Development and Validation of a Reversed-Phase HPLC Method for Determination of Elaidic Acid in Oils and Fats

Fuad Al-Rimawi[1*], Jihad Abadi[2] and Ibraheem Afaneh[3]

[1]Chemistry Department, Faculty of Science and Technology, Al-Quds University, P.O.Box 20002, East Jerusalem, Palestine.
[2]Biology Department, Faculty of Science and Technology, Al-Quds University, P.O.Box 20002, East Jerusalem, Palestine.
[3]Department of Food Technology, Faculty of Science and Technology, Al-Quds University, P.O.Box 20002, East Jerusalem, Palestine.

Authors' contributions

This work was carried out in collaboration between all authors. Author FAR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors JA and IA managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

A simple, precise, accurate, and selective method with low limit of quantitation (LOQ) was developed and validated for analysis of elaidic acid which is the predominant trans fatty acids in partially hydrogenated vegetable oils. Separation was achieved on a reversed-phase C18 column, using mobile phase consisting of acetonitrile/water (80:20, v/v) containing 0.1% acetic acid, and using UV detection at 205 nm. This method was validated according to the requirements for new methods, which include accuracy, precision, selectivity, robustness, limit of detection (LOD), LOQ, linearity and range. The current method demonstrates good linearity over the range of 3-1000 mg L^{-1} of elaidic acid with r^2 greater than 0.999. The recovery of elaidic acid in oils and fats ranges

Corresponding author: E-mail: falrimawi@science.alquds.edu, fuad_12345@yahoo.com;

from 94.5 to 98.7%. The method is selective where elaidic acid is good separated from oleic acid and other components of fats and oils with good resolution. The method is also precise where the RSD of the peak areas of replicate injections of elaidic acid solution is less than 1%. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and rugged.

ABBREVIATIONS

LOD   : Limit of Detection
LOQ   : Limit of Quantitation
TFAS   : Trans Fatty Acids
PHVO   : Partially Hydrogenated Vegetable Oils
SD   : Standard Deviation
RSD   : Relative Standard Deviation
GC   : Gas Chromatography
HPLC   : High Performance Liquid Chromato-
               graphy
IR   : Infrared Spectrometry

1. INTRODUCTION

Trans Fatty Acids (TFAS) are unsaturated fatty acids with at least one double bond in the trans configuration. Trans fatty acids are found in two major sources, natural and industrial sources. Trans fatty acids from industrial sources are mainly generated from vegetable oil polyunsaturated fatty acids, either during partial hydrogenation or during refining processes. With partially hydrogenated vegetable oils (PHVO), cis and trans isomers of oleic acid are the main components with double bond positions located from $\Delta 5$ to $\Delta 16$. Elaidic acid which is the trans isomer of oleic acid (cis) is considered as the main trans fatty acids in these commercially partially hydrogenated fatty acids [1]. Cis oleic acid is represented as 18:1 $\Delta 9c$ while elaidic acid as 18:1 $\Delta 9t$. Additionally, partially hydrogenated vegetable oils contain various positional and geometric isomers of linoleic acid with trans or non-methylene interrupted double bonds. Levels up to 7% of TFAs have been found in some margarines. The widespread use of partially hydrogenated vegetable oils, mainly as a substitute for saturated fats has raised questions concerning the health consequences of intake of TFA's. Reports indicate that TFAs are hypercholestrol as saturated fatty acids affect adversely the LDL/HDL cholesterol ratio. Additionally TFAs as compared to oleic acid and linoleic acid increase serum levels of lipoprotein (a) where high lipoprotein (a) is a greater risk factor than is high serum cholesterol for coronary heart disease [2]. In Canada and in some European countries, the voluntary nutritional labeling regulations of food requires that monounsaturated only of the cis configuration be declared on the label. According to Canadian regulations, polyunsaturated fatty acids are restricted to cis, cis methylene-interrupted structures. These labeling regulations necessitates the determination of total trans fatty acids content as well as accurate determination of cis and trans-monounsaturated fatty acids and the fatty acid composition of food fats [1-2]. As elaidic acid is the main trans fatty acid present in PHVO, the current work focuses on development and validation of a simple LC method for determination of elaidic acid in fats (PHVO) and oils.

