GC-MS Determination and Identification of Eleven Fatty Acids in Triglycerides Isolated from the Seeds of Traditional Kurdish Medicinal Plant *Anchusa azurea* Mill.

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Abstract: *Anchusa azurea* Mill. is naturally grown in Iraqi Kurdistan, and it belongs to the Boraginaceae family. The species *A. azurea* is eaten by Kurdish people and from a phytochemical point of view it has been almost neglected so far and it was thus considered worthy of study. In this investigation the lipids were extracted from seeds and leaves of *A. azurea* plant with petroleum ether, followed by chloroform and methanol using soxhlet apparatus. The lipid percentage from seeds and leaves were (7.03%) and (1.17%) respectively. Preliminary work on the first extract allowed for the isolation of a mixture of triglycerides, which were submitted to hydrolysis, followed by methylation of free fatty acids. GC-MS analysis showed that the main components were oleic, palmitic, palmitoleic, 11-eicosenoic, erucic and two ω-9 acids. Totally, eleven fatty acids were analyzed from the seeds of the studied plant using GC-MS analysis. The results showed that the plant seeds contain high percentage of elaidic acid (46.42%), palmitic acid (18.9%), linoleic acid (14.59%), and the other main fatty acids (FAs) are erucic acid (6.33%), 11-eicosenoic acid (5.02%), stearic acid (4.55%) and 6,9,12-octadecatrienoic acid (2.43%). The percentage of minor FAs is (0.78%) nervonic acid, (0.46%) myristic acid, (0.38%) palmitoleic acid and (0.14%) for 11-hexadecenoic acid. The total percentages of polyunsaturated (PUFAs), monounsaturated (MUFAs) and saturated fatty acids (SFAs) from the seeds of *A. azurea* are (17.02, 59.07, 23.91%) respectively. It was found that the percentage and type of fatty acid constituents from *A. azurea* seeds oil in the present study varied from the same plant in other places and countries.

Keywords: Boraginaceae, Anchusa Azurea, Fatty Acids, GC-MS

1. Introduction

Fatty acids, both free and as part of complex lipids, play a number of key roles in metabolism – major metabolic fuel (storage and transport of energy), as essential components of all membranes, and as gene regulators. In addition, dietary lipids provide polyunsaturated fatty acids (PUFAs) that are precursors of powerful locally acting metabolites, i.e. the eicosanoids. As part of complex lipids, fatty acids are also important for thermal and electrical insulation, and for mechanical protection. Moreover, free fatty acids and their salts may function as detergents and soaps owing to their

amphipathic properties and the formation of micelles (Arild & Christian, 2005).

Iraqi Kurdistan region is considered as a rich area for the medicinal herbs, which are used in many ways, as food, spices, perfumes and drugs. In spring, some plants can be used as a food source. The *Anchusa azurea* is one of those plants that is used by many Kurdish people for eating especially in the villages. *Anchusa azurea* Mill. (Synonym: *Anchusa italica* Retz.) is a known species of flowering plant with a common name as Italian bugloss (Samuelsson, 1999). It belongs to the Boraginaceae family, which included a variety of shrubs, trees, and herbs, totaling about 2000 species. In worldwide 146 genera were found, while it is represented in wild Iraq with only 26 genera and approximately 93 species (Al-Mussawy, 1987). It is a perennial plant (Tutin et al., 1972) and locally it has many names like: Gormza, Kawlla Shina, Chizy (FAW, 2003), Lsan Al-thor, Ward Mawe (Al-Mussawy, 1987) (Figure 1). *Anchusa azurea* Mill. is a native plant throughout whole Europe, especially the southern and central parts (e.g. Italy, Greece, Hungary, Romania, France, Portugal, Spain, Ukraine, and also Russia). It is also found in Western Asia (Iraq, Pakistan, Israel, Cyprus, Turkey, etc.), Caucasus (e.g. Azerbaijan, Soviet & Middle Asia: Kazakhstan etc.), and Tropical Asia (e.g. Pakistan) (USDA, 1984). The plant is present in Iraq especially it is widely distributed in Kurdistan region (Al-Mussawy, 1987) like; Kirkuk, Persian Foothill, Mosul, Upper Jazira, Amadia, Rawanduz and Sulaimaniya Districts (Al-Rawi & Chakravarty, 1988).

