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Original Article

TRANSESTERIFICATION OF LINOLEIC ACID IN GRAPE SEED [*VITIS VINIFERA L.*]OILAND ITS ANALYTICAL METHOD DEVELOPMENT USING GAS CHROMATOGRAPHY

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ABSTRACT

Objective: Linoleic acid is an essential fatty acid that humans need to ingest. It is the main compound of grape seed [*Vitis vinifera L.*] oil which is known as inhibitor of hyperproduction of melanin pigment. The objective of this research is to obtain volatile transesterified linoleic acid and to develop its analytical method.

Methods: Sample A was obtained by soxhletation with n-hexane at 60°C and sample B was commercial grape seed oil obtained mechanically by expeller pressure. Linoleic acid methyl ester was obtained by acid pretreatment with sulfuric acid-methanol and base transesterification with sodium hydroxide-methanol.

Results: The optimized condition was resulted at an isothermal column temperature at 190°C for 12 minute whereas the injector and detector were kept at each 250°C and 280°C.Linearity was confirmed over a calibration range with regression coefficients [r] of 0.999 and coefficient variance of linear regression [V_x0] of 1.670%. The limit of detection [LOD] and limit of quantitation [LOQ] of this method were determined from the coefficient of variation of a known concentration of reference standards resulting 38.480 ppm and 116.620 ppm, respectively. Precision test revealed a coefficent of variance of 1.910% for the first day, 0.850% for the second day, and 1.980% for the third day. Accuracy revealed a percentage of recovery in a range of 98.510-99.830 %.

Conclusion: Linoleic acid in grape seed [*Vitis vinifera L.*] oil was succesfully transesterified and its analytical method development using gas chromatography was validated. Concentration of linoleic acid in sample A and B were 60.233 and 70.393%, respectively.

Keywords*:* Grape seed oil, Linoleic acid, Transesterification, Gas Chromatography, Validation of Analytical method

INTRODUCTION

Oleic acid, linoleic acid and alpha linoleic acid are three main compounds of unsaturated fatty acids. Unsaturated fatty acids have many benefits for the body, one of which is for skin health. Several studies have shown that linoleic acid may act as a proinflammatory agent during the inflammatory phase of the wound healing processas well as overcome hyperpigmentation due to the effects of ultraviolet light-induced skin [1, 2, 3]. Grape seed oil is obtained from grape seed extract. It is one source that has abundant linoleic acid content [4,5]. According to the Codex [6], the levels of linoleic acid in grape seed oil can reach 58-78%. Stearic, oleic, and linoleic contained in grape seed oil differ only in the degree of unsaturation anda narrowboiling points difference. Therefore, it is too difficult to separate the mixture of fatty by only simple extraction [7]. The common chosen method of determination of linoleic acid is using gas chromatography. Prior to the analysis, linoleic acid is derivated to increase its volatility i.e ester compound by transesterification [8, 9, 10, 11]. This study aims to obtain volatile transesterified linoleic acid and to perform development of analytical methods for determining the levels of linoleic acid in grape seed oil samples using gas chromatography.

MATERIALS AND METHODS

Materials

Grape seed, grape seed oil commercial, standard linoleic acid 99% w / v were obtained from Sigma-Aldrich, 95% methanol, n-hexane, distilled water, concentrated sulfuric acid 98%, sodium hydroxide, and butyl hydroxy toluene [BHT].

Instrumentation

Hitachi D 2500 gas chromatograph with OV-1 column [25m × 0.53 mm] and a flame ionization detector [FID], rotavapor, and glasses apparatus.

Sample Preparation

Grape seed was determinated at the School of Life Sciences and Technology ITB. Grape seed is dried, pulverized, weighed and extracted using Soxhlet with solvent n-hexane. Hexane extract was evaporated with a rotary evaporator at 60°C until all the solvent evaporated then the oil was stored in closed containers and protected from light.

Characterization of Grape Seed Oil

Characterization of grape seed oil included the determination of specific gravity, free fatty acid, saponification number, iodine number, and index of refraction. Characterization were done using quality parameters of grape seed oil.

Preparation of Standard Solution Methyl Ester-Linoleic acid

A total of 100 mL standard solution of 99% linoleic acid was dissolved in 5 mL of n-hexane and added to 5 mL of 1% sulfuric acid-methanol, and 100 mg of BHT. This mixture was shaken and heated at 60 °C for 2 hours using a thermostat and appliance reflux. The n-hexane phase and methanol were then separated using liquid-liquid. N-hexane phase was taken and filtered using a 0.22 µm membrane paper.

Preparation of Methyl Esters Linoleic Acid Solution of Samples

A total of 100 mL of oil was dissolved in 5 ml of n-hexane and added to 5 mL of 1% sulfuric acid-methanol, and 100 mg of BHT. This mixture was shaken and heated at 60°C for 2 hours using a thermostat and appliance reflux. Then, the extraction was carried out as above.

Test Method Validation

The parameter of validation method consisted of linearity, precision, accuracy, and ruggedness[12,13,14].