Many methods were used for analysis of trans fatty acids in fats and oils, and including GC [3-4], IR [5-6], and silver ion chromatography [7-8]. HPLC was also used for analysis of trans fatty acids in fats and oils [9-10]. However, a simple reversed phase HPLC with UV detector was not used before for determination of elaidic acid. Therefore, the objective of the current work is to develop and validate a simple reversed-phase mode with isocratic elution and UV detection for determination of elaidic acid in oils and fats.

2. EXPERIMENTAL

2.1 Materials

2.1.1 Chemicals

Acetonitrile HPLC grade is from J.T Baker (NJ, USA). Acetic acid, and n-hexane are from Merck (Darmstadt, Germany). Oil (olive) and fat (margarines) samples were purchased from local market in Palestine.

2.1.2 Apparatus

HPLC system (Merck Hitachi LachromeElite HPLC system, Japan) with an L-2130pump, an L-2200 autosampler, L-2300 column oven, and L-2490 UV detector was employed. The Ezochrom Elite software was employed. The C18 column (150 × 4.6 mm I.D., 5 µm) is from Waters Corporation (Milford, Massachusetts, USA).

2.2 Methods

2.2.1 HPLC conditions

C18 column (150 × 4.6 mm I.D., 5 μm) was used for chromatographic separation, UV detection was employed at 205 nm, isocratic elution was used at a flow rate of 2.0 ml/min, and injection volume was set to 50 μl.

2.2.2 Preparation of the mobile phase and standard solutions

The mobile phase was prepared by mixing 800 ml of acetonitrile with 200 ml of water for HPLC, and adding 1 ml of acetic acid.

Stock standard solution of elaidic acid with a concentration of 1000 mg L^{-1} was prepared by dissolving 100 mg of elaidic acid in 100 mL of n-hexane. Six solutions of elaidic acid with concentrations: 3, 5, 100, 300, 500, and 800 mg L^{-1} were prepared from the stock standard solution by dilution using n-hexane as diluent. These solutions were used for linearity and range study of the method. For recovery of elaidic acid in olive oil, three solutions of elaidic acid spiked in olive oil at three concentrations (0.1%, 1.0%, and 5.0%) were prepared. 0.1% of elaidic acid in olive oil was prepared by spiking 10 mg of elaidic acid in 10 g of olive oil and dissolving in n-hexane, and dilution to 100 ml with n-hexane. The resulting solution is 0.1% elaidic acid in olive oil, and with a concentration of 100 mg L^{-1} elaidic acid in n-hexane. 1.0% of elaidic acid in olive oil was prepared by spiking 10 mg of elaidic acid in 1.0 g of olive oil and dissolving in n-hexane, and dilution to 100 ml with n-hexane. The resulting solution is 1.0% elaidic acid in olive oil, and with a concentration of 100 mg L^{-1} elaidic acid in n-hexane. 5.0% of elaidic acid in olive oil was prepared by spiking 10 mg of elaidic acid in 0.2 g of olive oil and dissolving in n-hexane, and dilution to 100 ml with n-hexane. The resulting solution is 5.0% elaidic acid in olive oil, and with a concentration of 100 mg L^{-1} elaidic acid in n-hexane. For recovery of elaidic acid in margarine, the same procedure was conducted using margarine instead of olive oil.

3. RESULTS

3.1 Method Development

Preliminary studies involved trying C8 and C18 reversed-phase columns and testing several mobile phase compositions were conducted for the separation of elaidic acid (trans oleic acid) from oleic acid (cis) with good chromatographic parameters (e.g. minimized peak tailing, good symmetry, good resolution between oleic acid and elaidic acid). A C18 column (5 μm, 150 × 4.6mm I.D.) as a stationary phase with a mobile phase of acetonitrile/water (80:20, v/v) containing 0.1% of acetic acid at a flow rate of 2.0 mL/min and a detection wavelength of 205 nm afforded the best separation of oleic acid and elaidic acid. Acetic acid in the mobile phase gave sharper peaks for both oleic and elaidic acid (high efficiency), while the mobile phase without acetic acid gave very broad peaks (low theoretical plates) with very poor resolution between oleic acid and elaidic acid. Fig. 1 shows a chromatogram of a standard solution of oleic acid and elaidic acid with retention times of 11.2 and 12.6 minutes for oleic acid and elaidic acid, respectively.

3.2 Method Validation

After method development, validation of the method for elaidic acid was performed in accordance with requirements for new methods which include accuracy, precision, selectivity, robustness, linearity and range, LOD, and LOQ [11-12].