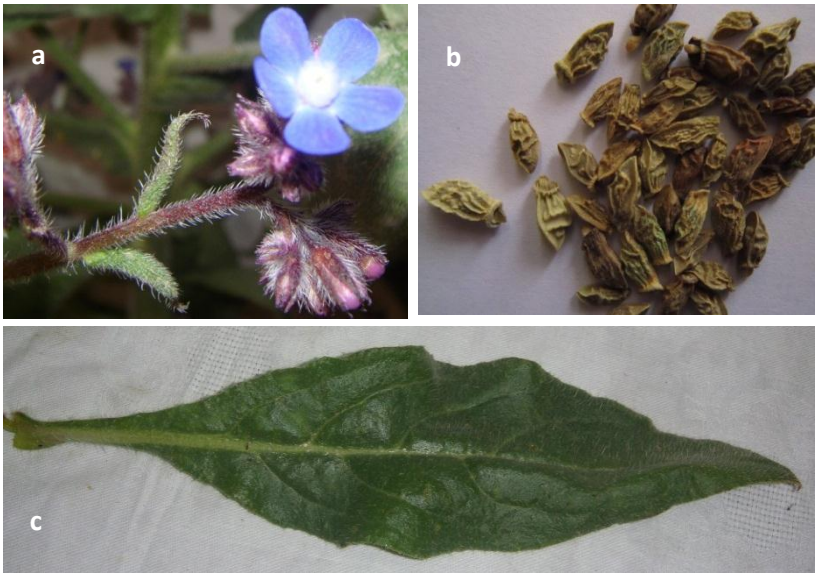


Figure 1: *Anchusa azurea* Mill.: a- flowers; b- seeds and c- leaf

Anchusa species are used in folk medicine for wound healing and as a diuretic agent (Yeşilada et al., 1995). The whole plant is antitussive, depurative, and diuretic. It is harvested when in flower and dried for later use. The dried and powdered herb is used, internally with caution, as a poultice to treat inflammations.The plant contains alkaloid cynoglossine which can have a paralyzing effect (Chiej, 1984) and carcinogenic (Harpestreng, 2004). The seeds of *Anchusa azurea* Mill. are rich sources of many unsaturated fatty acids like; linoleic acid, oleic acid, γ-linolenic acid, eicosenoic acid and several saturated fatty acids like palmitic acid (López et al., 2005; Guil et al., 2001). Also the plant contains some other compounds such as triterpene glycosides (saponins), polyphenols (flavonoids) (Kuruuzum et al., 2010), toxic and untoxic pyrrolizidine alkaloids (ESCO, 2009), vitamin E and

tannins (Khare, 2007).

Anchusa azurea Mill. is naturally occurring plant in Kurdistan region. To the best of our knowledge of the open literature, no previous study was done on *A. azurea* in Iraq. Therefore, we decided to start the study of the plant in terms of extraction, isolation and identification of fatty acids in the seeds of *A. azurea*, using different techniques such as GC-MS and ^{1}H-NMR.

2. Materials and Methods

2.1 Plant Material (Collection)

Anchusa azurea Mill. was collected during May 2009 from the Garota village (Safeen Mountain) which belongs to Shaqlawa-Erbil/Kurdistan Region. The collected plant materials were identified and classified from ESUH (Education Salahaddin University Herbarium). The plant raw materials were washed and air-dried under shade at room temperature. After drying, the plant parts separately were ground into fine powder using a laboratory blender, passed through a 0.71 mm mesh sieve, to provide homogeneous powder for the analysis. Powdered materials were stored in dark bottles and maintained at room temperature until required for analysis.

2.2 Lipid Extraction

Lipids were extracted from *A. azurea* parts especially seeds and leaves according to Harborne method with slight modification. One hundred gram dry powder seeds of the plant were extracted with 300 ml of petroleum ether 60-80 $^{\circ}$C using soxhlet system for 8 hr. to obtain 'extract I'. The residue was followed by re-extraction with 300 ml of $CHCl_3$–MeOH (2:1) under same condition to give 'extract II'. The 'extract I & II' were combined and then evaporated to dryness under reduced pressure at 40 $^{\circ}$C using rotary evaporator and extract (**L**) was obtained (Harborne, 1998). Also lipids were extracted from leaves part of *A. azurea* using same procedure.

