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Extraction and Analysis of Conjugated Linoleic Acid and Its Isomer from Local Cheese Sample by Using Various Chromatographic Technique

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Abstract: *It is evident natural products, specifically dairy fats contain different CLA(conjugated linoleic acid) isomers and among them (cis9,trans11 conjugated linoleic acid)is dominant. Conjugated fatty acids ,specially octadecadienoic acid (18:2) is difficult from analysis point of view due to the mixture of positional and geometrical isomers of linoleic acid(9cis,12cis 18:2) known as CLA.CLA is formed when reaction shift one or both of the double bonds of linoleic acid. In the present investigation different isomers of CLA are separated by the formation of DMOX (dimethyl oxazoline) derivative and identified by different analytical methods. For this purpose local cheese is used as a product of dairy.The analytical methods include HPLC for identification of CLA and UV detector is used for the confirmation, silver ion TLC, FT-IR for the identification of cis and trans form and structural determination of isomer by GC-MS is used. In proposed work , dairy product local cheese is used and (18:2, 8,10 ; 18:2, 9,11 ; 18:2, 10,12 ; 18:2 , 11,13 ;) CLAs are found. This method is good to evaluate dairy product for CLA contents to design the experimental diet.*

Keywords: *FAME, CLA, HPLC, GC-MS, CLA Isomer, DMOX derivative*.

I. Introduction

The chemistry of conjugated fatty acids, specially octadecadienoic acid (18:2) is difficult from analysis point of view due to mixture of positional and geometric isomers of linoleic (9cis 12cis 18:2) acid – known as Conjugated Linoleic Acid (CLA).[(1)] CLA is formed when reactions shift one or both of the double bonds of linoleic acid so that the two double bonds are no longer separated by two single bonds.[(2)] Each double bond can be in cis or trans configuration and in any position on carbon chain. Heat treatment,[(3)] Isomerization[(4)] and Microbial reaction[(5)] contribute to formation of CLA from linoleic acid – which is an essential fatty acid supplied by food and not synthesised in the body. The occurrence of CLA in biological systems is taken as evidence of ongoing lipoperoxidation process.[(6)] CLA has been claimed to be involved in oxidative stress as an antioxidant.[(7)] Upsurge in the interest of CLA has started after the report that these are associated with anticarcinogenic activity.[(8)] Several additional physiological and pathological effects[(9-12)] have also been attributed to these. It has been found that different CLA isomers have distinct different biochemical properties.[(13)] Dietary CLA isomers reduce adipose tissue in animals.[(14-16)]

II. Material And Methods

2.1 Extraction and analysis of Conjugated linoleic acid isomer from local cheese

2.1.1 Experimental

For extraction grated cheese (15gm) and sodium sulphate (15gm) were homogenised in isopropanol (60ml). After 10 minuts of magnetic stirring the supernatant was removed and filtered. The extraction was repeated twice. The filtered supernatant were chromatographed on column protected with glass wool plug and containing a lower layer of anhydrous sodium sulphate (60gm), separated from upper layer of celite (15gm) capped with glass wool. Elution was done using hexane. Hexane fraction having fatty acid content was converted to fatty acid methyl ester (FAME) content using methanol and sulphuric acid. For HPLC (Waters 8700 with ODS column, Rheodyne injector) analysis (Fig 1) of methyl esters, these were taken in minimum amount of acetone (10µl/mg), analyzed isocratically at room temperature with acetonitrile (4ml/min) as mobile phase using RI detector.

2.1.2. Analysis of linoleic acid isomer

Palmitoleic acid (16:1) and linoleic acid (18:2) also co eluted along with isomer fraction and were collected. Their identities were confirmed by TLC with standard.

For enrichment of linoleic acid isomer fraction, silver nitrate impregnated (10% in acetonitrile) TLC plates were used. Toluene was used for development and the band was compared with authentic.

The isomer content band was scraped off and eluted with hexane. The eluted portion (methyl ester) was saponified with 2ml KOH (in 95% ethanol) for 3 hours to convert the methyl ester in to free fatty acid. The free

fatty acid content was separated by 5ml of hexane: ether(1:1). The free fatty acid was subjected to reduction by hydrazine (1ml) at 40 ^{0}C for 1 hour to convert dine in to monoene. Methyl ester of monoene was formed using methanol/sulphuric acid. It was divided into two portions. One portion was subjected to IR (Perkin Elmer RXI) analysis (Fig 3A and 3B) and another to DMOX derivatization (100 mg Methyl ester + 0.25 ml 2-amino2-methyl-Propanol at 170^{0}C for 3 hours). The derivative was subjected to GC (Varian Vista 6000, with N_2 carrier gas and FID) analysis (Fig 4).

