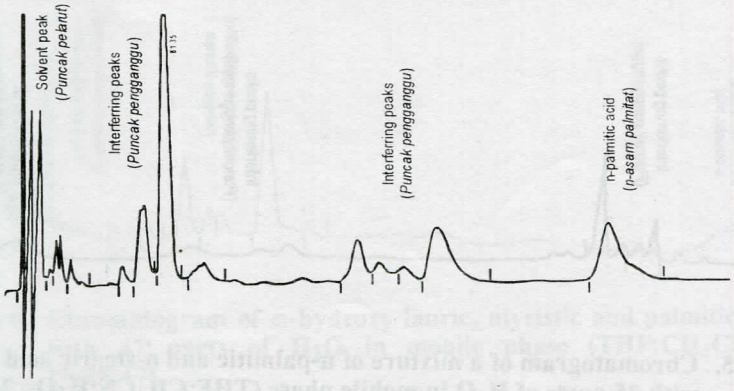
Appendix 1. Chromatogram of n-palmitic acid injected, with 35 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:35).

Lampiran 1. Kromatogram n-asam palmitat, 35 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:35).

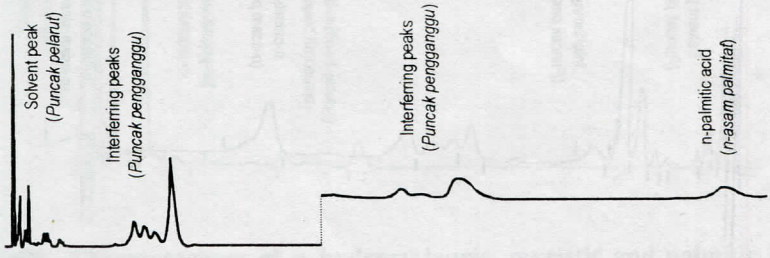
Appendix 1. Chromatogram of n-palmitic acid injected, with 35 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:35).

Lampiran 1. Kromatogram n-asam palmitat, 35 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:35).



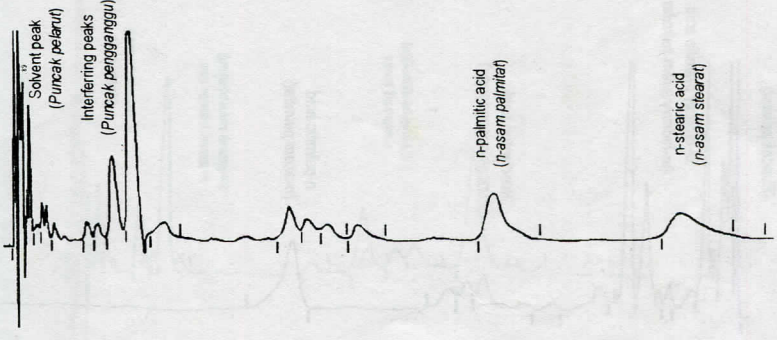
Appendix 2. Chromatogram of n-palmitic acid injected, with 45 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:45).

Lampiran 2. Kromatogram n-asam palmitat, 45 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:45).



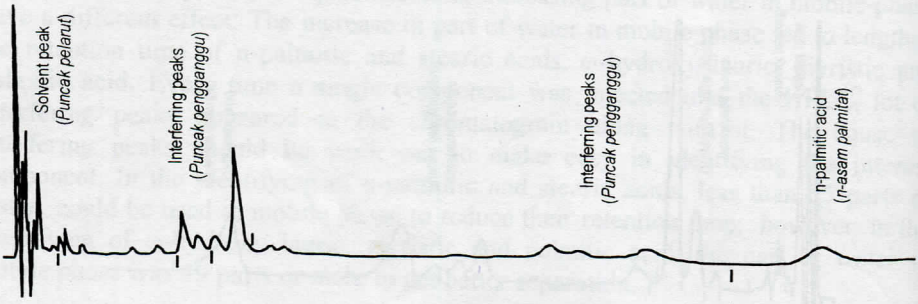
Appendix 3. Chromatogram of n-stearic acid injected, with 35 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:35).

Lampiran 3. Kromatogram n-asam stearat, 35 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:35).