2.2.1 Transesterification of Triglycerides to FAMEs and Identification by GC-MS Analysis

Initially the extract (**L**) was dissolved in a small portion of *n*-hexane and then purified with a chromatographic column of silica gel using a gradient from *n*-hexane-EtOAc (9:1) to EtOAc 100% to EtOAc-MeOH (8:2). Fraction 1 from the eluent was not free of fatty acids but it was triglycerides according to the ^{1}H-NMR spectrum as shown in (Figure 2A), and the triglycerides then transesterified to FAMEs as follows: to Fraction 1, 0.25 g of NaOMe was added which was previously dissolved in 10 ml of MeOH. The reaction was left under magnetic stirring at r.t. for 30 hr. and then the solvent was evaporated under vacuum. After evaporation of most of MeOH, the fraction was dissolved in Et_2O and then extracted with acidulated H_2O. After checking that the organic phase had a neutral pH and evaporation of the Et_2O, the ^{1}H-NMR spectrum (Figure 2B) indicated that only a small portion of the fatty acids were methylated, and glycerin did not remain, so the rest of the fraction was methylated using diazomethane under stirring at r.t. for 4 hr. The methylation was monitored by taking a direct phase TLC using *n*-hexane-EtOAc (8:2) as shown in (Figure 3).

The methylated product was purified with a column chromatography of silica gel using *n*-hexane-EtOAc (9:1). The methylated fatty acids were injected in GC-MS (Thermo DSQ, with an HP5 30m x 0.25mm column): FOCUS GC: (Inlet Temperature 250°C, carrier gas Helium 1 ml/min in constant

flow, ramp: 60°C x 1 min, 10°C/min to 260°C, 260°C x 15min), DSQ: (ion source 250°C, positive ions, mass range 45-400 full scan).

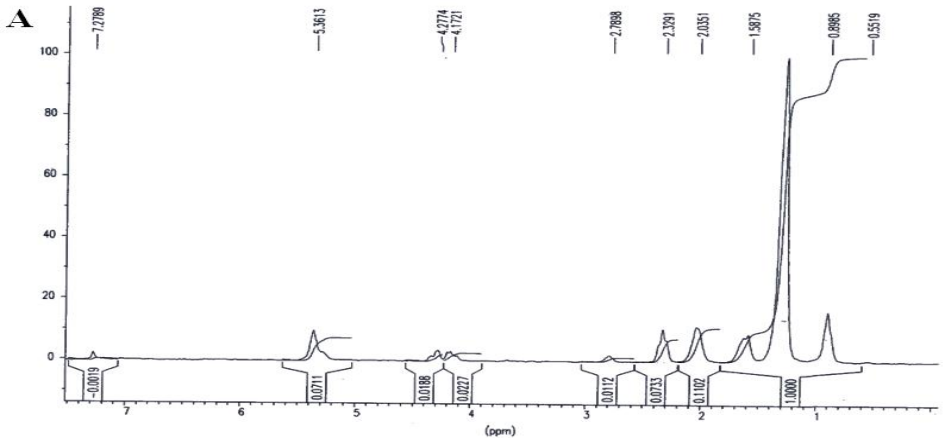
As a result we obtained eleven FAMEs which are consisting from three saturated FAMEs and eight unsaturated FAMEs as shown in (Table 1) and their GC-MS spectra in (Figure 4).

3. Results and Discussion

Lipids were extracted from seeds and leaves of *A. azurea* plant according to Harborne method with slight modification. The percentage of lipids from seeds of the plant was (7.03%) and it is higher than that of leaves part which was (1.17%).

3.1 Transesterification of Triglycerides to FAMEs

The ^{1}H-NMR spectroscopy was applied to monitoring the conversion of triglycerides to FAMEs (fatty acid methyl esters) by means of transesterification. Before transesterification, the following chemical shifts (δ, ppm) were performed in deuterized $CHCl_3$ as follows: (0.89 ppm) –C**H$_3$** of the beginning fatty acid chains, (1.40 ppm) –(**CH$_2$**)$_n$– of the fatty acid chains, (1.58 ppm) –C**H$_2$**–CH$_2$–COO– (β-protons to the carboxylic group), (2.03 ppm) –C**H$_2$**–CH=CH– (α-protons to the double bond), (2.32 ppm) –C**H$_2$**–COO– (α-protons to the carboxylic group), (2.78 ppm) =CH–C**H$_2$**–CH= protons between two double bonds, (4.17 and 4.27 ppm) –C**H$_2$**– of the glycerol, (5.36 ppm) –C**H**– of the glycerol and–C**H**=C**H**– protons on double bonds, (7.27 ppm) traces of C**H**Cl$_3$ in the $CDCl_3$, (Figure 2A). During transesterification, the signals at (0.89, 1.40, 1.58, 2.03, 2.32, 2.78 ppm) were unchanged, while the signals at (4.17 and 4.27 ppm) disappeared. Also the intensity of the signal at (5.36 ppm) decreased and a new peak appeared at (3.67 ppm) with low intensity, these changes indicate the formation of methyl esters, (Figure 2B). Eventually, at the end of transesterification, it was the same as the second step, but here the intensity of the peak at (3.67 ppm) highly increased and the intensity of the signal at (5.36 ppm) further decreased, which mean that all fatty acids changed to FAMEs, (Figure 2C).



