Extraction and Purification of Conjugated Linoleic Acid (CLA) Using Lactobacillus paracasei and Safflower Oil

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Abstract

The present study aims to use Lactobacillus paracasei and test its ability to produce conjugated linoleic acid (CLA) using safflower oil. Safflower oil, known by the scientific name Carthamus tinctorius was obtained by cold-pressing safflower seeds, and the linoleic acid content was measured using a GC, which was 17.59%. MRS-Tween-80 fermentation medium was prepared, and safflower oil was added as a source of free linoleic acid (LA). The fermentation medium was inoculated with Lactobacillus paracasei and incubated under optimal conditions (safflower oil concentration 50% g/mlμ, 37°C, pH 24, 6 h incubation time). Linoleic acid was then extracted chemically by adding solvents. The purity of the extracted conjugated linoleic acid was measured using HPLC. This technique was used to detect the extracted conjugated linoleic acid, which showed a retention time of 20.71, which was close to the retention time of the standard linoleic acid of 20.29. FTIR showed that the extracted conjugated acid was similar to the standard conjugated linoleic acid, with slight variations due to the use of either impure acid or different concentrations, which led to the peaks shifting slightly from their natural positions– .

Introduction

Conjugated linoleic acid (CLA) is a fatty acid that has received increasing attention in recent decades due to its numerous health benefits. It acts as an anti-cancer agent, protects the immune system, reduces body fat, and reduces the risk of heart disease and atherosclerosis. [7].Conjugated linoleic acid (CLA) is an essential polyunsaturated fatty acid composed of a group of C18:2 octadecenoic acid isomers, most of which contain double bonds. This acid belongs to the omega-6 group. [19.[

Safflower oil is considered a vegetable oil rich in linoleic acid, reaching approximately 70-75% when analyzed by GC. Therefore, safflower oil is considered an ideal vegetable oil for the production of CLA using Lactobacillus Paracasei bacteria. [15.[

This study aims to add safflower oil to the MRS-Tween80 fermentation medium as a source of pure linoleic acid (LA). Lactobacillus Paracasei bacteria were used to produce conjugated linoleic acid (CLA), followed by extraction of the conjugated acid using organic solvents. The conversion efficiency of the conjugated linoleic acid was then measured and its purity was tested using HPLC and FTIR techniques.

Materials and Methods

2-1Isolation Activation:

Lactobacillus paracasei was obtained from Al-Amin Laboratories. A series of 110-910 decimal dilutions of sterile peptone water were performed. Lactobacillus paracasei was then

ISSN 2072-3857

added, and 1 ml of the culture was cultured on MRS agar in three replicates for activation. The plates were incubated in aerobic and anaerobic conditions using Anaerobic-Jar at 37°C for 48 hours. The process was repeated several times to obtain an activated isolate and identify it using the Vitek2 device [5.[

:2-2Oil Preparation and Pressing

:2-2-1Safflower Oil by Cold Pressing

Dried safflower seeds were brought from local markets in Najaf Governorate and cleaned of impurities. The seeds were then pressed by mechanical cold pressing according to the described method[4] The quality and quantity of the oil's active compounds were determined using a GC device.

:2-2-2-Determination of Active Compounds Using GC in Oils

The active compounds present in safflower oil were detected by analysis using a gas chromatograph connected to a mass spectrometer equipped with an electronic calculator. The mass of the compounds was measured in a laboratory affiliated with the Ministry of Science and Technology. 200 microliters of oil were taken, then 4 ml of potassium hydroxide (KOH) was added. The mixture was placed in a water bath at 50°C in a shaking incubator for 30 minutes and allowed to cool to room temperature. 1 ml of distilled water was then added and placed on a glass slide for one minute. 1 ml of hexane was added to the mixture. The mixture was centrifuged for ten minutes at 10,000 rpm. The supernatant (upper layer) was withdrawn, the sample filtered, and injected into a GC device to display the active compounds on the calculator screen [6]. The active compounds were estimated according to the following equation: C sample = (A sample)/(A standardized) x Cst