2.2. Isomer identification by GC-MS of DMOX derivative

Present work, has been made to extend GC-MS in the analysis of dimethyl oxazoline (DMOX) derivative.

2.2.1. Experimental

The CLA rich fraction (enriched through $AgNO_3$ TLC scrapings) methyl ester was converted to DMOX derivative by reacting 100 mg of the fatty acid methyl ester (FAME) with 0.25 ml of 2-amino-2-methyl propanol in a capped tube (in the atmosphere of N_2). The tube was heated at 180^{0}C in oil bath for 4 hours.[23] The reaction mixture was cooled and mixed with 2 ml methylene chloride. After removal of the solvent, it was taken in 2 ml of hexane and dried over anhydrous Na_2SO_4. Hexane was removed and the sample was taken for analysis in chloroform. The sample was analysed by GC-MS (Fig 5) on Hewlett-Packard 5890A GC fitted with injector and column connected to Hewlett-Packard 5989A MS.

III. Results

3.1. Extraction of grated cheese

Fig.1 shows general HPLC profile of fatty acid methyl ester of hexane fraction of fatty acid content of grated cheese, followed by column chromatography. **Peak 1: Palmitic (16:0), Peak 2: Palmitoleic (16:1), Peak 3: Linoleic (18:2) and Peak 4: CLA content.** For the confirmation of Conjugated Linoleic Acid (CLA) UV detector is also used shown in Fig. 2

Fig.2 shows HPLC profile where the presence of linoleic acid isomer content (absorption at 234nm) is seen. (Fraction 4 of the general profile – **Fig.1**).

3.2. Analysis of linoleic acid isomer

Fig.3. shows FT IR profile of methyl ester of monoene is shown in **(A and B)**. In **Fig. 3.-3A** Peak at 3013 cm^{-1} is due to cis-monoene double bond. In **Fig. 3.-3B** Peak at 966 cm^{-1} is due to trans-monoene **double bond.**

In GC profile of DMOX derivative **(Fig. 4.)** four isomers [18:2, 8,10; 18:2, 9, 11; 18:2, 10, 12, and 18:2, 11, 13 corresponding to Peak nos.1 to 4 (UN denotes unidentified)] have been identified. No commercial standards for each isomer are available; therefore resolution of all isomers has been based on reported retention times.

3.3. Isomer identification by GC.MS. of DMOX derivative

The GC-MS profile **(Fig.5.)** shows molecular ion at m/z 333 and base ion at m/z 126. There are ions with m/z 12 difference between 196 and 208 (for the double bond at C9) and another m/z 12 difference between m/z 222 and 234 (for the double bond at C11).

IV. Discussion

The isomers of linoleic acid (9cis, 12cis octadecadienoic acid) are probably formed from the disruption of methylene – interrupted sequence by migration of the double bond along the carbon chain, with a modification of double bond geometry.

The cis/trans structure of the conjugated double bonds in these fatty acids have a Literature reveals that CLA content absorb in UV region [17] at around 230 nm and doublet in IR region.[18] In the present case, there is absorbtion at 234 nm in one of the collected fractions (fraction 4) of HPLC and there are specific absorptions in IR (conforming to cis and trans regions). Four positional isomers [18:2 – 8, 10; (Peak 1) 18:2 – 9, 11; (Peak 2) 18:2 – 10,12 (Peak 3) and 18:2 – 11, 13 (Peak 4)] have been identified. Except for standard for 18:2 – 9, 11, no standards for different isomers are available, therefore resolution becomes all the more difficult.[19] Recently, 18:2 – t10, c12 have been synthesized in laboratory.[16] Mechanisms of body fat modulation by CLA,[20] effect of processing on CLA content[21] and CLA production by ruminal microbes[22] are some recent areas of work in the field.

On the basis of the findings of GC-MS of DMOX derivative, the isomer has been identified to be that of CLA (9, 11 octadecadienoic) acid **(Fig.6.)**.