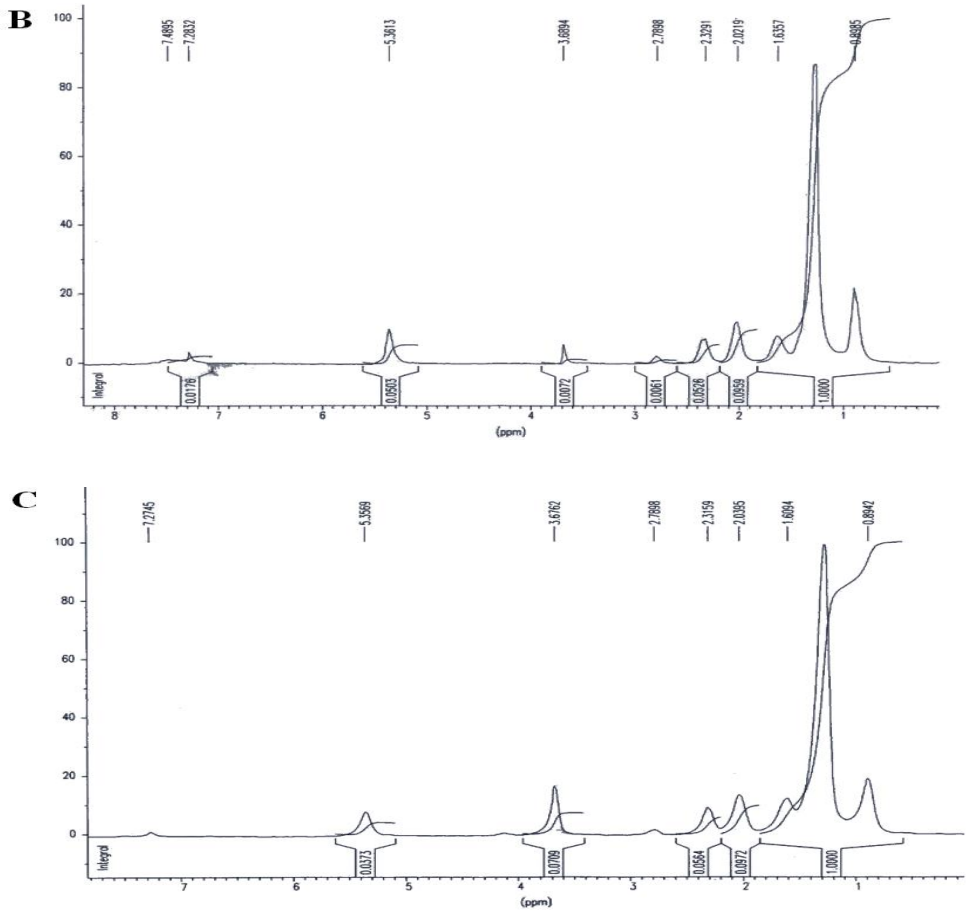


Figure 2: ^{1}H-NMR spectrum of FAMEs mixture; A-before, B-during and C-end of (transesterification)

In addition, TLC (thin layer chromatography) was applied to monitor the conversion of triglycerides to FAMEs. After reaction of fatty acids with diazomethane under stirring at r.t. for 4 hr., the direct phase TLC of silica gel in *n*-hexane–EtOAc (8:2) was used. The "start" spot was the sample before the methylation reaction with diazomethane and the "end" spot was at the end of the reaction. It seems that in the beginning there was a big purple spot in the middle which was due to the presence of free fatty acids, and it disappeared at the end of the spot, because the fatty acids were completely methylated and migrated above, (Figure 3).

