RESEARCH ARTICLE

Estimation of Linoleic Acid in Flax Seed Oil Formulation using a Validated High-performance Thin-layer Chromatograph Method

Lata Kothapalli*, Rahul Tupe, Asha Thomas, Vaidehi Raut

Department of Pharmaceutical Chemistry, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, Maharashtra, India.

Received: 10th October, 2022; Revised: 07th November, 2022; Accepted: 29th November, 2022; Available Online: 25th December, 2022

ABSTRACT

Linoleic acid, an omega-6 fatty acid present widely in plant seeds and one of the major components among the fatty acids in *Linum Usitatissimum L*., commonly called as flax seed and represents the content of 12–19% out of the total lipids in flax seed. This work attempted to quantify the Linoleic acid from flax seed oil using the high-performance thin layer chromatography (HPTLC) method with mobile phase, methanol: toluene: formic acid (3:7:1 v/v), and the wavelength used is 254 nm. The method gave a sharp, symmetrical peak at Rf value 0.63 ± 0.02. The method was validated using intracerebral brain hemorrhage (ICH) guidelines and applied to the assay of marketed capsules of flaxseed. It is confirmed that the reported method can help analyze flax seed oil in different dosage forms, along with cosmetic products containing linoleic acid-rich oils.

Keywords: Flax seed oil, High-performance thin layer chromatography, Linoleic acid, Validation

International Journal of Pharmaceutical Quality Assurance (2022); DOI: 10.25258/ijpqa.13.4.13

How to cite this article: Kothapalli L, Tupe R, Thomas A, Raut V. Estimation of Linoleic Acid in Flax Seed Oil Formulation using a Validated High-performance Thin-layer Chromatograph Method. International Journal of Pharmaceutical Quality Assurance. 2022;13(4):421-425.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION-

Flax seed is known for its oil content (40%) equivalent to fish oil; since 5000 BC, flax seed is known for its nutritional value as a food ingredient and medicinal utility[1] Flax seed also named *Linum usitatissimum* L.) has brown and yellow or golden varieties.[2,3] The Flax seed contains ω-3 fatty acids, linolenic acid (ALA) and ω-6 fatty acid, linoleic acid, fibers, lignans, vitamins, minerals, and proteins.[4-6] $C_{18:3}$ α-linolenic acid (ALA) is a building block for anti-inflammatory mediators of the cell.[7] The oil also has saturated fatty acids (9-12%) and polyunsaturated fatty acids (82-91 %).[8] Flax seed oil with a dosage of 25 g/day can reduce serum lipids in humans.[9] The oil also contains antioxidants like tocopherols and β-carotene which help augment the oxidative stability of oil.[10]

Presently analysis of flaxseed for the presence of various constituents reported in the literature includes the work done by Lan *et al*. and Pali *et al.* for the determination of fatty acids and proteins.[11,12] High-performance liquid chromatography (HPLC) method for polyphenols and lignan,[13,14] HPLC with gas chromatography helped the analysis of fatty acids after hydrolyzing the triglyceride,[15] and chromatographic separation linked with bioactivity using high-performance thin-layer chromatograph methods (HPTLC)[16,17] are reported for the flaxseed and linoleic acid (LA) in another plant source. Andronie *et al.*[18] FSO reports characterization of flax seed oil (FSO) by spectrometric method with excess oleic acid and LA indicates possible adulteration with hydrogenated seed oils as reviewed by Herchi *et al.*[19] Hence, the objective of the present work was to develop a precise and accurate HPTLC method for the estimation of LA in flax seed oil formulation.

MATERIALS AND METHODS

The standard LA was purchased from HiMedia, and the FSO formulation was purchased from the local market (INLIFE® Flax seed oil supplements of INlife Health Care, Hyderabad). All reagents of analytical grade and HPLC grade solvents were used. Chromatographic separation was achieved using an HPTLC system (CAmag) equipped with Camag TLC Scanner -3, Win CAT software version 1.4.3.6336.

EXPERIMENTATION

Physicochemical Parameters

All physicochemical parameters were evaluated as per procedures mentioned in Indian Pharmacopoeia.[20]

HPTLC Method

Preparation of Standard and Sample Solution

Standard solutions were prepared by accurately taking about 100 mg of LA in a 100 mL volumetric flask and dissolved in methanol, and the volume was made up to 100 mL (1000 ug/mL) with methanol.