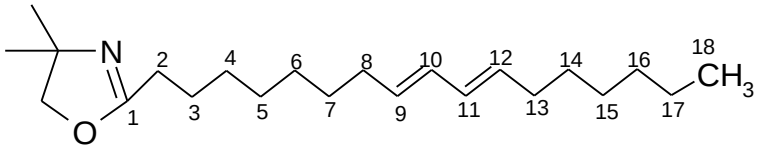

Fig.6

Acknowledgement

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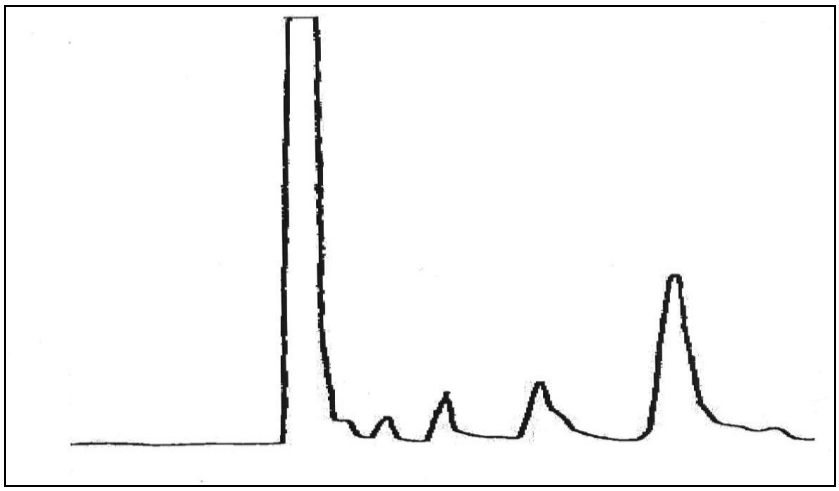