Figure 3: Qualitative TLC analysis, at start and end of transesterification of triglycerides

3.2 GC-MS Analysis for FAMEs from Seeds Oil of the Plant

Qualitative and quantitative analysis of the methyl esters of fatty acids showed the presence of seven main FAMEs and four minor FAMEs of the studied oil of *A. azurea* seeds as shown in (Table 1). The results showed that the seeds of the studied plant contain high percentage of elaidic acid methyl ester (46.42%), palmitic acid methyl ester (18.9%), linoleic acid methyl ester (14.59%), and the other main FAMEs are erucic acid methyl ester (6.33%), 11-eicosenoic acid methyl ester (5.02%), stearic acid methyl ester (4.55%) and 6,9,12-octadecatrienoic acid methyl ester (2.43%). The percentage of minor FAMEs were (0.78%) nervonic acid methyl ester, (0.46%) myristic acid methyl ester, (0.38%) palmitoleic acid methyl ester and (0.14%) for 11-hexadecenoic acid methyl ester. The gas chromatography (GC) spectrum of FAMEs, (Figure 4) showed relative abundance of eleven FAMEs with their retention times (R_t). Mass spectroscopy showed that the seeds of *A. azurea* consist of three saturated FAMEs and eight unsaturated FAMEs (six-monoenes + one-diene + one-triene) (Figure 5-12).

Table 1: Percentage of FAMEs of *A. azurea* seeds oil obtained from GC-MS analysis

No.	Common name (—acid methyl ester)	Chemical Formula	Δ^{x*}	M. wt.	Apex R_t	Start R_t	End R_t	% Area
1	Myristic acid	$C_{15}H_{30}O_2$	----------	242	14.36	14.34	14.4	**0.46**
2	Palmitoleic	$C_{17}H_{32}O_2$	*c*-Δ^9	268	16.26	16.22	16.28	**0.38**
3	11Z-Hexadecenoic	$C_{17}H_{32}O_2$	*c*-Δ^{11}	268	16.38	16.36	16.4	**0.14**
4	Palmitic	$C_{17}H_{34}O_2$	----------	270	16.48	16.42	16.54	**18.9**
5	6,9,12-Octadecatrienoic	$C_{19}H_{32}O_2$	*t,t,t*-$\Delta^6,\Delta^9,\Delta^{12}$	292	17.95	17.92	17.98	**2.43**
6	Linoleic	$C_{19}H_{34}O_2$	*c,c*-Δ^9,Δ^{12}	294	18.1	18.06	18.13	**14.59**
7	Elaidic	$C_{19}H_{36}O_2$	*t*-Δ^9	296	18.19	18.14	18.24	**46.42**
8	Stearic	$C_{19}H_{38}O_2$	----------	298	18.37	18.34	18.4	**4.55**
9	11-Eicosenoic	$C_{21}H_{40}O_2$	*t*-Δ^{11}	324	19.91	19.88	20.01	**5.02**
10	Erucic	$C_{23}H_{44}O_2$	*c*-Δ^{13}	352	21.6	21.54	21.75	**6.33**
11	Nervonic	$C_{25}H_{48}O_2$	*c*-Δ^{15}	380	23.85	23.79	23.99	**0.78**

***** : Δ = double bond, x = position of double bond, t = trans isomer, c = cis isomer

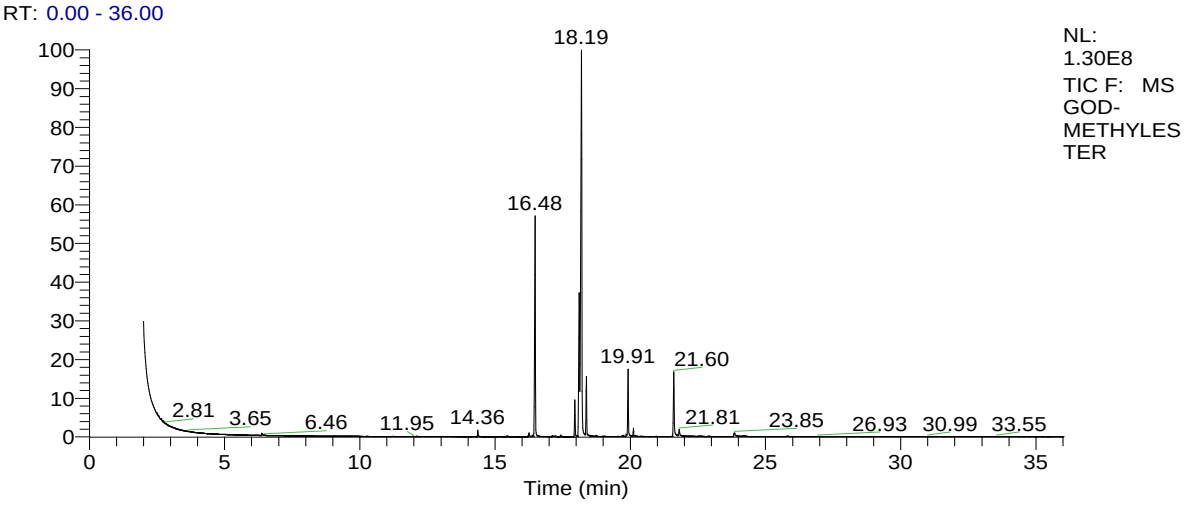


Figure 4: GC chromatogram of fatty acid methyl esters (FAMEs)

