Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Scholars Research Library

Annals of Biological Research, 2013, 4 (11):18-24
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Identification and estimation of fatty acids in fresh water fish *Anabus testudineous*

K. S. Barma and B. C. Goswami

Department of Chemistry, Gauhati University, Guwahati

ABSTRACT

Fatty acid content of the fresh water fish Anabus testudineous (Local Name: Kawoi) were examined by TLC and GCMS. It has been confirmed that the fish lipid contains approximately 20.72% saturated fatty acid(SFA), 41.98% monounsaturated fatty acid(MUFA) and 1% polyunsaturated fatty acid(PUFA). The components of SFA are Palmitic acid C16:0(19%), Tetradecanoic acid C14:0(1.8%) and Stearic acid C18:0(0.76%). The components of MUFA are Oleic acid C18:1(40.4%) and Eicosaenoic acid C20:1(1.5%). The fish contains 1% of Arachidonic acid C20:4 which is an important natural product.

Keywords: FA, LIPID, TLC, GCMS.

INTRODUCTION

A fatty acid has a carboxylic acid at one end and a methyl group at the other end. Unsaturated fatty acids are fatty acids in which one or more than one double bond exists within the molecule. Essential fatty acids are fats that are extremely important nutrients for health but cannot be made in our body from other components and therefore must be obtained from the diet. In the body, essential fatty acid supplementation is recommended for more than sixty heath conditions. They must be obtained from foods such as nuts, oilseeds and their products and oil rich fish. [1] Scientists classify essential fatty acids into two types depending on their chemical composition. [2] They are omega - 3- fatty acids and omega -6- fatty acids. In a long chain fatty acid, the carbon atom in the methyl group is called the omega carbon, because omega is the last letter of the Greek Alphabet. Omega -3-fatty acids have a double bond three carbons away from the methyl carbon and omega -6- fatty acids have a double bond six carbon away from the methyl carbon.

My investigation deals with the extraction, purification and esterification of fish lipid, estimations and identification of methyl ester mixture by TLC and GCMS. These essential nutrients are found in high quantities in oily fish. Fish does not produce omega -3-fatty acids, but accumulate them from their foods. [3] I have extracted the lipids from edible part of Kawoi fish *(Anabus Testudineous*)

The extract is then methylated and analysed by TLC and GCMS. The analysis is repeated 5 to 8 times.

Esterification, GCMS and TLC estimations are also carried out for standard acids like stearic acid, palmitic acid etc. and are used as standard methyl ester in TLC.

This fish is found in canals, lakes, ponds, swamps and estuaries. Adults occur in medium to large rivers, flooded fields and stagnant water bodies including sluggish flowering canals. Often found in areas with dense vegetation. Can tolerate extremely unfavorable water conditions and is associated mainly with turbid, stagnant waters.

MATERIALS AND METHODS

(a) Extraction of lipid from fish:
The lipid is extracted from the body muscle (edible portion) of fish by the method cited in the literature. [4]

About 20gm of wet fish is ground well in a homogenizer with about 200ml distilled water to make pulp. The pulp is transferred to a volumetric flask and 800 ml of chloroform – methanol (2:1v/v) mixture is added and shaken well for about 3h. For complete extraction, it is kept overnight at room temperature, preferably in the dark. The resulting suspension is subjected to centrifugation where three layers are found. Chloroform layer with lipid is separated. To ensure complete extraction, the process is repeated and the combined extract is dried with a flow of nitrogen gas.

(b)Conversion of lipid into corresponding fatty acid methyl esters (FAMES) [4] :
The dried chloroform layer with lipid (by flow of N_2 gas) is evaporated to dryness in a rotary evaporator. It is dissolved in 2ml of freshly prepared mixture of acetyl chloride and methanol at a ratio of 1: 20 (v/v). The mixture with very small amount of BHT (used as an antioxidant) is placed in Teflon- capped Pyrex Tube and the reaction is continued at 100^0C for 1 hour under an atmosphere of nitrogen in darkness. After cooling to 30-40^0C, 1 ml of extracting solvent (hexane) is added and then vortexes for about 20 sec). Purification of the solution is achieved either by salting out (using 1 ml of saturated sodium chloride solution) or washing (using 1ml of water), causing the formation of two immiscible phases, which are then allowed to separate. The upper extracted solvent phase is recovered, dried over anhydrous sodium sulphate and analyzed using TLC, GCMS.

