

Nuclear Magnetic Resonance Properties of Conjugated Linoleic Acid (CLA) Synthesized from *Clarias macrocephalus* Oil

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ABSTRACT

*This research characterizes fish oil extracted from catfish (*Clarias macrocephalus*) to verify the production of conjugated linoleic acid (CLA). Conjugated linoleic acid is a positional and geometric isomer for linoleic acid (LA) distinguished by its conjugated double bond system. The study utilized Nuclear Magnetic Resonance Spectroscopy (NMR) in both 1D and 2D dimensions to identify the formation of conjugated olefin proton and carbon in C18:2 (9Z,11E). The findings shed light on the use of spectroscopy in understanding the structural changes accruing during the alkali isomerization process. This method offers a non-destructive and simple procedure that does not require complex sample preparation techniques. Additionally, it provides valuable insights into the transformation of fatty acids from local fish oil and their potential application in food industry.*

INTRODUCTION

As consumers become more conscious of the connection between diet and health, nutritional quality becomes a more significant factor in food selection. Conjugated linoleic acid (CLA), often referred to as "functional food" components, is one such ingredient found in foods made from ruminants. CLA has attracted considerable attention as a biologically beneficial essential fatty acid. In vivo and in vitro studies have revealed various biological activities of CLA, including increase the level of antioxidant Glutathione peroxidase (GPx) in atherosclerosis patients, controlling inflammation and immune system, antidiabetic, and antiobesity properties, [1]

In chemistry, the term "CLA" describes a blend of linoleic acid's positional and geometric isomers with a conjugated double-bond system (*cis*-9, *trans*-11 octadecadienoic acid). Figure 1 shows the structure of two CLA isomers in comparison to vaccenic and linoleic acids. The rumen's biohydrogenation of polyunsaturated fatty acids (PUFA) is linked to the existence of CLA isomers in bovine fat. The biohydrogenation of dietary polyunsaturated fats (PUFA) by rumen bacteria is one of the reason ruminant lipids contained *trans*-fat content which commonly viewed as harmful. However, in 2008, the FDA designated CLA as Generally Regarded as Safe (GRAS), removing the need for it to be listed on food labels as a *trans*-fat. CLA has been demonstrated to have several positive health effects [2]

In this study, oil from *Clarias macrocephalus*, a species of catfish, was utilized as the starting material for the synthesis of CLA. Despite being a freshwater fish with high demand in the Southeast Asia market, catfish has received minimal attention from researchers regarding its nutritional value. For instance, Thailand's annual farmed catfish yield is projected to reach 110,000 tons in 2020, with a market value surpassing 160 million US dollars yet the majority of it is allocated for surimi production. Only recently have freshwater fish become increasingly recognized as part of a nutritionally sound food system. Therefore, the enrichment of feed for freshwater fish has been reported by several researchers, including the addition of linolenic acid, fish oil, and soybean oil to Nile Tilapia fish feed [3-4].