:2-3Investigation of conjugated linoleic acid (CLA(

:2-3 -1-Preparation of the fermentation medium for the bacteria and their ability to produce conjugated linoleic acid

The bacteria were activated on MRS broth medium. The bacteria were transferred Lactobacillus paracasei was added to the fermentation medium prepared in paragraph (1-2) and 10 ml of MRS agar, 100 microliters of Tween-80, 100 microliters of oil, and 500 microliters of bacteria were added to it. The plates were incubated at 37°C in a shaking incubator at 120 rpm for 72 hours under anaerobic conditions. After that, the production of conjugated linoleic acid was measured using a UV spectrophotometer at a wavelength of 233 nm [17[

:2-3-2 .Preparation: Standard curve for conjugated linoleic acid (CLA(

The standard curve was prepared according to the method used by [21], with modifications. A series of concentrations of standard conjugated linoleic acid were prepared, ranging from (5, 2.5, 1.25, 0.625, 0.312, 0.156) g/mlμ dissolved in hexane. The absorbance was measured at a wavelength of 233 nm, according to the table below

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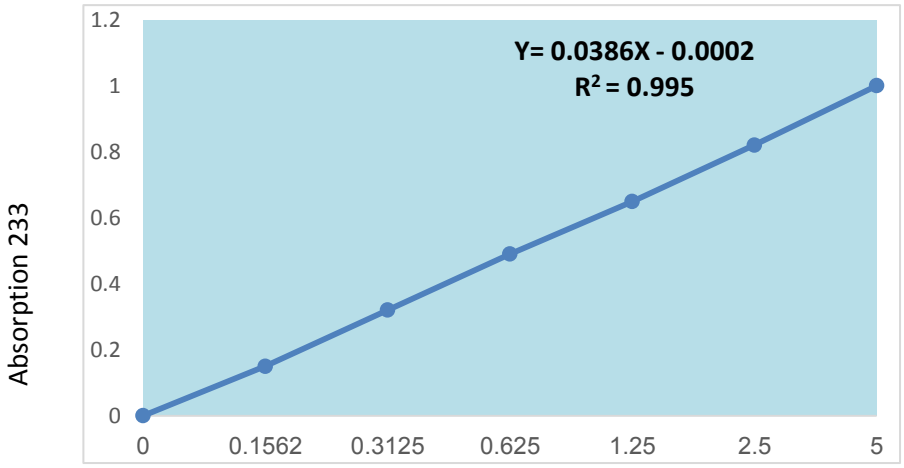

CLA concentration µg/ml

Figure (2-1) Absorption measurement of CLA using UV

Table (2-1) Preparation of standard curve dilutions for the determination of conjugated linoleic acid

concentration µg/ml	Absorbance 233nm
5	1.136
2.5	0.675
1.25	0.328
0.625	0.141
0.3125	0.072
0.1562	0.007

The percentage of conversion of linoleic acid to conjugated linoleic acid was calculated according to the method [18.[

%CLA acid percentage=(Amount of conjugated linoleic acid obtained from the standard curve)/(Amount of added linoleic acid (ml or g) remaining standard)×100

:2-4Conjugated Linoleic Acid (CLA) Extraction and Purification

After determining the optimal conditions for CLA production (pH, temperature, oil concentration, incubation time), conjugated linoleic acid (CLA) was extracted according to the method of Christie (2003) with modification.

:2-4-1Lipid Separation

Lips were separated in the fermentation medium containing L. paracasei bacteria using a centrifuge at 10,000 rpm at 4°C for 10 minutes. The precipitate containing the bacterial cells was then separated from the filtrate containing the lipids. Ethanol was added to the supernatant at a 1:1 ratio and

ISSN 2072-3857

mixed using an orbital shaker at room temperature for 30 minutes to extract the lipid containing linoleic acid from the ethanol layer. A separating funnel was used to separate the alcoholic layers containing the dissolved lipids. The process was repeated several times to ensure maximum fat extraction. A rotary evaporator was used to concentrate the solvent (ethanol 98%) to obtain a lipid extract at a low temperature (30-40°C). The samples were placed in a lipid separating funnel. The process was repeated several times to extract the maximum amount of lipid. The sample was then concentrated in a rotary evaporator to obtain a pure lipid extract for purification.