(c) TLC examination of methyl ester:
Thin layer chromatography is an excellent tool for micro preparative separation of mixture. The methyl ester mixtures are charged on thin layer of silica gel and are developed by ascending technique using the following solvent systems.
a. Petroleum ether (40-60^0c): ether (60:40) (v/v)
b. Petroleum ether (40-60^0c): ether (80:20) (v/v)
c. Hexane : ether (80 :20) (v/v)

After developing, the plates are dried at room temperature and placed in iodine chamber. The fatty acids methyl ester gave violet colored spot with this iodine vapour. The coloured spots are marked and the R_f values of the spots are calculated. Chromatography with standard methyl ester is carried out and R_f values are compared whenever possible.

Table [1]: R_f values of methyl ester of standard fatty acids composition in different solvents

Developing solvent system	Methyl Palmitate	Methyl Stearate	Methyl Oleate
P:E (80:20)	0.552	0.471	0.460
P:E (60:40)	0.600	0.728	0.552
H:E (80:20)	0.671	0.752	0.822

(P: E=Petroleum Ether: Ether; H: E= Hexane: Ether)

(d) Fatty acids analysis by GC-MS:
Instrument – Perkin Elmer; Model No. – 600 GC-MS; Column- Elite 5 MS column diameter 30m x 0.25.

Gas Chromatographic conditions-
Initial – 190^{0}c, Hold time-2min; Ramp -20^0 c/min to 280^{0}c; Hold time-10 min at 260^{0}c Total programming time = 12 min; Injector temperature- 290^0 c; Auxiliary, i.e. MS transferred time temperature = 250^0 c; Split ratio- 50:1; Split flow -50 ml/min.

MS Conditions:-
Mass range- 40-450; Solvent delay-1.5 min; ion source 1.5min; ion source temperature 200^{0}c ; Carrier gas- Helium.

(e) Identification of Fatty Acids:
Peak identification of fatty acids in the analyzed samples are carried out by comparing with the retention time and molecular mass of mass spectra of standard obtained from library (Wiley and NIST) of the GC-MS instrument.

Table [2]: Gas chromatographic retention time rule and mass spectrometric characteristic ions of fatty acids methyl esters

Fatty acids	Shorter form	Double-bond	Base ion peak (m/z)	Characteristic ion peaks
1 Decanoic acid	C10:0	0	74	74,143, 186
2 Dodecanoic acid	C12:0	0	74	74,171, 214
3 Tetradecanoic acid	C14:0	0	74	74,242, 199
4 trans-Tetradecenoic acid	C14:1	1	55	55,208, 240
5 cis-Tetradecenoic acid	C14:1	1	55	55,208, 240
6 Palmitic acid	C16:0	0	74	74,227, 270
7 trans-Hexadecenoic acid	C16:1	1	55	55,236, 268
8 cis-Hexadecenoic acid	C16:1	1	55	55,236, 268
9 Stearic acid	C18:0	0	74	74,255, 298
10 trans-Oleic acid	C18:1	1	55	55,264, 296
11 cis-Oleic acid	C18:1	1	55	55,264, 296
12 trans,trans-Linoleic acid	C18:2	2	67	67,263, 294
13 cis,trans-Linoleic acid	C18:2	2	67	67,263, 294
14 cis,cis-Linoleic acid	C18:2	2	67	67,263, 294
15 trans,trans,trans-Linolenic acid	C18:3	3	79	79,261, 292
16 trans,trans,cis-Linolenic acid	C18:3	3	79	79,261, 292
17*trans,cis,cis*-Linolenic acid	C18:3	3	79	79,261, 292
18 *cis,cis,cis*-Linolenic acid	C18:3	3	79	79,261, 292
19 Eicosanoic acid	C20:0	0	74	74,283, 326
20 *trans*-Eicosanoic acid	C20:1	1	55	55,292, 324
21 *cis*-Eicosanoic acid	C20:1	1	55	55,292, 324
22 *trans,trans,trans,trans*-Arachidonic acid	C20:4	4	79	79,318
23 *trans,trans,trans,cis*-Arachidonic acid	C20:4	4	79	79,287, 316
24 *trans,trans,cis,cis*-Arachidonic acid	C20:4	4	79	79,287, 316
25 *trans,cis,cis,cis*-Arachidonic acid	C20:4	4	79	79,287, 316
26 *cis,cis,cis,cis*-Arachidonic acid	C20:4	4	79	79,287, 316
27 *trans,trans,trans,trans,trans*-Eicosapentaenoic Acid	C20:5	5	79	79,287, 316
28 *trans,trans,trans,trans,cis*- Eicosapentaenoic Acid	C20:5	5	79	79,287, 316
29 *trans,trans,trans,cis,cis*-Eicosapentaenoic Acid	C20:5	5	79	79,287, 316
30 *trans,trans,cis,cis,cis*-Eicosapentaenoic Acid	C20:5	5	79	79,287, 316
31 *trans,cis,cis,cis,cis*-Eicosapentaenoic Acid	C20:5	5	79	79,287, 316
32 *cis,cis,cis,cis,cis*-Eicosapentaenoic Acid	C20:5	5	79	79,287, 316
33 *trans,trans,trans,trans,trans, trans*-Docosahexaenoic Acid	C22:6	6	79	79,313,342
34 *trans,trans,trans,trans,trans,cis*-Docosahexaenoic Acid	C22:6	6	79	79,313,342
35 *trans,trans,trans,trans,cis,cis*-Docosahexaenoic Acid	C22:6	6	79	79,313,342
36 *trans,trans,trans,cis,cis,cis*-Docosahexaenoic Acid	C22:6	6	79	79,313,342
37 *trans,trans,cis,cis,cis,cis*-Docosahexaenoic Acid	C22:6	6	79	79,313,342
38 *trans,cis,cis,cis,cis,cis*-Docosahexaenoic Acid	C22:6	6	79	79,313,342
39cis,cis,cis,cis,cis,cis-Docosahexaenoic Acid	C22:6	6	79	79,313,342