3.2.1 Linearity and range

To evaluate linearity of the current method, seven calibration standards of elaidic acid with concentrations 3, 5, 100, 300, 500, 800, and 1000 mg L^{-1} were analyzed by the method developed in this study, and the peak areas of elaidic acid were recorded. A plot of peak areas versus elaidic acid concentration was linear in the range of 3-1000 mg L^{-1} with a correlation coefficient of 0.9999. This result demonstrates linearity of this method with wide concentration range.

3.2.2 Accuracy (percentage recovery)

The percentage recovery of elaidic acid was determined in both oil (olive oil) and fat (margarine).

3.2.2.1 Recovery from olive oil

For determination of the percentage recovery of elaidic acid in olive oil, elaidic acid was spiked in olive oil at three concentrations, and analyzed by HPLC. The % recovery for each level was calculated by proportion of the area of the peak of elaidic acid resulted from the spiked solution to

the area of the peak of elaidic acid resulted from a standard solution. Results showed that the % recovery of elaidic acid from oil at the three concentration levels (0.1%, 1.0%, and 5.0%) ranges from 96.3% to 98.7%, and with a relative standard deviation lower than 1.0%, see Table 1. Figs. 2-3 show chromatograms for elaidic acid spiked in olive oil at 0.1 and 1.0% concentrations, respectively.

3.2.2.2 Recovery from margarine

The same procedure was followed as in section 3.2.2.1 but using margarine instead of olive oil,

and results are shown in Table 2. As it is seen in Table 2, the % recovery of elaidic acid in margarine using this method is from 94.5% to 97.6%.

3.2.3 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the RSD for a statistically significant number of samples. There are two types of precision: repeatability and intermediate precision (ruggedness).
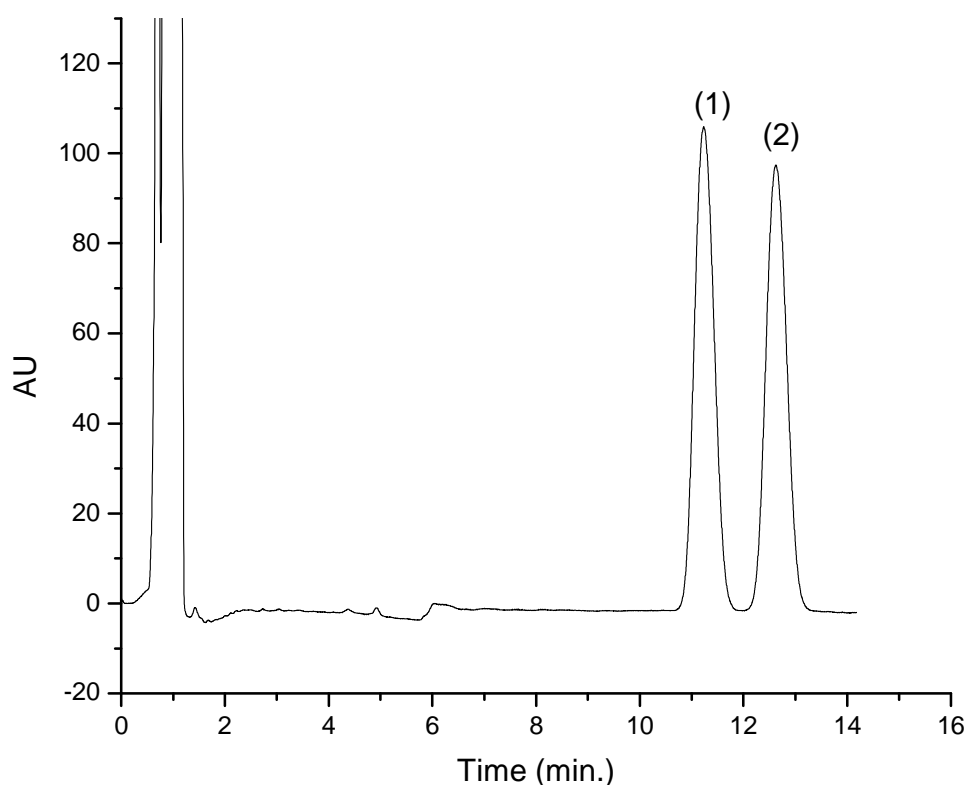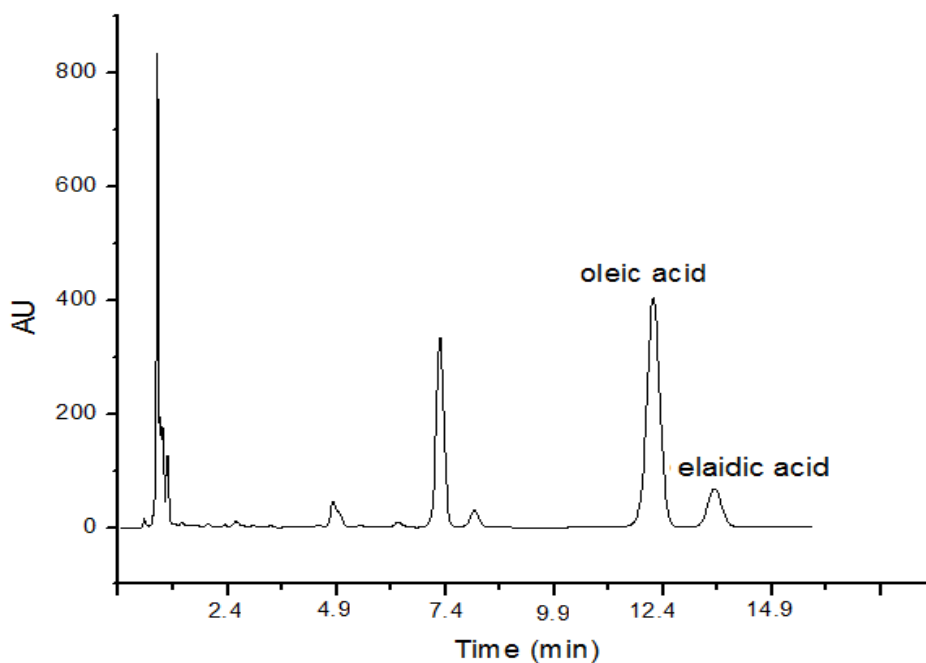