*Author for Correspondence: latapk19@gmail.com

The sample solution was prepared by taking 20 capsules of INLIFE® Flax seed oil supplements which were weighed; oil was collected in a flask and hydrolyzed with 50 mL of 0.5M potassium hydroxide under reflux for one hour. The mixture was extracted with 50 mL n-hexane twice to extract out the unhydrolyzed part of the oil, and the aqueous layer was treated with 1M HCl to neutralize the fatty acid salts. The free fatty acids were then extracted again using 50 mL n-hexane twice. The organic layer was removed using a rotary evaporator at 40ºC and then recovered in 100 mL methanol to get approximately 100 mg oil/ml of stock solution. Further, suitable dilutions were done using methanol for further analysis. (10 mg/ml oil) and CAMAG LINOMAT-V automatic sample applicator was used for the application of standard and sample stock solution on the thin-layer chromatograph methods (TLC) plate for further analysis.

Optimization of Mobile Phase

An important component of method development is the mobile phase, given in different proportions. To start with the optimization of a method, a solvent system is required for good extraction efficiency, which will give a compact spot and no interference for the determination of actives in the formulation. Several trials using a combination of solvent systems in different proportions, like toluene: ethanol; toluene: ethanol: ammonia; toluene: methanol: glacial acetic acid; toluene: methanol: triethylamine; n-butanol: methanol, was done to get the analyte peak as a sharp band with accepted Rf value (0.2–0.8). Methanol: toluene: formic acid (3:7:1 v/v) was then selected as the mobile phase for further analysis.

Selection of Analytical Wavelength for Densitometry Evaluation

The optimized mobile phase was placed in a twin-through glass chamber and kept for saturation for 10 minutes, and then the plate was placed for the partitioning of the drug in the mobile phase for a run of 8 cm length. The plates were then removed from the chamber and air-dried. The plate was scanned in the UV-visible range of 200–700 nm. LA exhibited strong absorbance at 254.0 nm.

Optimization of Chromatographic Conditions

Suitable sample dilutions were applied as 6 mm bands on a precoated silica gel plate 60 F_{254}. The slit dimension was kept at 5 × 0.45 mm. The sample application volume was 4.0 µL. Plates were developed under similar conditions as mentioned for analytical wavelength determination under uniform

Table 1: Characterization of flaxseed oil

S. no.	*Physicochemical parameters*	*Flax seed oil*	
		Literature value[23,24]	*Observed value (Mean* ± S.D)*
1.	Acid value (mg KOH/g)	0.80–1.5	0.34 ± 0.092
2.	Saponification value (mg/g)	190–210	172.35 ± 0.881
3.	Iodine value (mg/100g)	177–180	113.78 ± 0.293
4.	Refractive index	1.46–1.48	1.49 ± 0.856

*Mean of three readings.

Table 2: Results of assay of flax seed oil in capsule formulation

S. no	*Flaxseed oil (ng/band)*	*Peak area*	*Amount of LA estimated (ng)*	*%of linoleic acid*
1	5002	5564.17	884.11	17.68
2	5004	5578.16	886.05	17.72
3	5003	5580.20	886.33	17.72
4	5001	5678.17	899.96	17.99
5	5004	5519.00	877.82	17.55
6	5003	5517.07	877.56	17.55
Mean ± S.D		5572.795 ± 53.45	885.35 ± 7.45	17.70 ± 0.136

Table 3: Recovery and robustness studies for analysis of flax seed oil

S. no.	*Recovery studies*		*Robustness studies*		
	Level of recovery %	*Mean percent recovery* ± S. D*	*Parameter*	*Rf (mL)*	*Mean area* ± S.D*
1	80	99.49 ± 0.46	The volume of the Mobile phase (10 ± 1)	0.64 ± 0.32	5283.19 ± 6.7539
2	100	99.76 ± 1.72	Absorbance maxima (254 ± 2nm)	0.65 ± 0.008	5352.71 ± 45.3206
3	120	99.33 ± 1.09	Saturation Time (10 min)	0.63 ± 0.012	5350.273 ± 36.3769

*Mean of three readings

Table 4: System and method precision for linoleic acid in flax seed oil

S. no	System precision				Method Precision		
	LA (ng/band)	AUC	Amount of LA estimated (ng)	%of LA	AUC	Amount of LA estimated (ng)	% of LA
1	600	3564.17	606.0234	101.0039	3548.8	603.8863	100.60
2	600	3578.16	607.9686	101.3281	3655.6	618.7361	103.1227
3	600	3580.20	608.2522	101.3754	3522.2	600.1877	100.0313
4	600	3678.17	621.8743	103.6457	3508.6	598.2967	99.7161
5	600	3519.00	599.7428	99.95713	3681.2	622.2956	103.7159
6	600	3517.07	599.4744	99.9124	3566.3	606.3195	101.0533
Mean ± S.D*		*3572.795 ± 53.636*	*607.2226 ± 7.4577*	*101.2038 ± 1.2429*	*3580.45 ± 65.2629*	*608.287 ± 9.0743*	*101.2038 ± 1.51*