:2-4-2Purification of conjugated linoleic acid

Sodium hydroxide (NaOH) was added to the lipid extract at a rate of 10% to perform the alkali cracking (saponification) process. The mixture was heated in a water bath at 60-70°C for 60 minutes. (This reaction will convert the triglycerides to the sodium salts of the fatty acids, soap, and glycerol.) Concentrated hydrochloric acid (HCL) was added until the pH of the medium reached 2 to extract the free fatty acids. Then, Hexane was added at a ratio of 1:1 to the fatty layer to extract conjugated linoleic acid. The mixture was shaken well and the organic layer was separated using a separating funnel. Then, a rotary evaporator was used to obtain the free fatty acids [3.[

:2-5Conjugated Linoleic Acid Identification:

2-5-1Conjugated Linoleic Acid Identification Using FT-IR

This test was conducted according to the method of [16]. 185 mg of anhydrous potassium bromide (KBr) was weighed, and 50 microliters of the sample (purified and standardized conjugated linoleic acid, separately) were added to the anhydrous potassium bromide. The mixture was thoroughly mixed until it formed a solid. The mixture was then finely ground to form a powder. The final amount of powder (mixture) was then placed on a double-sided iron disc, and the disc was pressed and compressed. The sample was then placed in the device to read the active aggregates at a wavelength of 233 nm for each sample separately. The readings for each sample were then recorded.

:2-5-2Using HPLC (High-throughput liquid chromatography) technology

200microliters of conjugated linoleic acid were injected using Column 18, with the water as the mobile phase and phosphoric acid at a concentration of 0.1%, and silica as the stationary phase. The flow rate was 0.8 ml/min, at 25 °C, and the wavelength was 210 nm. The samples were then filled into 20-microliter HPLC tubes and placed in the device for diagnostic purposes after establishing all optimal diagnostic conditions [11]. The active compounds were estimated using the following equation:

) =A sample)/(A standared) xCst sample C

:3Results and Discussion

:1-3Estimation of active ingredients in safflower oil using GC

The results (Figure 3-1) indicate that natural cold-pressed safflower oil contains ten fatty acid compounds, which were estimated using gas chromatography coupled with a mass spectrometer. The chromatographic analysis results revealed the presence of linoleic acid at 17.5%, followed by lactic, palmitic, and butyric acids at 13.4%, 5.04%, and 3.55%,

ISSN 2072-3857

respectively. In addition, the results showed that safflower oil contained stearic acid and acetic acid at 1.1% and 1.5%, respectively, as well as linolenic acid, propionic acid, oleic acid, and formic acid at 3.17%, 3.06%, 2.52%, and 2.41%, respectively. Other fatty acids also appeared in very small proportions. Salma Wahba Al-Rahman (2021) explained that safflower oil contains approximately 6-8% palmitic acid, 2-3% fatty acids, 16-20% oleic acid, and 71-75% linoleic acid. Linoleic acid ranks first among vegetable oils, and safflower oil is often blended with other vegetable oils because of its high linoleic acid content, improving their nutritional properties. In addition to the high percentage of linoleic acid, the oil contains three types of tocopherols in varying quantities (β-tocopherol, α-tocopherol, and γ-tocopherol), ranging in quantity from 46.05 to 70.93, 0.85 to 2.16, and 0.45 gμ/gm100, respectively. These are in addition to the presence of carotenoids and phenocetrols [13]. The amount of tocopherol in the oil is one of the most important characteristics determining its quality, depending on the type of oil extraction [20.[