Quantitation of linoleic acid in the sample

Levels of linoleic acid in samples were determined by injecting 2 mL of transesterified sample solution into the gas chromatograph with a system that has been optimized and validated.

RESULTS AND DISCUSSION

The results of determination confirmed that the grape seed species is *Vitis vinifera L.* Grape seed oil was further made by soxhletation with solvent n-hexane at 60°C until all of the content was extracted. The yield of oil was 12.307%. The amount is still fit into the range of grape seed oil content were that is stated the literature[11.6 to 19.6 %] [6,15].Based on the test results, the quality of the oil samples through soxhletation met the standard literature on the parameters except the level of free fatty acid [Table 1].

The involvement of heating process may cause oxidation which results in an increase of free fatty acids and decreasing numbers of iodine. The phenomena occcurs due to reduction of double bond [16].

The oil in the n-hexane phase was transesterified by refluxing for two hours at 60 °C using methanol with sodium hydroxide catalyst. was chosen as a solvent because it is a non-polar to dissolve the oil, linoleic acid and methyl ester form. Moreover, N-hexane has a low partition coefficient that tends to be partitioned. It is suitable as a carrier solvent in the analysis by gas chromatography. BHT was added as an antioxidant to reduce the occurrence of autooxidation of unsaturated fatty acids [17]. Optimization of gas chromatography system was done to obtain a reliable system that can be used for qualitative and quantitative analysis. The result can be seen in Table 2.

Table 1: Results of Grape Seed Oil's Characterization.

Parameter	Grape seed oil	
	Sample	Literature [6]
Density	0.925	0.920 – 0.926
% free fatty acid	1.4	< 0.5
Saponification number	190	188-194
Iodium number	133	128-150
Refractive index	1.473	1.467-1.477

Table 2: Results of Gas Chromatography System Optimization

Parameter	Result
Column	OV- 1 25 m x 0,53 mm
Mobile phase	Nitrogen
Detector	Flame Ionization Detector
Injector temperature	250°C
Detector temperature	280°C
Column temperature	190°C hold for 12 minutes
Hydrogen pressure	1.5 bar
Sensitivity of recorder	30
Noise	30
Atenuation	9
Chart speed	2.5 mm/minute

Conformance test is intended to ensure the effectiveness of the system prior to analysis. Repeatability test obtained coefficient of variance [CV] of AUC values of 1.517% and that of retention time of 0.435% [Table 3]. This value fulfilled the requirements of the system suitability test which require maximum value of 2% [12,13,14].

Table 3: Result of Suitability System Test

Parameter	TR [minutes]	AUC
	9.690	134793
	9.703	135468
	9.606	134609
	9.676	131647
	9.716	136248
	9.720	137799
Mean	9.685	135094
Standard deviation	0.042	2049.207
CV [%]	0.435	1.517

Selectivity requires a minimum value of 1, whereas in this study, it showed a value of 6.615. Value of the resolution requires in the system suitability test is a minimum of 1.5, while the value obtained from the experimental resolution was 9.5. Furthermore, the symmetry showed the ideal value of 1 meaning 100% symmetry [Fig.1]. Linearity parameters was obtained by testing six standard concentrations of linoleic acid methyl ester in a concentration range of 20 -120 % of the actual concentration of the sample. The concentration of each standard was plotted against the response to produce a calibration curve. Linearity was evaluated from the correlation coefficient [r] and the regression coefficient of variance [V_X0] [Table 4, Figure 2]. Regression equation [r value] was in a value of 0.999 and V_x0 of 1.67%. Theoretically, good linearity system has a minimum r value of 0.999 and a $V_x0$maximum value of 2%. The limit of detection [LOD] and limit of quantitation [LOQ] of this method were determined from the coefficient of variation of a known concentration of reference standards resulting 38.480 ppm and 116.620 ppm, respectively. Accuracy of the method was determined by standard addition method. A number of standards with concentration of 800, 1000, and 1200 ppm were added respectively to the sample and treated the same as sample preparation. This procedure was performed in three repetitions. Accuracy is expressed as the average percent recovery. Percent recovery of the three solutions were in a range of 98.510 to 99.830% [Table 5].

Precision testing included intra and inter day measurements. The inter day was performed for three days. Based on the results of the experiment, the recovery value increased from the first to the third day. This may occur as a result of solvent n-hexane may volatile and obtain a concentrated solution. Precision values obtained still met the requirements of the CV [Tables 6]. Determination of linoleic acid levels in the sample A [grape seed oil] and B [commercial grape seed oil with 75% on the label] were done by three times of measurements. Levels of linoleic acid in the sample are presented in the table-7.