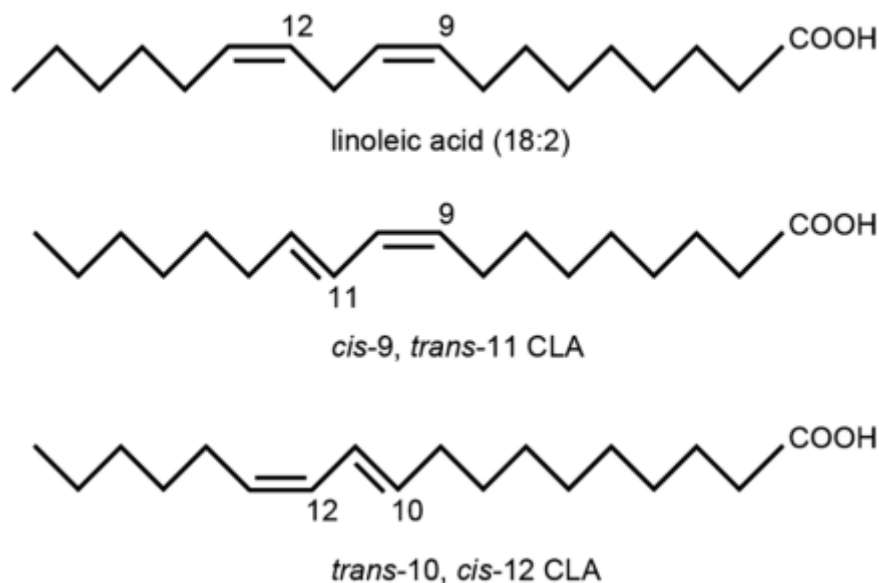


Figure 1. Chemical Structures of; (A) Linoleic Acid (*Cis*-9, *Cis*-12 18:2); (B) *Cis*-9, *Trans*-11 Conjugated Linoleic Acid; and (C) *Trans*-10, *Cis*-12 Conjugated Linoleic Acid [1].

Nuclear magnetic resonance (NMR) spectroscopy is a widely used technique for elucidating the structure and conducting qualitative and quantitative analysis of lipid molecules. It is a powerful analytical technique available to the organic chemist who allowed kinetic, structural, and equilibrium studies of individual compounds, both mixture or pure in comparison to standard chromatographic analysis techniques. In one study conducted by Arain et al. 2023 [5], the identification of CLA isomer was conducted by using gas chromatography mass spectrometry. However the method is destructive and requires the used of standard. In contrast, NMR spectroscopy offers several advantages including a non-destructive method, requires no standards for quantification, and relies on the nucleus and its surroundings to determine chemical shifts and coupling constants. Due to significant advancements in high field instrumentation, the use of extremely sensitive cryogenic probes, sophisticated data processing, enhanced automated methods for assignment, and the wide range of 1D and 2D NMR techniques, high-resolution NMR spectroscopy has grown in popularity in the study of lipids [6]. NMR was claimed to be an applicable instrument in determination of CLA isomer as well as quantification analysis.

EXPERIMENTAL DESIGN

Alkali Isomerization of Linoleic Acid

Linoleic acid was extracted and isolated from *Clarias macrocephalus* oil by referring to method describe by [7]. The alkali isomerization was conducted following the methodology outlined in the study by Lin Yang *et al.* [8]. Linoleic acid (1 g) was mixed with 2.6 g KOH in 10 ml of ethylene glycol in a trineck round bottom flask and refluxed at 160 °C under nitrogen for 4 h. 20 ml of methanol was added to the reaction mixture and followed by acidification with 40 ml of 3 N HCl. The reaction mixture was then extracted three times with 20 ml hexane. The hexane extract was washed thrice with 15 ml of 30% methanol in water, three times with 15 ml of distilled water, and then dried over anhydrous sodium sulfate. Hexane was removed in a rotary evaporator under vacuum at 60 °C.

Nuclear Magnetic Resonance (NMR) Analysis

LA and CLA was measured by Bruker 600MHz NMR spectrometer. Spectra were acquired in CDCl₃ solvent and were referenced against internal TMS.

RESULTS AND DISCUSSION

NMR One-Dimensional (1D)

The ¹H NMR spectrum of CLA showed four distinct signals for the four olefinic protons which shows an early indication to the present of conjugated double bond. This contrasts with linoleic acid with only one signal that represents the double bond (Figure 2.) The signals for CLA protons at the olefin position appeared to have two multiplets (5.30 ppm and 5.65 ppm) and two triplets (5.94 ppm and 6.30 ppm). Respectively, this signal was expected to correspond to the shifts of the “outer” (9-H, 12-H) and “inner” (10-H, 11-H) olefinic protons of the diene system. The large J constant of the triplet at 6.30 ppm was an indication that this signal arose most probably from one of the protons of the *trans*-double bond. The proton shifts of the olefinic protons are presented in Tab. 1.