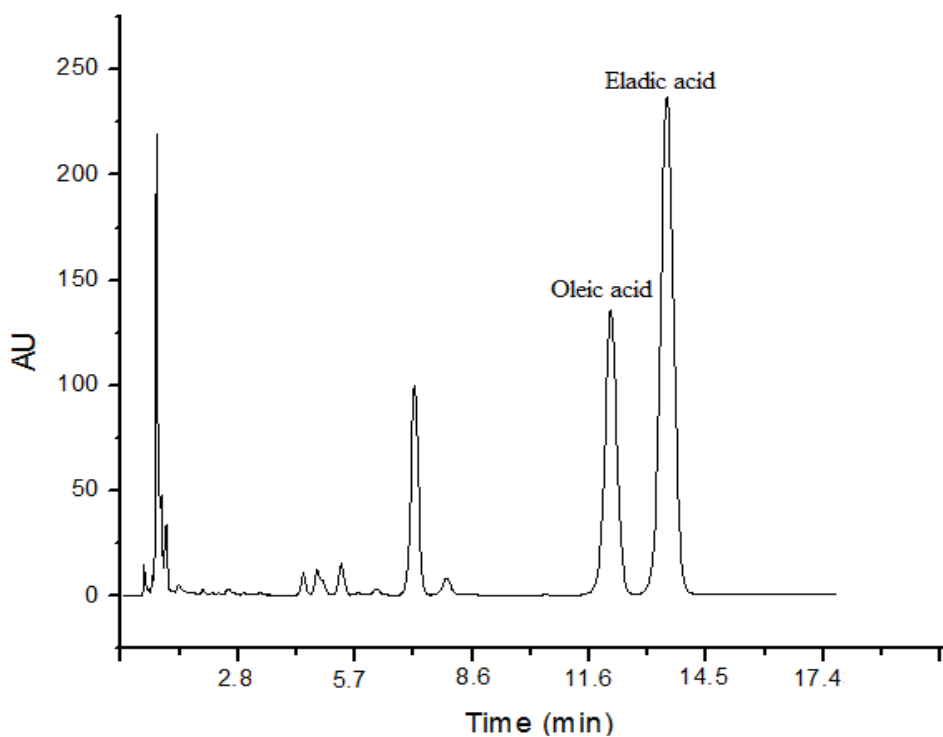**Fig. 1. Chromatogram of oleic acid (1) and elaidic acid (2) with a concentration of 100 mg L^{-1} of each**
Mobile phase: Acetonitrile: water (80:20, V/V) containing 0.1% Acetic acid. Flow rate 2.0 mLmin–1, injection volume 50 µL. Column: C18, 5 µm, 15cm length, 4.6 mm inner diameter, UV detection: 205 nm. Retention time of oleic acid and elaidic acid are 11.2 and 12.6 minutes, respectively