The fatty acid constituents from *A. azurea* seed oils in the present study varied from same plant in other places and countries. Four references are mentioned here for comparison and illustration of these differences as shown in (Table 2) (Guil-Guerrero et al., 2000 **(I)**; Guil-Guerrero et al., 2001 **(II)**; López et al., 2005 **(III)**; Nurgiin et al., 2013 **(VI)**). Accordingly, the percentages of some fatty acids in most references were close to each other, while some others far. Also it was found that some fatty acids were present only in one reference.

Table 2: A comparison between fatty acid percentages from present study with other four references

Fatty acids	**[% of total fatty acids] in *A. azurea***				
	Present study	**Ref. (I)**	**Ref. (II)**	**Ref. (III)**	**Ref. (IV)**
C8:0	nd	nd	nd	nd	0.6
C14:0	0.46	0.12	0.09	nd	0.1
C16:0	18.9	8.93	8.63	9.22	8.0
C16:1ω5	0.14	nd	nd	nd	nd
C16:1ω7	0.38	0.22	0.35	nd	0.1
C16:1ω9	nd	nd	nd	0.16	nd
C18:0	4.55	4.32	2.19	2.18	2.7
C18:1ω7	nd	nd	0.43	nd	nd
C18:1ω9	46.42	34.3	24.10	30.65	31.0
C18:2ω6	14.59	31.7	41.78	37.00	32.0
C18:3ω6	2.43	7.85	11.11	8.68	6.2
C18:3ω3	nd	0.43	0.43	0.21	0.3
C18:4ω3	nd	0.10	0.08	nd	nd
C20:0	nd	0.28	0.23	0.23	nd
C20:1ω9	5.02	3.57	3.57	4.47	0.3
C20:2ω6	nd	nd	0.18	nd	nd
C21:0	nd	nd	nd	nd	0.1
C22:0	nd	0.42	0.37	0.31	0.5
C22:1ω9	6.33	6.25	nd	6.37	10.3
C24:1ω9	0.78	0.81	nd	nd	1.2
Others	0.00	0.70	6.46	6.89	6.5
Saturated (SFA)	23.91	14.1	11.51	11.94	12.1
Monounsaturated	59.07	}	28.45	35.28	}
(MUFA)	17.02	} 85.2	53.58	45.89	} 81.4
Polyunsaturated (PUFA)					

4. Conclusion

In this investigation, the oil obtained from seeds of *Anchusa azurea* contains high percentage of many unsaturated fatty acids like; oleic, palmitic, palmitoleic, 11-eicosenoic, erucic and two ω-9 acids. Overall, the eleven fatty acids were detected and analyzed from the seeds of *A. azurea* using GC-MS analysis. Fatty acids that present in the seed oils of the studied plant were different from other countries. The high percentage of these unsaturated fatty acids led to the suggestion for further investigation about the ability to use it as healthy oil in food.

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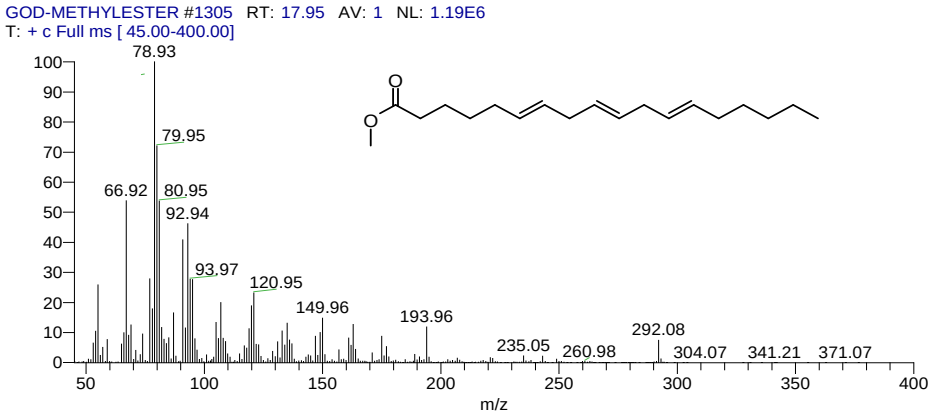