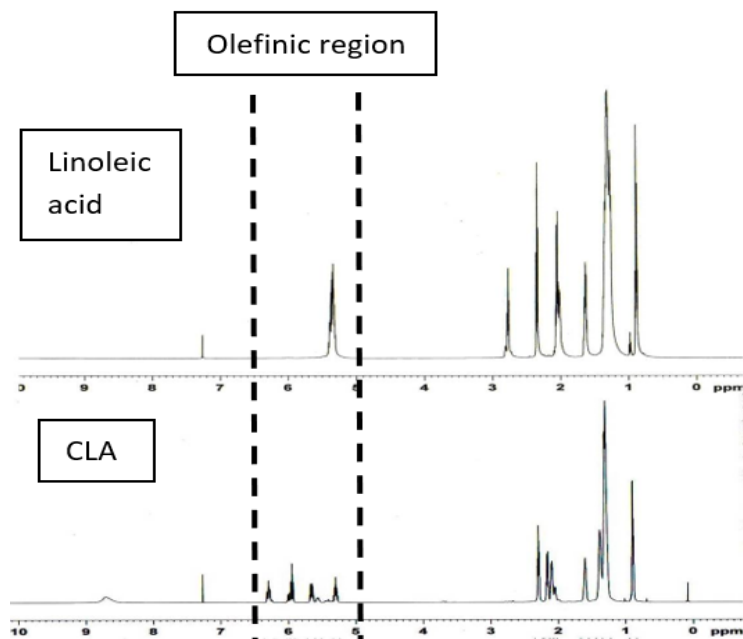


Figure 2. ¹H NMR spectrum of linoleic acid and CLA.

Table 1 ¹H NMR shift of the CLA olefinic protons

Signal (ppm)	Multiplicity	J Coupling constant (Hz)
5.30	multiplet	5.04
5.65	multiplet	14.88
5.94	triplet	10.98
6.30	triplet	14.85

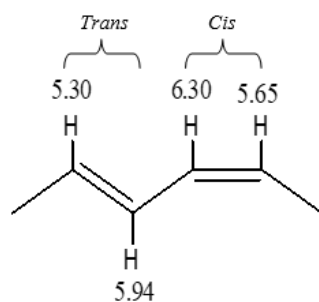


Figure 3. The arrangement of protons at the olefinic position with its geometric isomer.

According to ^{13}C NMR spectrum, four signals in the olefinic region can be clearly observed at 125.78, 128.91, 130.06, and 134.95 ppm (Figure 4). The reading was compared to study from previous researcher [9], [10]. Comparison shows that ^{13}C NMR reading in this study was situated at carbon 9 and 11 with 9Z11E isomer. Table 2 shows the ^{13}C NMR shift of the synthesized CLA and references correlated to CLA structure in Figure 5.

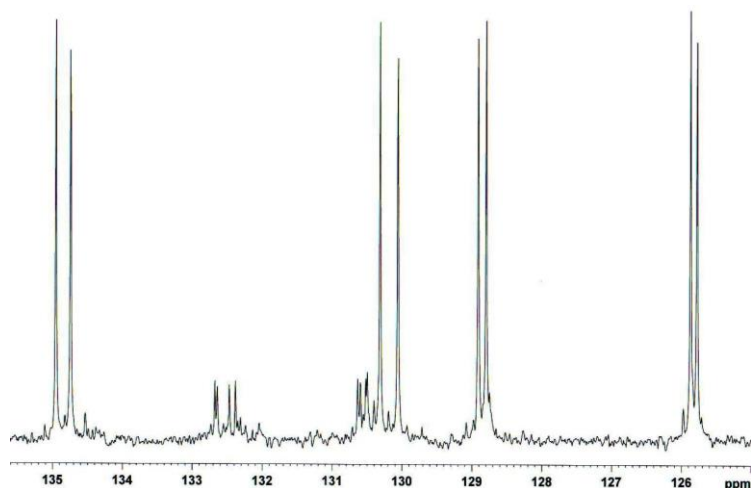


Figure 4. ^{13}C NMR spectrum of CLA with four distinct signals in the olefinic region.