Fig. 1 : HPLC profile of local Cheese

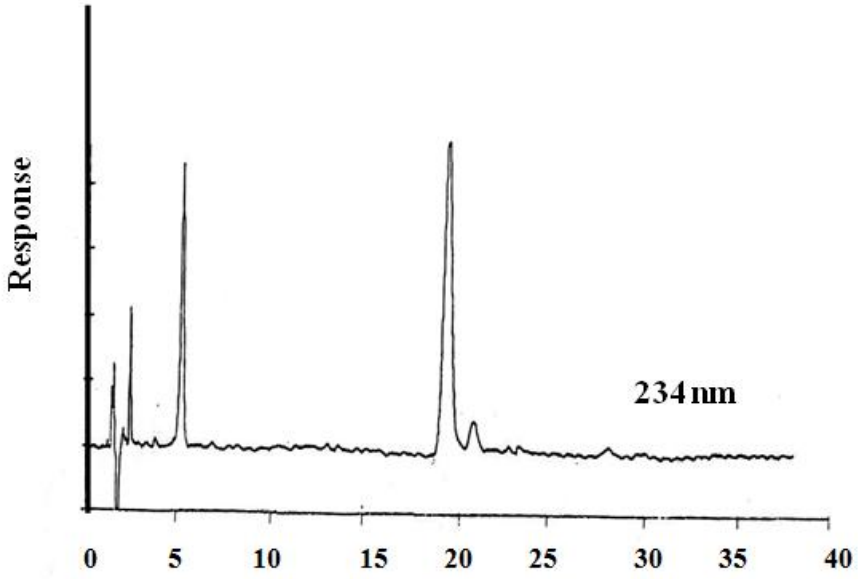

Fig. 2: CLA profile – HPLC detection

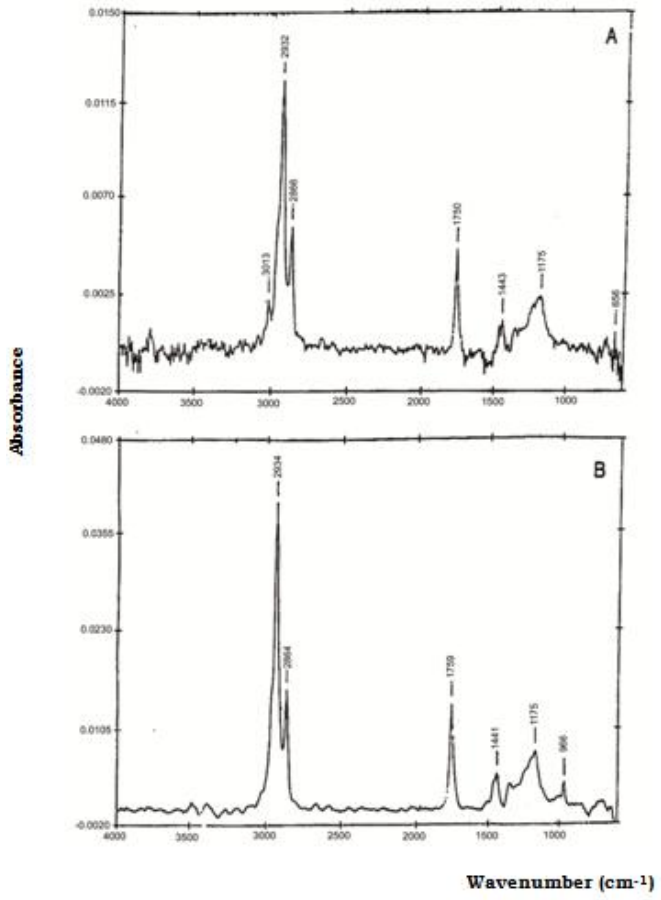

Fig. 3: (A & B) : FTIR profile of methyl ester of monoene

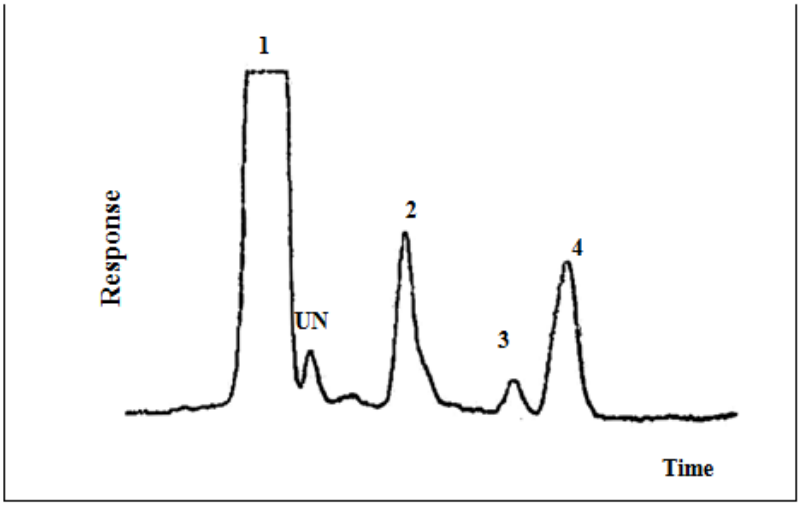

Fig. 4: GC profile of CLA

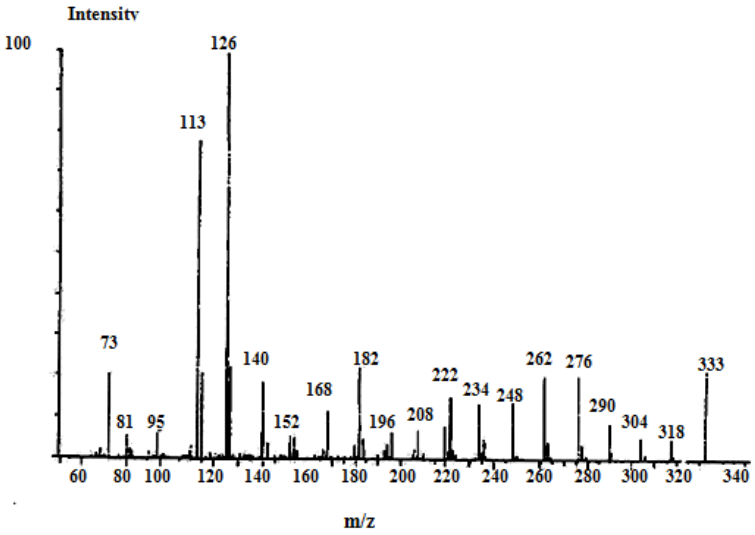

Fig.5 : GC-MS profile: CLA (9, 11, 18:2) DMOX