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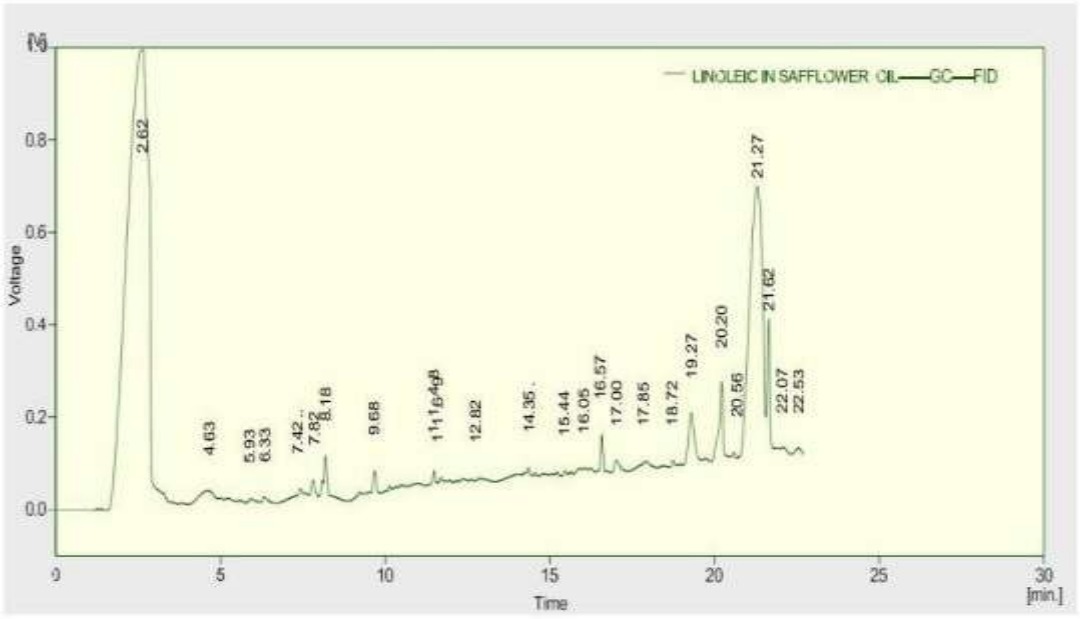


Result Table (Uncal – LINOLEIC IN SAFFLOWER OIL --- GC)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W.05 [min]	Compound Name
1	2.620	41470.515	944.438	0.0	65.0	0.76	
2	4.627	165.030	21.708	0.0	1.5	0.44	
3	5.927	18.921	4.124	0.0	0.3	0.09	
4	6.327	26.953	10.833	0.0	0.7	0.14	
5	7.417	51.630	14.794	0.0	1.0	0.11	
6	7.817	67.246	32.947	0.0	2.3	0.11	
7	8.183	244.250	67.542	0.0	4.7	0.08	PALMITIC
8	9.680	73.007	47.050	0.0	3.2	0.10	PROPIONIC
9	11.480	41.981	28.799	0.0	2.0	0.06	
10	11.687	36.497	10.843	0.0	0.7	0.05	
11	12.823	47.526	8.616	0.0	0.6	0.07	ACETIC
12	14.347	99.064	16.941	0.0	1.2	0.07	BUTYRIC
13	15.443	44.358	9.483	0.0	0.7	0.08	STEARIC
14	16.047	85.528	3.769	0.0	0.3	0.09	OLEIC
15	16.567	139.023	79.078	0.0	5.4	0.07	
16	17.000	63.044	4.786	0.0	0.3	0.06	FORMIC
17	17.853	46.099	6.438	0.0	0.4	0.15	
18	18.723	60.533	13.162	0.0	0.9	0.08	
19	19.273	259.391	16.889	0.0	1.2	0.07	LACTEIC
20	20.197	263.604	39.147	0.0	2.7	0.04	LINOLEIC
21	20.563	52.219	11.745	0.0	0.8	0.08	
22	21.273	461.839	12.403	0.0	0.9	0.06	
23	21.623	246.035	33.249	0.0	2.3	0.03	LINOLENIC
24	22.073	14.713	1.354	0.0	0.1	0.06	
25	22.527	15.635	11.792	0.0	0.8	0.16	
	Total						

Figure (3-1) shows the active compounds of safflower oil using GC.