Moreover identification is confirmed by studying the Gas Chromatographic Retention Time Rule and Mass Spectrometric Fragmentation Rule of Fatty acids [5] as mentioned below.

(f) Calculation of total lipid:
The lipid extract is evaporated to dryness; the amount of fat can be obtained by weighing. The total content of fat can be calculated by dividing the amount of fat to the weight of the sample [6].

RESULTS AND DISCUSSION

(a) Results of TLC:-
The methyl ester (FAME) of this fish lipid was charged on the thin layer of silica gel –G. The plates are developed using three different solvent systems.

(1)Results on TLC separation with Petroleum ether: Ether 80:20 (v/v)

Four different spots are found and their R_f values are recorded. Comparing this R_f values with the standard value I have found
Spot 1 with R_f value 0.318 could not be identified.
Spot 2 with R_f value 0.450 is close to the standard R_f value of methyl stearate.
Spot 3 with R_f value 0.538 is close to the standard R_f value of methyl palmitate.
Spot 4 with R_f value 0.835 could not be identified.

Table [3]: TLC separation of fatty acid methyl ester

Spots of FAME	R_f –Values of standard compounds	R_f -values of TLC separation	Inference
1.	-	0.318	Could not be identified
2	0.471	0.450	May be methyl stearate
3	0.552	0.538	May be methyl palmitate
4	-	0.835	Could not be identified

(2) Results on TLC separation with Petroleum ether: 60:40 (v/v)

Four different spots are found and their R_f values are recorded. Comparing this R_f values with the standard value I have found.
Spot 2 with R_f value 0.592 is close to the standard R_f value of methyl palmitate.
Spot 3 with R_f value 0.634 could not be identified.
Spot 4 with R_f value 0.734 is close to the standard R_f value of methyl palmitate
Spot 1 with R_f value 0.479 could not be identified.

Table [4]: TLC separation of fatty acid methyl ester

Spots of FAME	R_f –Values of standard compounds	R_f -values of TLC separation	Inference
1.	-	0.479	Could not be identified
2	0.600	0.592	May be methyl palmitate
3	-	0.634	Could not be identified
4	0.728	0.734	May be methyl stearate

(3) Results on TLC separation with Hexane: Ether: 80:20 (v/v)

Four different spots are found and their R_f values are recorded. Comparing these values with the standard value, we get-
Spot 1 with R_f value 0.692 could not be identified.
Spot 2 with R_f value 0.740 is close to the standard R_f value of methyl stearate.
Spot 3 with R_f value 0.772is close to the standard R_f value of methyl palmitate.
Spot 4 with R_f value 0.835 could not be identified.