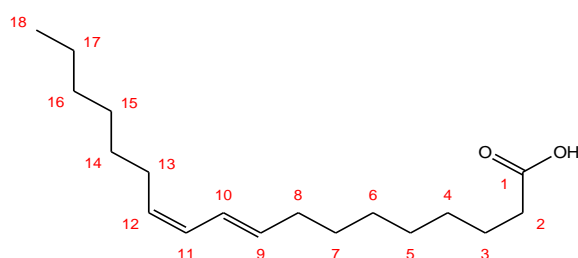


Figure 5. The arrangement of 18 carbon in molecular structure of CLA with 18 carbon isomer 9Z11E.

Table 2 ^{13}C NMR shift of the synthesized CLA and references

Atom	9Z11E (this study)	9Z11E ^a	9Z11E ^b	9E11Z ^a
1	180.49	174.32	180.09	174.34
2	34.95	34.1	34.05	34.1
3	25.17	24.95	24.67	24.95
4	29.33	29.06	29.03	28.97
5	29.36-29.92	29.12-29.67	29.12	29.13-29.45
6	29.36-29.92	29.12-29.67	29.03	29.13-29.45
7	29.36-29.92	29.12-29.67	29.66	29.13-29.45
8	27.87	27.66	27.65	32.86
9	130.06	129.89	129.89	134.51
10	128.91	128.71	128.73	125.72
11	125.78	125.58	125.58	128.57
12	134.95	134.76	134.8	130.17
13	33.1	32.92	32.9	27.72
14	29.41	29.41	29.4	29.73
15	29.13	28.95	28.93	28.97
16	31.69	31.77	31.76	31.77
17	22.77	22.65	22.63	22.65
18	14.26	14.12	14.1	14.12

Sources : a-[10] b- [9]

NMR Two-Dimensional (2D)

To assign the correlation of proton and carbon atoms of the conjugated diene system, it was necessary to conduct 2D NMR experiment. The experiments included ^1H - ^1H correlation spectroscopy (COSY), Heteronuclear Multiple-Quantum Correlation spectroscopy (HMQC), and Heteronuclear Multiple Bond correlation (HMBC). From the ^1H - ^1H NMR COSY correlation spectrum (Fig. 6), it is possible to assign the order of four olefinic proton by interconnection of one proton to its neighbors. According to the spectrum, all four protons can be arranged in position of proton multiplet at 5.65 ppm.

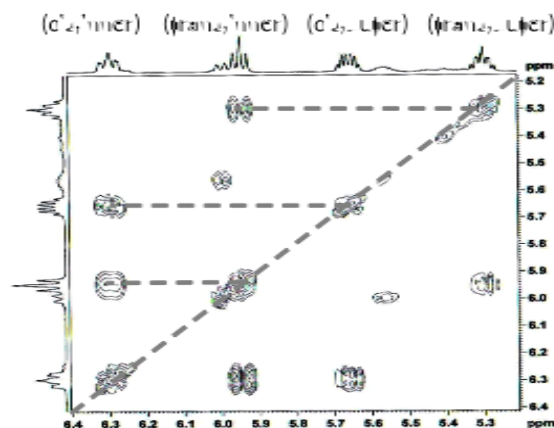


Figure 6. ^1H - ^1H NMR COSY at the olefinic position.

The HMQC correlation spectrum (Fig. 7), allows carbon to be correlated with its directly attached proton. The signal at 134.95 ppm (C-9) was correlated to proton signal at 5.65 ppm, signal at 125.78 ppm (C-10) was correlated to the proton signal at 6.30 ppm, signal at 128.91 ppm (C-11) was correlated to the proton signal at 5.94 ppm, and the signal at 130.06 ppm (C-12) was correlated to the proton at 5.30 ppm. These correlations thus determined the shifts of the “inner” carbon atoms (C-10, C-11) at 125.78 ppm and 128.91 ppm, and the shifts of the “outer” positioned carbon atoms (C-9, C-12) was at 134.95 ppm and 130.06 ppm.