Table (3-1) shows the active compounds of safflower oil using GC.

No.	Name of Compound	Molecular Weight	Percentage%	Molecular Formula
1	Linoleic	263.604	17.59	C18H32O2
2	Linolenic	246.035	3.17	C18H30O2
3	Lacteic	259.391	13.4	C3H6O3
4	Formic	63.044	2.41	CH2O2
5	Oleic	85.528	2.56	C18H34O2
6	Stearic	44.358	1.17	C18H36O2
7	Butyric	99.064	3.55	C4H8O2.
8	Acetic	47.526	1.57	C2H4O2
9	Propionic	73.007	3.06	C3H6O2
10	Palmitic	244.250	5.04	C16H32O2

:2-3Testing the ability of L. paracasei to produce conjugated linoleic acid (CLA(

After activating L. paracasei on MRS-Tween80 medium, its ability to produce conjugated linoleic acid (CLA) was tested. The addition of safflower oil to the fermentation medium with L. paracasei yielded the highest amount of CLA, 296.6 g/mlµ. Although many bacterial isolates have the ability to convert linoleic acid to CLA, the type of substrate on which the bacteria work varies greatly in their CLA production. Linoleic acid toxicity may be due to the presence of double bonds, which alter the acid's molecular shape. The combination of unsaturated fatty acids can disrupt the lipid bilayer structure, and the diffusion of fatty acids across the membrane can impede osmosis or separate transmembrane pathways. Therefore, the concentration of the substrate can affect the levels of conjugated linoleic acid production. Additionally, the ability of bacterial isolates to produce the acid varies depending on the type of oil added to the fermentation medium[12.[

A study conducted by [10] showed that using F0221 L. acidophilus in CLA production yielded the highest concentrations of conjugated linoleic acid in MRS medium supplemented with 0.5 mg/ml of linoleic acid, while adding a concentration of 2.0 mg/ml of linoleic acid was more effective in enhancing the production of conjugated linoleic acid inoculated with L. acidophilus.

[1]also showed that CCRC14079 L. acidophilus produced relatively large amounts of conjugated linoleic acid after adding 1.0 µg/ml of linoleic acid.Lactic acid bacteria also have the ability to convert fatty linoleic acid to conjugated linoleic acid by secreting enzymes that assist in the conversion. Therefore, the type of bacteria has a significant impact on the production of conjugated linoleic acid (CLA). [2.[

:3-3Detection of functional groups in conjugated linoleic acid (CLA(

:3-1FT-IR examination

After extracting conjugated linoleic acid (CLA), the results of the identification of conjugated linoleic acid extracted from the MRS-Tween80 fermentation medium, on which L. paracasei bacteria were grown, and

the standard linoleic acid (SLA) was shown in Figure (3-2). To detect the functional groups in the chemical structure of linoleic acid, FT-IR spectroscopy was used and then compared with the standard groups. The peak wavelength of 3010 cm-1 indicates the presence of C-H double bonds, indicating unsaturation in the fatty acids, while the wavelengths of 2926 cm-1 and 2854 cm-1 indicate unsaturation. cm-1 reflects the stretching of aliphatic bonds and indicates the presence of hydrocarbon structures. The peak wavelength of 1740 cm-1 indicates the presence of the carbonyl group (C=O), while wavelengths of 1458 cm-1 and 1377 cm-1 represent the reflection of the bending vibration of the hydrocarbon bonds. Wavelengths of 1000 cm-1 and 1160 cm-1 represent the C-O stretching of the carboxyl group. Interpreting the above wavelengths reveals a similarity between the standard acid and the purified acid, with slight variations. This variation is either due to the impurity of the purified sample or due to different concentrations that cause the peaks to shift [14.[