*Mean of six readings

conditions with a temperature of 25 ± 2°C and %RH: 60 ± 5. Developed plates were scanned at 254 nm, and peak area was recorded for each standard concentration to plot calibration curves of concentration vs. peak area for LA. The mobile phase consisted of methanol: toluene: formic acid (3:7:1, v/v). Varying compositions of the mobile phase were used for optimization, and a good resolution was achieved using methanol: toluene: formic acid with an Rf value of LA as 0.63 ± 0.02.

Assay

From the sample stock solution, a 5 µL sample was applied (n=6) to the TLC plate and an optimized chromatographic condition was used for separation, the percentage content of LA was calculated from the calibration curve.

METHOD VALIDATION[21,22]

Linearity

Linearity was determined for LA by plotting the calibration curve of the concentration of LA against the peak area for the response obtained in the concentration ranging from 200-1200 ng/band, and the regression coefficient (r^2) was calculated with the slope and constant.

Accuracy

The accuracy of the method was evaluated by adding standard solution at 80, 100, and 120% of the test concentration, and the dilutions were further analyzed using optimized chromatographic conditions.

Robustness Studies

The variation in the volume of the mobile phase (10 ± 1-mL), absorbance maxima with wavelength 254 ± 2 nm, and chamber saturation time of 10 ± 5 minutes from development to scanning was done to understand the robustness of the proposed method. The effect of these changes was analyzed for Rf values of LA and the peak area of the analyte.

Precision

The method developed was used to understand precision, wherein the analysis of the test solutions after every two hours on the same day (intraday precision) and over a period of 24 hours (interday precision) was repeated using optimized analytical conditions.

Logarithm of the Odds and Limit of Quantitation

The limits of detection (LoD) and limits of quantification (LoQ) are calculated based on the standard deviation of the response of the calibration curve and the slope of the calibration curves. The LoD and LoQ were determined using the equations (1) and (2):

$$LoD = 3.3\sigma/S \quad (1); \quad LoQ = 10\sigma/S \quad (2)$$

RESULT AND DISCUSSION

Characterization of Flax Seed Oil

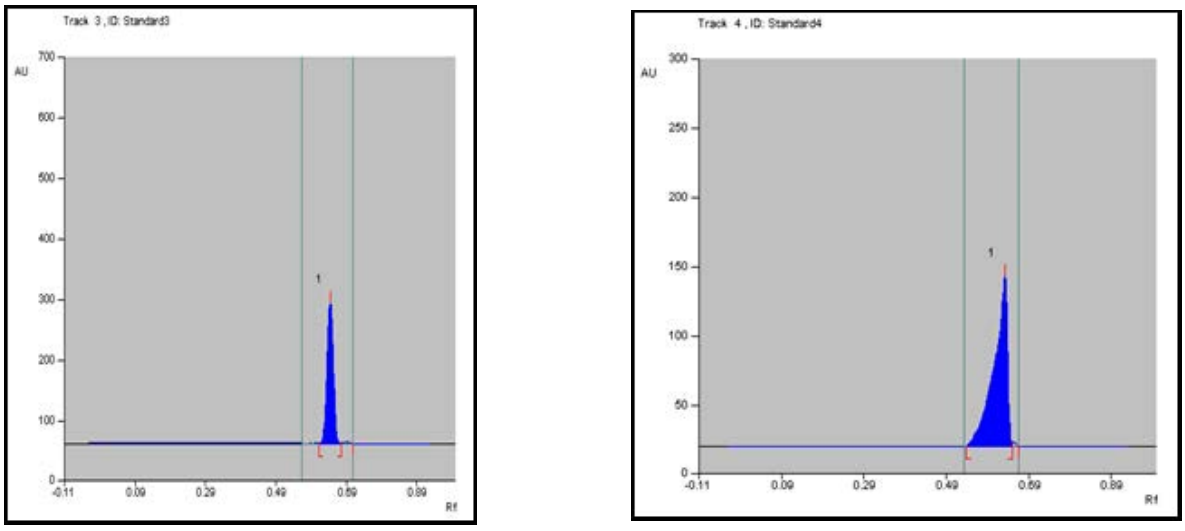
The FSO was tested for its physical characteristics as well as physicochemical parameters, as shown in Table 1. The oil in the formulation appeared yellowish with a characteristic odor. The pH of 1% oil in methanol was 6.9.