Table 1. % Recovery of elaidic acid in olive oil at three concentration levels

Concentration level (%)	% Recovery			Mean±SD	RSD (%)
	Sample 1	Sample 2	Sample 3		
0.1	96.3	97.2	97.5	97.0±0.62	0.64
1.0	97.5	97.9	98.1	97.8±0.31	0.31
5.0	97.9	98.7	98.1	98.2±0.42	0.42

Fig. 2. Chromatogram of elaidic acid spiked in olive oil (for recovery study), concentration level 0.1% (0.1 g of elaidic acid in 100 g of olive oil). For HPLC conditions, see Fig. 1
* The oleic acid peak as well as the other peaks appeared are from the olive oil

Fig. 3. Chromatogram of elaidic acid spiked in olive oil (for recovery study), concentration level 1% (1.0 g of elaidic acid in 100 g of olive oil). For HPLC conditions, see Fig. 1
* The oleic acid peak as well as the other peaks are from olive oil

3.2.3.1 Repeatability

Repeatability of the current method was evaluated by calculating the RSD of the peak areas of six replicate injections for the standard concentration (100mg L^{-1}) of elaidic acid, which was found to be 0.51%. Furthermore, the RSD of the percentage recovery of elaidic acid for three samples (see tables 1 and 2) at each concentration level was calculated, and it was found to be less than 1.0%. These results show that the current method for elaidic acid analysis is repeatable.

3.2.3.2 Intermediate precision (Ruggedness)

Intermediate precision (also called ruggedness) of a method measures the repeatability of the result obtained with the same method, on the same sample, in the same laboratory, but by different operators and in different day. Intermediate precision of the current method was evaluated by analyzing a solution which contains oleic acid and elaidic acid at 100mg L^{-1} concentration by another analyst in the same laboratory but using different HPLC systems and in a different day. Results of this study showed that elaidic acid is good separated from oleic acid with a good resolution by the second analysts, and the retention times of oleic acid and elaidic acid are repeatable (differs only slightly).

3.2.4 Selectivity

Selectivity of the current method was demonstrated by good separation (with good resolution) of elaidic acid from oleic acid as well as other peaks which present in oils (e.g. olive oil) and fats (e.g. margarine), see Figs. 1-4, and Table 3.

3.2.5 Robustness

Robustness measures how a method stands up to slight variations in the operating parameters of the method like flow rate, wavelength, percentage of mobile phase composition. Robustness of the current method was investigated by analyzing the standard solution of oleic acid and elaidic acid using the same method developed in this study but using (a) flow rate of 1.9 and 2.1 mL/min instead of 2.0 mL/min and (b) volume fraction of acetonitrile 82% and 78% instead of 80%. It was shown that the resolution between oleic acid and elaidic acid, theoretical plates, and asymmetry of oleic acid and elaidic acid peaks were not affected significantly as flow rate and volume fraction of acetonitrile was slightly and deliberately changed, see Table 4.

3.2.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ of elaidic acid using this method were determined by preparing dilute solutions of elaidic acid (1, 2, 3, 4, and 5 mg L-1) and injecting these solutions into the liquid chromatograph and recording the signal to noise ratio (S/N) for the elaidic acid peak at each concentration. LOD was selected to be the concentration of elaidic acid that gives a S/N ratio between 3 and 10, while LOQ was selected to be the concentration that gives a S/N ratio between 10 and 20. Results have shown that LOD and LOQ of elaidic acid are 1 and 3, respectively. This low LOD and LOQ permit the determination of elaidic acid in oils and fats at low concentration.

3.3 Analysis of Elaidic Acid in Real Fat and Oil Samples

After successful development and validation of this method, it was employed for analysis of elaidic acid in different types of oils and fats (e.g. margarine and shortening), see Fig. 4. Analysis of elaidic acid in a sample of margarine obtained from Palestinian market showed that elaidic acid present in this sample at 13.2% relative to oleic acid calculated by area normalization method (% of elaidic acid is 13.2% and % of oleic acid is 86.8%, see Fig. 4).