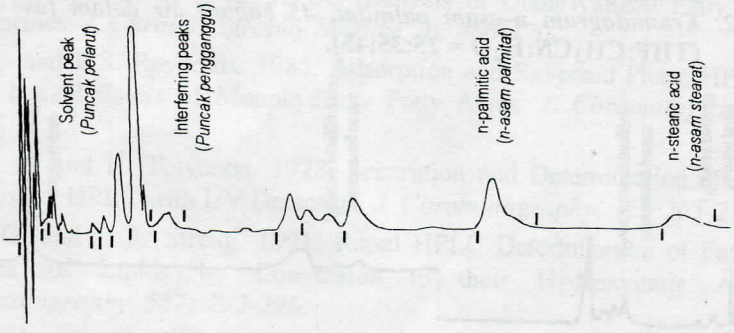
Appendix 4. **Chromatogram of n-stearic acid injected, with 45 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:45).**

Lampiran 4. ***Kromatogram n-asam stearat, 45 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:45).***



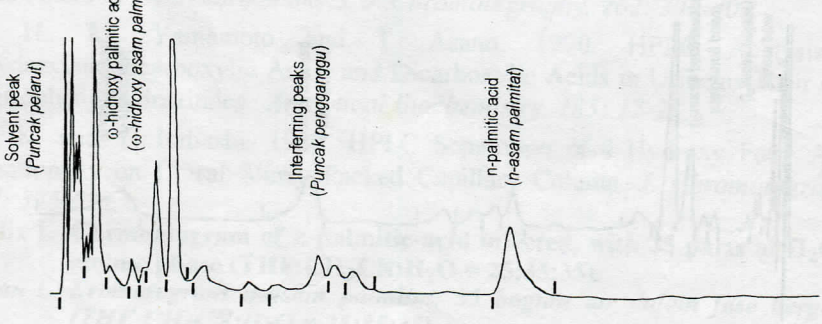
Appendix 5. **Chromatogram of a mixture of n-palmitic and n-stearic acid injected, with 35 parts of H_2O in mobile phase (THF:CH_3CN:H_2O= 25:35:35).**

Lampiran 5. ***Kromatogram campuran n-asam palmitat dan stearat, 35 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:35).***



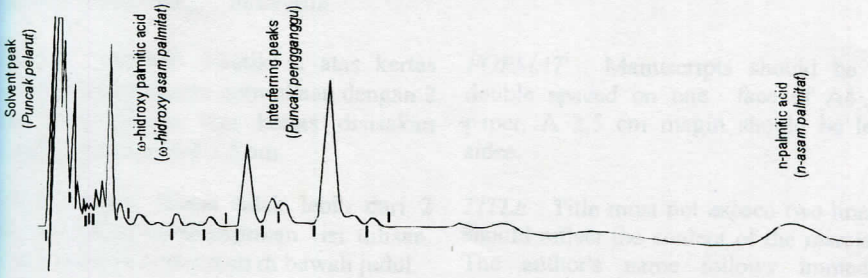
Appendix 6. **Chromatogram of ω-hydroxy palmitic acid injected, with 35 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:35).**

Lampiran 6. ***Kromatogram ω-hidroksi asam palmitat, 35 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:35).***



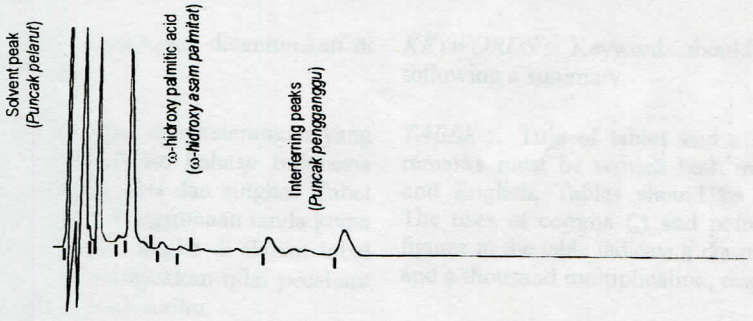
Appendix 7. Chromatogram of ω-hydroxy palmitic acid injected, with 49 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:49).

Lampiran 7. Kromatogram ω-hidroksi asam palmitat, 49 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:49).