Table [5]: TLC separation of fatty acid methyl ester

Spots of FAME	R_f-Values of standard compounds	R_f values of TLC separation	Inference
1.	0.671	0.692	May be methyl palmitate
2	0.752	0.740	May be methyl stearate
3	-	0.788	Could not be identified
4	-	0.875	Could not be identified

I have found from TLC that methyl ester mixture gave four spots. In petroleum ether –ether and hexane-ether solvent system, two spots are identified as palmitic acid and stearic acid. Thus, I may conclude that the fatty acid methyl ester mixture of this fish may contain palmitic acid and stearic acid. Separation is comparatively well with the hexane – ether solvent.

(b) Analysis of lipid extract by GC-MS:
Peak identification of fatty acids in the analyzed samples are carried out by comparing with the retention time and molecular mass of mass spectra of standard, obtained from library (Wiley and NIST) of the GCMS instrument and also confirmed by comparing the Mass Spectrometric Fragmentation Pattern with the standard[Table-2].

The peak corresponding to retention time 1.409 minute showed mass spectra with molecular mass 256 gm/mol., which is same as the molecular mass of methyl tetradecanoate. This is also confirmed by comparison of fragmentation pattern with general fragmentation rule of fatty acid [Table-2].

The peak corresponding to retention time 1.599 minute showed mass spectra with molecular mass 270 gm/mol., which is same as the molecular mass of methyl palmitate. This is also confirmed by comparison of fragmentation pattern with general fragmentation rule of fatty acid [Table-2].

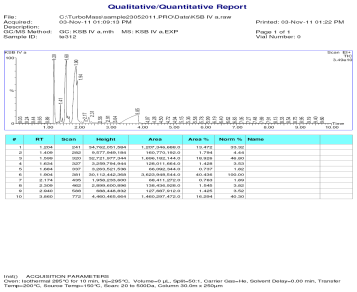
The peak corresponding to retention time 1.904 minute showed mass spectra with molecular mass 296 gm/mol., which is same as the molecular mass of methyl oleate. This is also confirmed by comparison of fragmentation pattern with general fragmentation rule of fatty acid [Table-2].

The peak corresponding to retention time 2.174 minute showed mass spectra with molecular mass 318 gm/mol., which is same as the molecular mass of methyl arachidonate. This is also confirmed by comparison of fragmentation pattern with general fragmentation rule of fatty acid [Table-2].

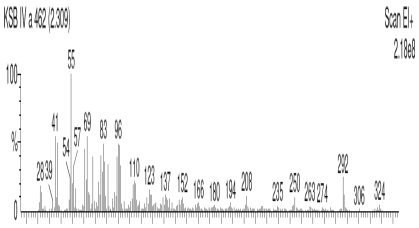
The peak corresponding to retention time 2.309 minute showed mass spectra with molecular mass 258 gm/mol., which is same as the molecular mass of methyl eicosonoate. This is also confirmed by comparison of fragmentation pattern with general fragmentation rule of fatty acid [Table-2].

(c)Calculation of total lipid of Kawoi fish:
Lipid of Kawoi fish accounts for 5.2% (=1.04gx100/20g) of the total fish weight &may be classified as medium fat fish.