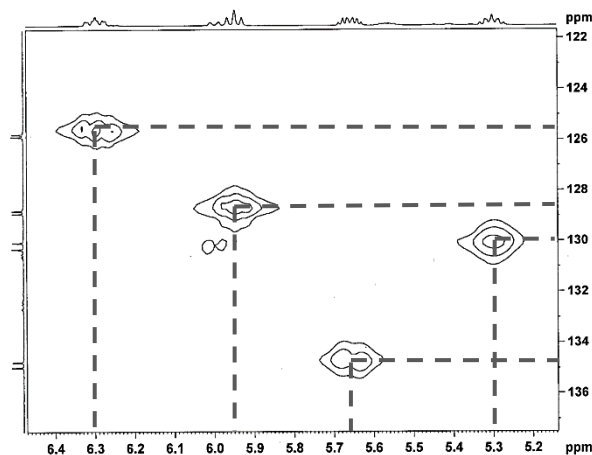


Figure 7. ^1H - ^{13}C NMR HMQC at the olefinic position.

To reconfirm these assignments, the HMBC spectrum was recorded (Fig. 8). This technique shows long range correlations of nuclei (carbon to carbon or carbon to proton) which are one, two or three bonds apart. The HMBC spectrum connection was summaries in Table 3. These two- or three-bond connections reconfirmed that CLA with 9Z11E isomer has been produced (Fig. 9).

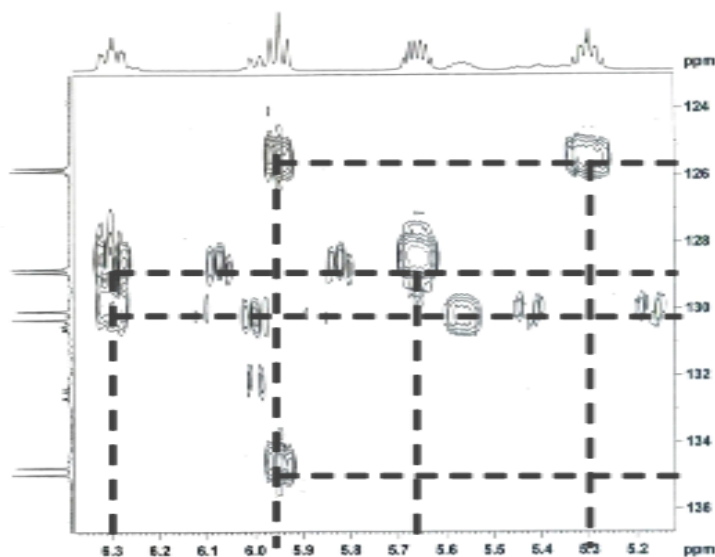


Figure 8. ^1H - ^{13}C NMR HMBC at the olefinic position.

Table 3 NMR HMBC correlations observed for the olefinic position

Atom	Carbon (ppm)	Proton (ppm)	Bond distance
9	134.95	5.94	3
10	125.78	5.94	2
		5.30	3
11	128.91	6.30	2
		5.94	1
		5.65	3
12	130.06	6.30	3
		5.30	1

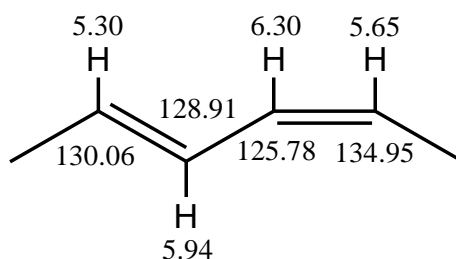


Figure 9. Proton and carbon arrangement at olefinic position for 9Z11E CLA.

CONCLUSION

CLA with 9E11Z isomer has been produced from catfish oil. NMR 1D and 2D have been shown to provide qualitative analysis and can be used to identify CLA isomer. H1 and C13 NMR analysis provides initial assumptions about the geometric isomers and positions in the synthesized CLA. Further analysis by 2D NMR through COSY, HMQC, and HMBC experiments has successfully confirmed the initial assumptions and established that the isomer produced by this synthesis is the c9t11 isomer.

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