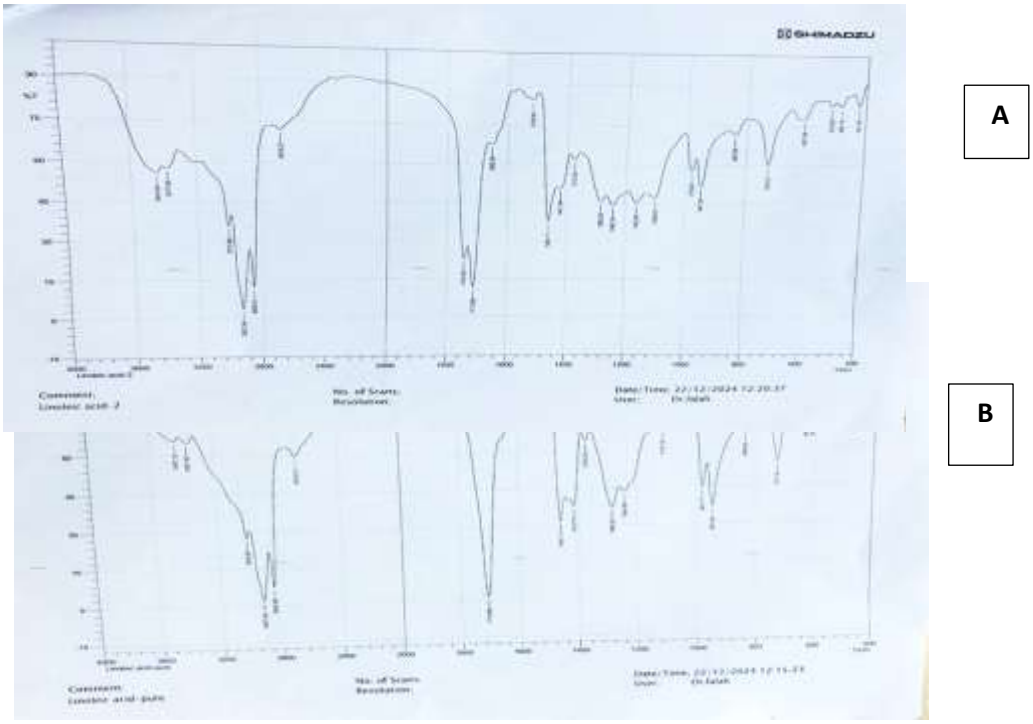


Figure (3-2) FT-IR examination of standard conjugated linoleic acid (A) and purified conjugated linoleic acid (CLA) from safflower oil.