4. DISCUSSION

There are many methods for analysis of trans fatty acids in fats and oils. The two common quantitative methods for total TFAs analysis are based on GC and IR.

Table 2. % Recovery of elaidic acid in margarine at three concentration levels

Concentration level (%)	% Recovery			Mean±SD	RSD (%)
	Sample 1	Sample 2	Sample 3		
0.1	95.3	94.5	96.0	95.3±0.75	0.79
1.0	97.2	97.0	96.8	97.0±0.20	0.21
5.0	97.0	97.6	97.2	97.3±0.31	0.31

Table 3. Chromatographic parameters of oleic acid and elaidic acid peaks separated in a standard solution (chromatogram is shown in Fig. 1)

Compound	Resolution	Asymmetry	Theoretical plates	Relative retention time
Oleic acid	3.6	1.12	2100	0.90
Elaidic acid		1.10	3400	1.0

Table 4. Robustness testing of the method for separation of oleic acid and elaidic acid

Parameter	Chromatographic parameters			
Flow rate (mL/min)	Resolution	Asymmetry		Theoretical plates	
		Oleic acid	Elaidic acid	Oleic acid	Elaidic acid
1.9	3.4	1.11	1.10	2200	3600
2.0	3.6	1.15	1.11	2100	3400
2.1	3.5	1.12	1.13	2500	3400
Acetonitrile (%)					
78	3.7	1.11	1.14	2000	3300
80	3.6	1.13	1.11	2100	3400
82	3.6	1.15	1.12	2700	3700

During GC analysis, fatty acid in samples need to be converted into its corresponding volatile methyl esters prior separation in a very long capillary column (100 m) which is coated with highly polar stationary phase [3-4]. The disadvantages of GC method are that it takes long analysis time, and the peaks of sample are usually overlapped. Another drawback of GC is the lack of chemical standards of all TFA isomers. Therefore, GC method is not the reference method for routine determination of TFAs for labeling purposes, although it was used in the scientific literature for analysis of TFAs in fats and oils [3-4]. The second major method for TFAs analysis is IR. This method which is a rapid method is based on the C-H out of plane deformation band observed at wavenumber 967 cm^{-1} which is a characteristic of isolated trans double bonds, regardless of the chain length or the position of the isolated trans double bond. The disadvantage of IR method is that only total TFAs can be determined by IR i.e. it is not specific for TFA components. Additionally, IR method is not accurate below 5% TFA and may be subject to interferences.
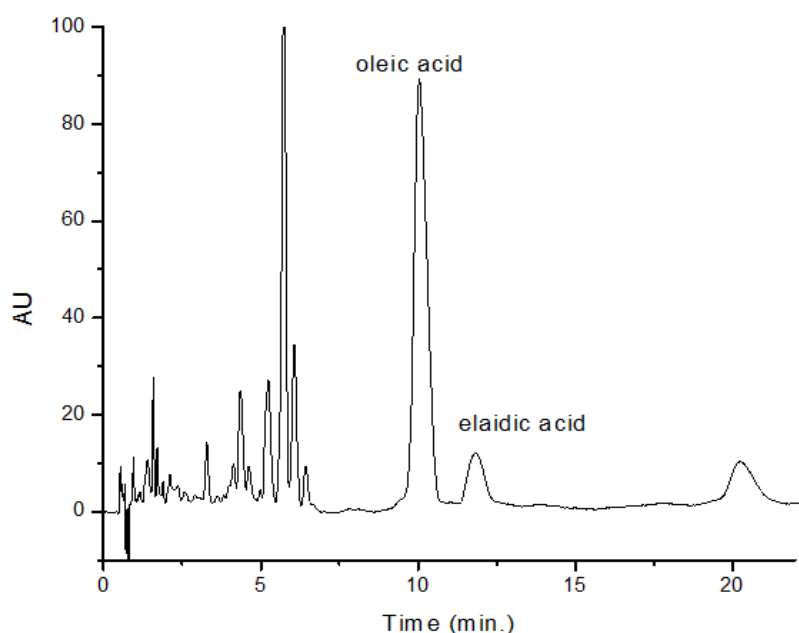
Fig. 4. Chromatogram of commercial margarine from Palestinian market (1.0 g of margarine in 10 mL of n-hexane). For HPLC conditions, see Fig. 1

In addition to GC and IR, silver ion chromatography is used for analysis of TFAs. HPLC technique in which silver ions are bound to an ion-exchange support afford better resolution, as an interaction between the silver ions and the bonds of TFA is observed and any adsorption effects are minimal. Silver ion chromatography is capable of better resolution of corresponding trans and cis forms of a given positional isomer of a monounsaturated fatty acid. This method was used for analysis of TFA in fats and oils [7-8]. The disadvantage of this method is that it requires a special column where silver is impeded in the stationary phase, and this column is very expensive.