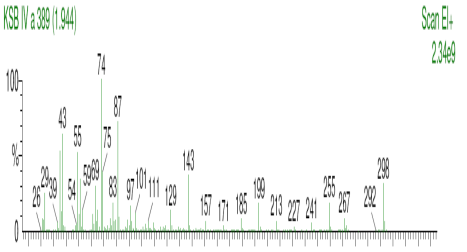
GC of Kawoi fish



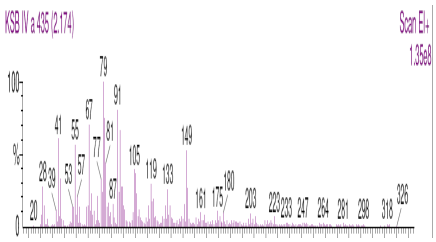
MS of Eicosaenoic acid 1.5%



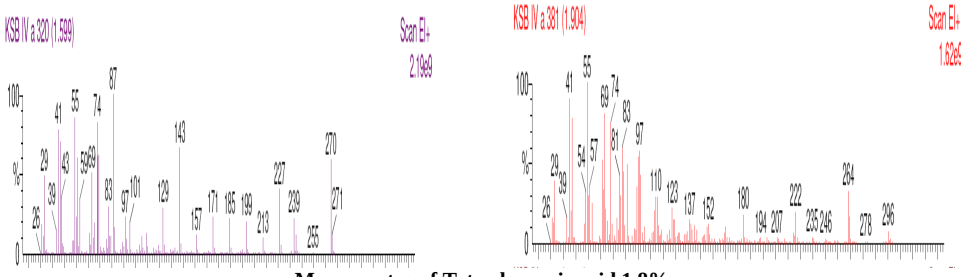
MS of Arachidonic acid 1%



MS of Stearic acid 0.7%



MS of Oleic acid 40.4% **MS Palmitic acid 19%**



Mass spectra of Tetradecanoic acid 1.8%

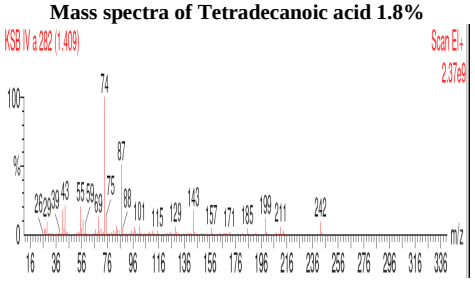


Table [6]

Name of FA	Set-1	Set-2	Set-3	Set-4	Set-5	Results(%age) (Mean ± Std. Dev.)
Palmitic Acid	19.5	19.0	18.8	18.5	19.2	19 ± 0.314
Tetradecanoic Acid	1.6	1.8	1.9	1.9	1.8	1.8 ±0.110
Oleic Acid	40.4	40.7	40.8	40.1	40.0	40.4 ± 0.100
Eicosaenoic Acid	1.8	1.7	1.5	1.3	1.2	1.5 ±0.052
Arachidonic Acid	0.76	0.85	0.98	1.2	1.24	1 ± 0.121

CONCLUSION

It is evident from TLC and GCMS that the fish lipid of Kawoi Fish *(Anabus Testudineous*) contains approximately 20.72% saturated fatty acids, 41.98% monounsaturated fatty acids (oleic acid & eicosanoic acid) and 1% polyunsaturated fatty acids (arachidonic acid). PUFA, namely Arachidonic acid found in above fish belongs to n-6 family has been designated as essential fatty acid for human. By repeating the same work got that amount of FA content varies to some extent which indicates that FA content of fish depends on the size and on the location from where it is collected. There is a future prospect of studies on PUFA content of fish under different stress conditions i.e. inhabitant, size, food of fish etc., since fish cannot synthesis PUFA but acquires from its food, namely algae [10].

From the foregoing evidences, it may be concluded that due to the presence of appreciable amount of mono and polyunsaturated fatty acids in the fish lipid under investigation, it is suitable for edible purposes owing to the important role it plays in the transport of cholesterol and thus preventing atherosclerosis, thrombosis and effectively involved in the transport of cholesterol from blood [11].

REFERENCES

[1] R.C. Eander et al. *Postaglandins, Leukotrienes and essential fatty acid* **1998**, 59 (2), 143-11.
[2] Harris, willium, *American Journal of clinical Nutrition,* 65 (5 supplant 1645-1654)
[3] M.S. Islam, M.T. Hussain Molla, M.T. Alam, *Pakistan Journal of Biological Sciences*, 11 (10): 1342-1347, **2008**.
[4] Cohen, Z. A. Vonshak and A. Richmond (**1988***) Effect of environmental condtions of fatty acid comporition of red alga porphyridium cruentum : correlation to growth rate. J. Phycol*. 24:328-332.
[5] Wu-Hui-Qin, Huang Xia-Lan, Lin Xiao –Shan, Huang Fang, Zhu zhi- xin, Ma ye-Fen ; *Chin J Anal Chem*, **2007**, *35 (7), 998-1003.*
[6] Conner, W.E. 2001. *ω- 3 fatty from fish and fish oil; Panacea or Nostrum Am. J. Clin. Nutr, 74: 415-416.*
[7] Gunstone, Frank *"Oilseed markets: Market update: Palm oil" INFORM (AOCS) 18 (12): December* **2007** *835-836.*
[8] Moghadasian MH. *" Advances in dietary enrichment with n-3 fatty acids" Critical Reviews in Food Science and Nutrition;* (May **2008**) 48 (5) : 402-10
[9] Soyland E; Funk J, Rajka G.et.al, *British Journal of Dematology* **2006**, 130 (6), 757-764.

[10] Pratoomyyol, J, Srivilas P. and noiraksar, T. Songklanakarin*; J. Sci. Technol,* **2005**, *27 (6*: 1179-1187.
[11] Hal- Link Tran, Seong- JooHong and Choul-Gyn Lee; *Biochemistry and Bioprocess Engineering*, **2009**, 14: 187-192