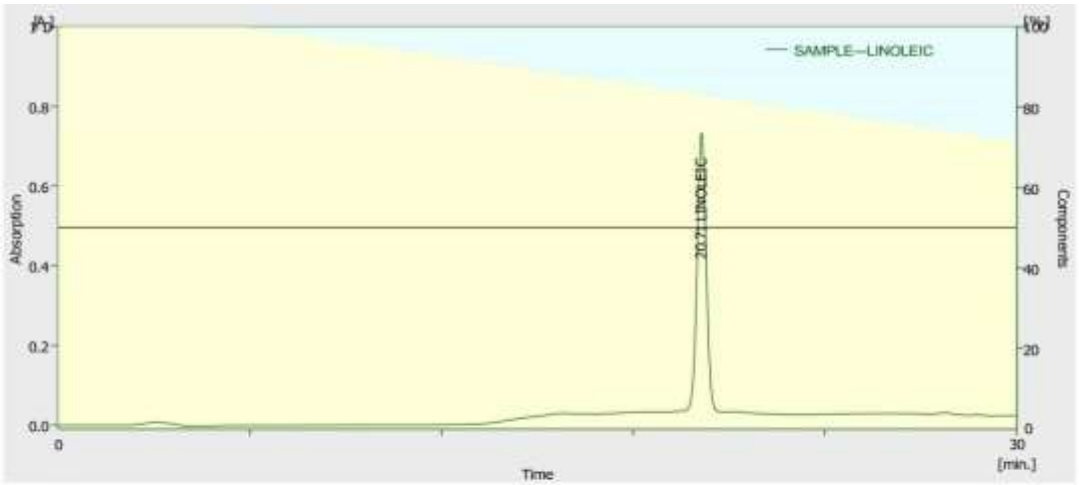
3-3 -2Using HPLC

The results shown in Figure 3-3 indicate that HPLC was used to identify conjugated linoleic acid extracted from a fermentation medium containing safflower oil and inoculated with L. paracasei bacteria. Based on the standard curve for CLA, the retention time of the extracted CLA was 20.71 minutes, which coincided with the appearance of another peak

ISSN 2072-3857

for the standard CLA with a retention time of 20.29 minutes in the pattern. This may be due to the fact that, after adding the bacteria to the extraction medium, the bacteria may have altered the chemical composition of the medium. In addition, studies have shown that lactic acid bacteria have the ability to produce the enzymes esterase and lipase, which modify fatty acids in triglycerides[8.[

On the other hand, adding lactic acid may alter the effectiveness of the medium as a result of biological and microbial interactions, which increases CLA in the medium. In addition, the substrate plays an important role, especially if it is LA, as the fatty acid is converted to 10-hydroxy-12-trans-Octadecadienoic acid (10-hydroxy-12-trans-Octadecadienoic acid (12-cis-Otadecadienoic acid (10-hydroxy-12-trans-Octadecadienoic acid (12-hydroxy-10-Octadecadienoic acid (12-cis-Otadecadieno ...8:2 cis 9,trans11(RA)]). It is generally agreed that lactic acid bacteria (LAB) convert polyunsaturated fatty acids into hydroxylated acids [9.[

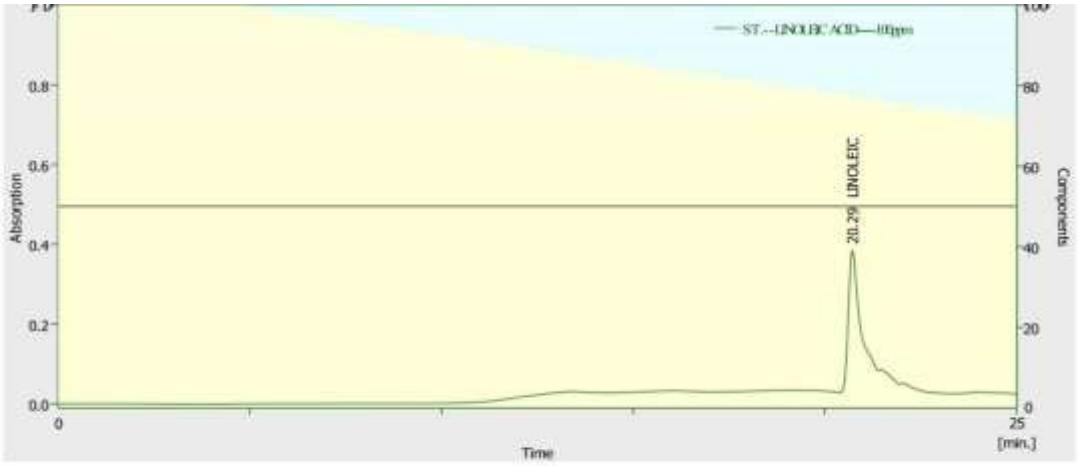


A

Result Table (SAMPLE--LINOLEIC)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	20.71	1095.251	213.460	100.0	100.0		LINOLEIC
	Total	1095.251	213.460	100.0	100.0		

ISSN 2072-3857



Result Table (Uncal - ST.-LINOLEIC ACID −100 ppm)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W.05 [min]	Compound Name
1	20.29	1502.905	187.205	100.0	100.0		LINOLEIC ACID
	Total	1502.905	187.205	100.0	100.0		

Figure (3-3) HPLC technique. (A) Extracted conjugated linoleic acid (CLA). (B) Standard linoleic acid

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.1
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ISSN 2072-3857