Figure 5: MS spectrum of 6,9,12-Octadecatrienoic acid methyl ester

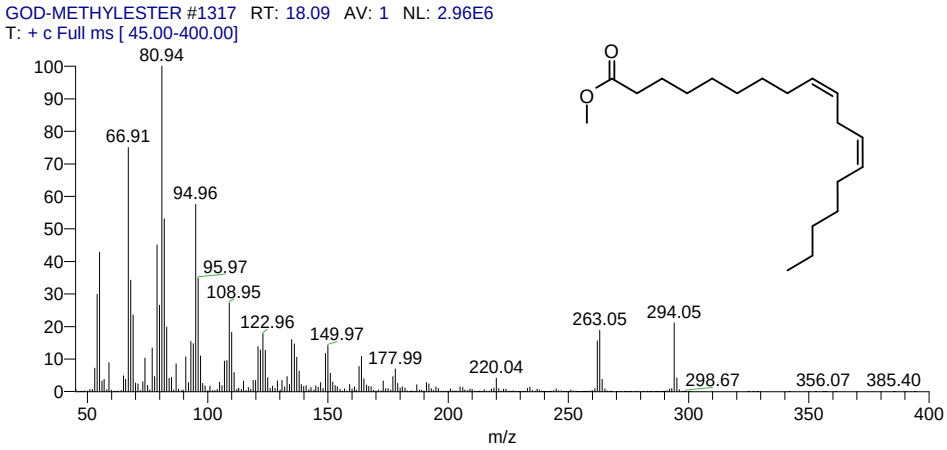

Figure 6: MS spectrum of Linoleic acid methyl ester

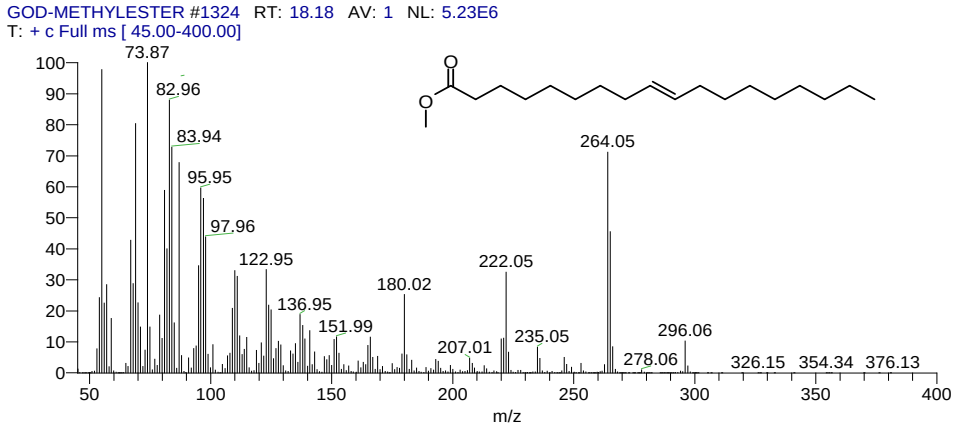

Figure 7: MS spectrum of Elaidic acid methyl ester

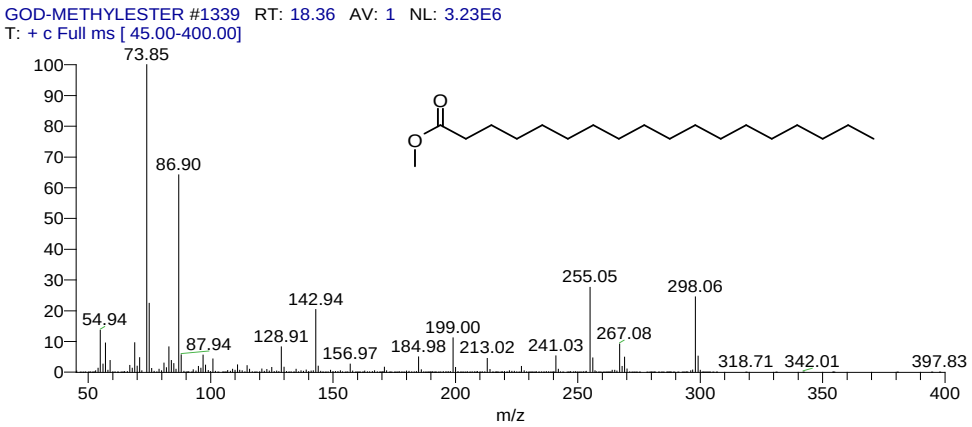