The method used in the current study is simple where reversed-phase mode is used with isocratic elution, and using UV detector which is available in most of analytical labs compared to other detectors like fluoresence and mass spectrometry which are not available in some labs. Additionally, derivatization of elaidic acid and oleic acid is not needed (i.e. detection is achieved for the free fatty acids) compared to GC and some HPLC methods which require derivatization of the fatty acids. Moreover, this method does not require a special column like silver-HPLC method. Furthermore, this reversed-phase HPLC method is more selective than IR method. Additionally, validation of the method is conducted in accordance with requirements of new methods which include linearity and range, accuracy, precision, selectivity, robustness, limit of detection, and limit of quantitation.

5. CONCLUSION

A simple, accurate, precise, and selective HPLC method was developed and validated for determination of elaidic acid in different types of oils and fats. The method is linear with a wide dynamic range. The method show good separation of elaidic acid from oleic acid with a good resolution. Low LOD and LOQ of elaidic acid enable the detection and quantitation of elaidic acid in oils and fats at low concentrations. The method of analysis is simple, the analysis time is short, elution is isocratic, and the detector is UV which is available in most of separation labs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Albuquerque TG, Costa HS, Castilho MC, Sanches-Silva A. Trends in the analytical methods for the determination of trans fatty acids content in foods. Trends Food Sci Technol. 2011;22(10):543-60.
2. Colon-Ramos U, Baylin A, Campos H. The relation between trans fatty acid levels and increased risk of myocardial infraction does not hold at lower levels of trans fatty acids in the costa Rican food supply. J. Nutr. 2006;136:2887-92.
3. Kravic S, Suturovic Z, Švarc-Gajic J, Stojanovic Z, Pucarevic M. Determination of trans fatty acids in foodstuffs by gas chromatography–mass spectrometry after simultaneous microwave-assisted extraction-esterification. J. Serb. Chem. Soc. 2010;75:803–12.
4. Ratnayake WMN. Overview of methods for the determination of trans fatty acids by gas chromatography, silver-ion thin-layer chromatography, silver-ion liquid chromategraphy, and gas chromatography/mass spectrometry. J AOAC Int. 2004;87:523-39.
5. Narkwichian N, Tongyong L, Pararapanich Ch. Analysis of trans fatty acid in some foods by attenuated total reflection-fourier transform infrared spectroscopy. J. Health Res. 2009;23:185-90.
6. Mossoba MM, Kramer JKG, Delmonte P, Yurawecz MP, Rader JI. Official methods for the determination of trans fat. AOCS Press. Champaign, IL (USA); 2003.
7. Rodrı´guez-Castan˜edas JL, Pen˜a-Egido MJ, Garcı´a-Marino M, Garcı´a-Moreno C. Quantitative determination of conjugated linoleic acid isomers by silver ion HPLC in ewe milk fat. J Food Compos Anal. 2011; 24:1004–8.
8. Nikolova-Damyanova B, Momchilova S, Christie WW. Silver ion high-performance liquid chromatographic separation of conjugated linoleic acid isomers, and other fatty acids, after conversion to p-methoxy-phenacyl derivatives. J. High Resol Chromatogr. 2000;23:348-52.
9. Miwa H. High-performance liquid chromategraphic determination of free fatty acids and esterified fatty acids in biological materials as their 2-nitrophenylhydrazides. Anal. Chim. Acta. 2002;465:237–55.
10. Sajiki J, Yonekubo J. Determination of free polyunsaturated fatty acids and their oxidative metabolites by high-performance

liquid chromatography (HPLC) and mass spectrometry (MS). Anal. Chim. Acta. 2002; 465:417–26.

11. International Conference on Harmonization (ICH), "Validation of Analytical Procedures-PA/PH/OMCL (05) 47 DEF", elaborated by OMCL Network/EDQM of the Council of Europe, June; 2005.

12. Wegscheider. Validation of analytical methods. In: Accreditation and quality assurance in analytical chemistry, edited by H. Guenzler, Springer Verlag, Berlin; 1996.

_____

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/12076