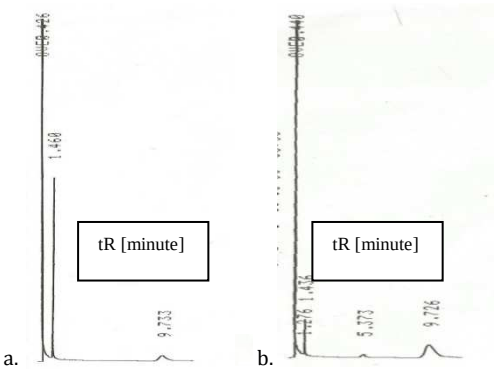

Fig. 1: Chromatogram of [a] standar methyl ester linoleic acid, [b] grape seed sampel

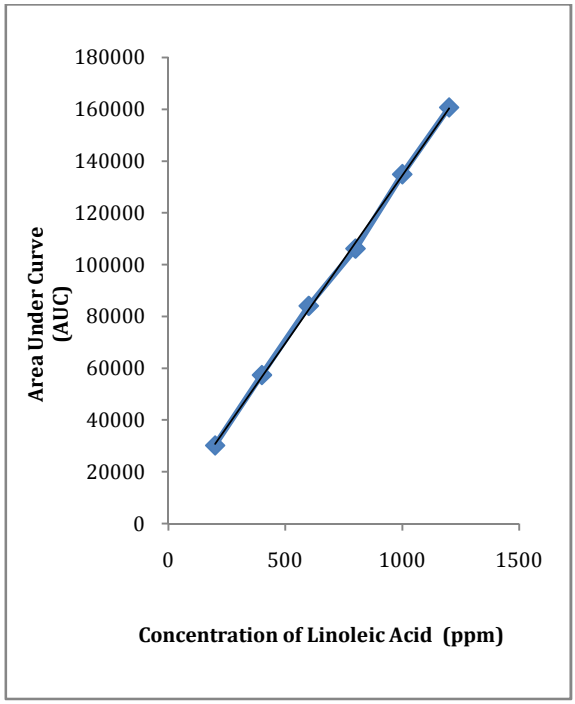

Fig. 2: Calibration Curve of Linoleic Acid Standard

Table 4: Linearity Test of Linoleic Acid Standard

Concentration of Linoleic acid [ppm]	tR [minute]	AUC
200	9.690	27196
	9.700	29933
	9.716	33755
400	9.703	55589
	9.686	56328
	9.726	60405
600	9.726	79071
	9.723	84506
	9.690	88801
800	9.700	104126
	9.720	105488
	9.676	109109
1000	9.606	134609
	9.690	134793
	9.703	135468
1200	9.686	157696
	9.733	158347
	9.666	166098
$S_{y/x}$		1507.680
V_x0[%]		1.670

Table 5: Accuracy Test of Linoleic Acid using Standard Addition Method

Theoritical concentration [ppm]	Measured concentration [ppm]	Recovery [%]	Mean [%]
800	762.580	95.320	99.830
	804.660	100.580	
	828.680	103.580	
1000	984.530	98.450	99.360
	991.750	99.170	
	1004.550	100.450	
1200	1175.210	97.930	98.510
	1184.500	98.710	
	1186.770	98.900	

Table 6: Precision test of linoleic acid for first, second and third day

Theoritical concentration [ppm]	Day-1	Day-2	Day-3
1000	97.940	107.510	111.000
	98.440	107.600	114.910
	98.450	107.890	116.930
	99.170	109.220	117.100
	100.450	109.330	116.370
	103.030	109.460	115.810
Mean	99.580	108.500	115.350
Standard deviation	1.899	0.926	2.278
CV [%]	1.910	0.850	1.980

Table 7: Levels of linoleic acid in the sample

Sample	Mean of AUC	Percentage [%]
A	115169.670	60.233
B	133394.000	70.393

Based on the test results [Table 7], the levels of linoleic acid in grape seed oil is still in the range of CODEX requirements, i.e. in a range of 58-78 %. The results of sample B in comparison to the label obtained a value of 93.858 %. Grape seed oil that was obtained by pressing [Sample B] showed higher levels of linoleic acid than those obtained with soxhletation oil [Sample A]. Effect of heating in soxhletation can cause oxidation reaction in linoleic acid. The more double bonds in the fatty acids, the rate of reaction with O_2 also higher. Linoleic acid has two double bonds with a fairly high rate of oxidation of 7.3 × 104 $M^{-1}s^{-1}$[18].

CONCLUSION

The method of gas chromatography with FID detector; OV-1 capillary column 25 mx 0.53 mm; using isothermal column temperature at 190°C for 12 min; injector temperature 250 °C; detector temperature 280°C can be used for identification and quantification of linoleic acid. Linearity was confirmed over a calibration range with regression coefficients [r] of 0.999 and coefficient variance of linear regression [V_x0] of 1.670%. The limit of detection [LOD] and limit of quantitation [LOQ] of this method were determined from the coefficient of variation of a known concentration of reference standards resulting 38.480 ppm and 116.620 ppm, respectively. Precision test revealed a coefficent of variance of 1.910% for the first day, 0.850% for the second day, and 1.980% for the third day. Accuracy revealed a percentage of recovery in a range of 98.510-99.830 %. Concentration of linoleic acid in sample A and B were 60.233% and 70.393%, respectively.

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