Figure 8: MS spectrum of Stearic acid methyl ester

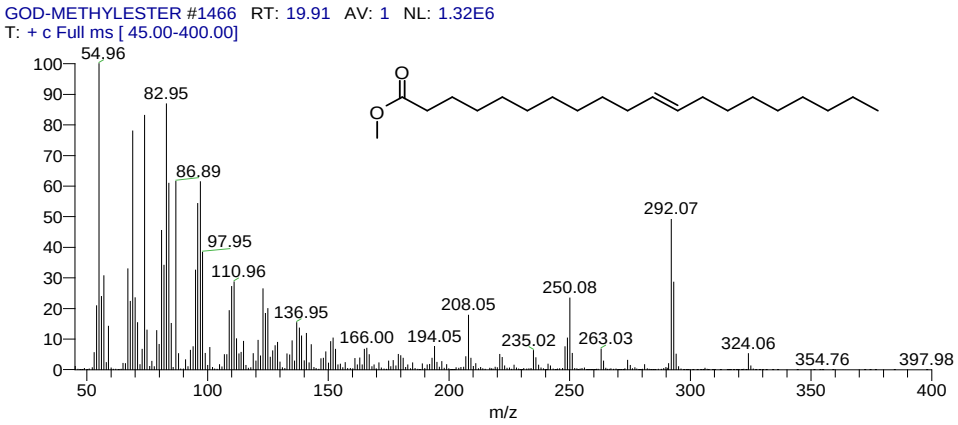

Figure 9: MS spectrum of 11-Eicosenoic acid methyl ester.

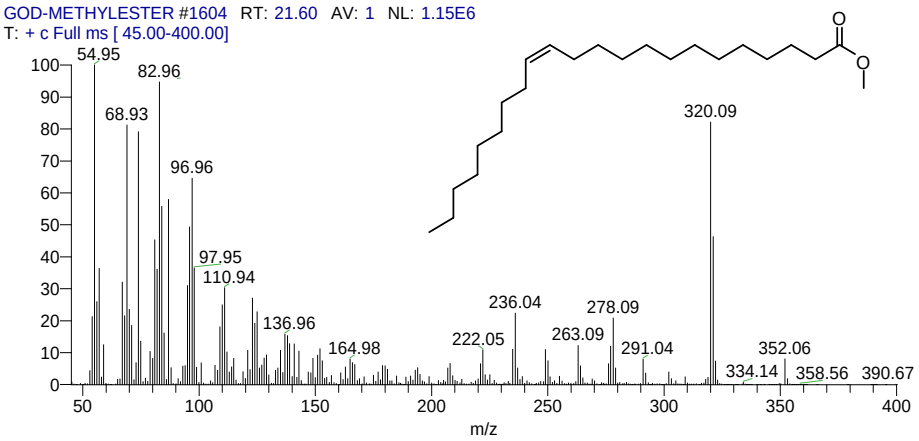

Figure 10: MS spectrum of Erucic acid methyl ester

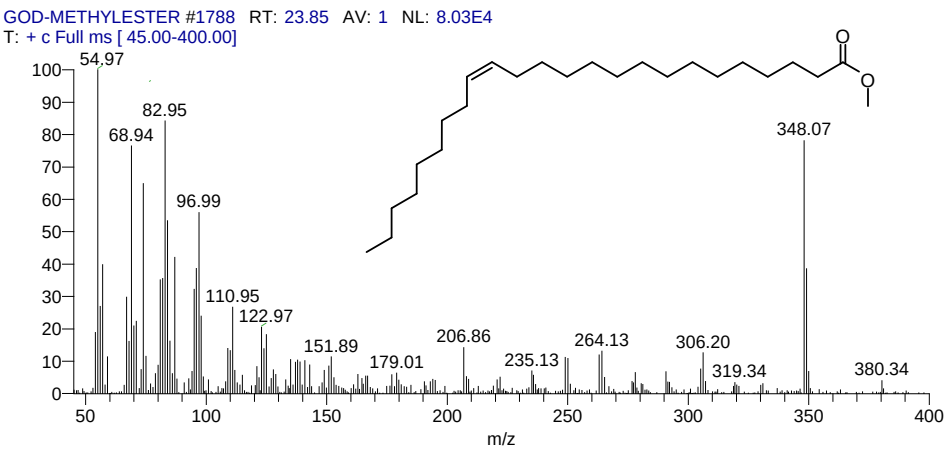

Figure 11: MS spectrum of Nervonic acid methyl