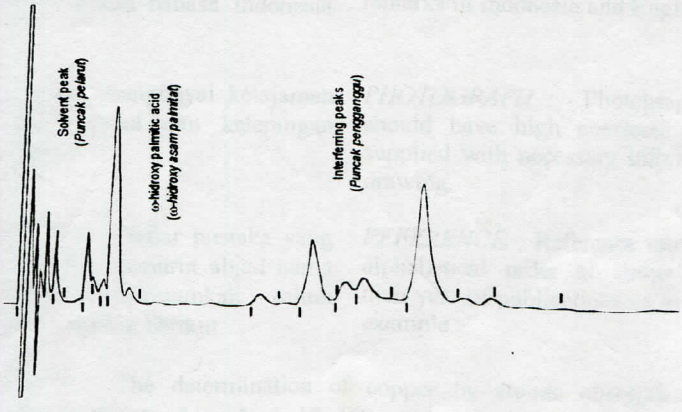
Appendix 8. Chromatogram of ω-hydroxy lauric, myristic and palmitic injected, with 47 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:47).

Lampiran 8. Kromatogram campuran ω-hidroksi asam laurat, miristat dan palmitat, 47 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:47).



Appendix 9. Chromatogram of ω-hydroxy lauric, myristic and palmitic injected, with 49 parts of H_2O in mobile phase (THF:CH_3CN:H_2O = 25:35:49).

Lampiran 9. Kromatogram campuran ω-hidroksi asam laurat, miristat dan palmitat, 49 bagian air dalam fase bergerak (THF:CH_3CN:H_2O = 25:35:49).



PETUNJUK BAGI PENULIS

BAHASA : Naskah ditulis dalam bahasa Indonesia dengan ringkasan dalam bahasa Inggris atau dalam bahasa Inggris dengan ringkasan dalam bahasa Indonesia.

FORMAT : Naskah diketik di atas kertas kuarto putih pada suatu permukaan dengan 2 spasi. Pada semua tepi kertas disisakan ruang kosong minimal 3,5 cm.

JUDUL : Judul dibuat tidak lebih dari 2 baris dan harus mencerminkan isi tulisan. Nama penulis dicantumkan di bawah judul.

RINGKASAN : Ringkasan dibuat tidak lebih dari 200 kata berupa intisari permasalahan secara menyeluruh, dan bersifat informatif mengenai hasil yang dicapai.

KATA KUNCI : Kata kunci dicantumkan di bawah ringkasan

TABEL : Judul Tabel dan keterangan yang diperlukan ditulis dalam bahasa Indonesia dan Inggris dengan jelas dan singkat. Tabel harus diberi nomor. Penggunaan tanda koma (,) dan titik (.) pada angka di dalam tabel masing-masing menunjukkan nilai pecahan/ desimal dan kebulatan seribu.

GAMBAR GARIS : Grafik dan ilustrasi lain yang berupa gambar garis harus kontras dan dibuat dengan tinta hitam. Setiap gambar garis harus diberi nomor, judul dan keterangan yang jelas dalam bahasa Indonesia dan Inggris.

FOTO : Foto harus mempunyai ketajaman yang baik, diberi judul dan keterangan seperti pada gambar.

DAFTAR PUSTAKA : Daftar pustaka yang dirujuk harus disusun menurut abjad nama pengarang dengan mencantumkan tahun penerbitan, seperti teladan berikut.

NOTES FOR AUTHORS

LANGUAGE : Manuscripts must be written in Indonesia with English summary or vice verse.

FORMAT : Manuscripts should be typed double spaced on one face of A4 white paper. A 3,5 cm magin should be left all sides.

TITLE : Title must not exceed two lines and should reflect the content of the manuscript. The author's name follows immediately under the title.

SUMMARY : Summary must not exceed 200 words, and should comprise informative essence of the entire content of the article.

KEYWORDS : Keywords should be written following a summary

TABLE : Title of tables and all necessary remarks must be written both in Indonesia and English, Tables should be numbered. The uses of comma (,) and point (.) in all figures in the table indicate a decimal fraction, and a thousand multiplication, respectively.

LINE DRAWING : Graphs and other line drawing illustrations must be drawn in high contrast black ink. Each drawing must be numbered, titled and supplied with necessary remarks in Indonesia and English.

PHOTOGRAPH : Photographs submitted should have high contrast, and must be supplied with necessary information as line drawing.

REFERENCE : Reference must be listed in alphabetical order of author's name with their year of publications as in the following example :

Allan, J.E. 1961. The determination of copper by atomic absorption spectrophotometry. Spectrochim. Acta, 17, 459 - 466.