HPTLC Method

HPTLC method was developed wherein optimization of a method with respect to the solvent system is the first step; for good extraction efficiency, the compact spot which quantifies the analyte with no interference of any excipient from the formulation. Several trials were done with a combination of solvent systems of low polarity to high polarity like toluene: ethanol; toluene: ethanol: ammonia; toluene: methanol: glacial acetic acid; toluene: methanol: triethylamine; n-butanol: methanol in different proportions, helped to understand the optimized conditions for the separation of the LA peak as a sharp band with accepted Rf value of 0.63 at a wavelength,254 nm as absorbance maxima. The standard and sample peaks are as shown in Figure 1.

Assay of Marketed Formulation

A total of 20 capsules were weighed to quantify LA in FSO herbal formulation, and appropriate dilution of oil in methanol was done to get 1 mg/mL of oil. From the above solution, 5 µL was applied (n = 6) on the TLC plate, developed under the optimized conditions to obtain sharp peaks of LA. The



(a) (b)

Figure 1: Typical Densitogram of Linoleic acid in a) standard and b) formulation of seed oil.

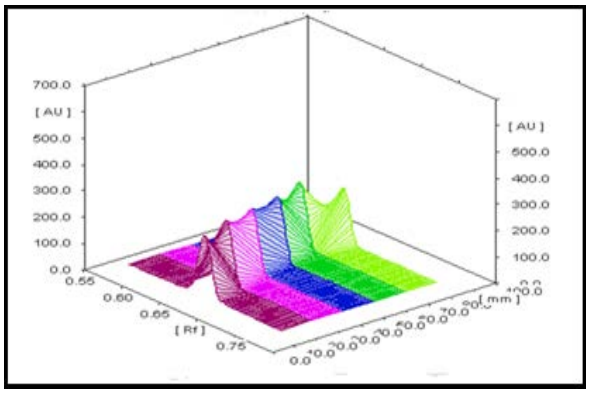


Figure 2: Peak Pattern for System Precision Study

percentage content of LA was calculated using the calibration curve. The results of the same are given in Table 2.

Method Validation[23,24]

Linearity and Range

The method developed showed that LA followed linearity in the range of 200–1200 ng/band. The linear regression equation was y = 7.192x-794.35, while the correlation coefficient (r2) was 0.99, with high reproducibility and accuracy.

Accuracy

The accuracy of the method was determined wherein standard LA was added to the sample in the proportion of 80 to 120%. Suitable dilutions of the test solution were applied on a TLC plate and analyzed using optimized chromatographic conditions. The recovery was obtained in the range of 98–102% as shown in Table 3.

Robustness Studies

Small changes were carried out using parameters like mobile phase volume, absorbance maxima, and saturation time to understand the robustness to estimate linoleic acid. The results of robustness studies and their statistical validation are shown in Table 3.

Precision

The analytical method is considered precise when the results are in close agreement between a set of analyses done on multiple sampling points under the prescribed conditions. The results indicate that method is precise with a %RSD of 0.94 and 1.04 significant for intra- and inter-day analysis of LA in marketed flax seed oil, as shown in Figure 2 and Table 4

LoD and LoQ

The LoD and LoQ for LA were determined using the calibration curve plotted using standard solutions at 254 nm. LoD and LoQ of LA were calculated to be 0.1 and 0.3 µg/mL, respectively.

CONCLUSION

Flax seed oil is known for its nutritional and medicinal value.[25-27] The proposed HPTLC method for the estimation of LA in FSO formulation was found to be specific, precise, and accurate. Thus it can be proposed for the analysis of LA in bulk and can help in the quantification of LA in different varieties of flax seed or other food ingredients. The developed HPTLC method can be used for analyzing the content of LA and applying the same for quality control of formulations with LA.

ACKNOWLEDGMENT

The authors express their gratitude to Dr. S.S. Chitlange, Principal, Dr. D.Y. Patil Institute of Pharmaceutical Science and Research, Pimpri, Pune, for the support and research facility

REFERENCE

1. Kajla P, Sharma A, Sood DR. Flaxseed-a potential functional food source. J Food Sci Technol. 2015 Apr;52(4):1857-71. doi: 10.1007/s13197-014-1293-y.

2. Soni RP, Katoch M, Kumar A, Verma P. Flaxseed–composition and its health benefits. Res. Environ. Life Sci. 2016 Mar; 9(3):310-6.
3. Goyal A, Sharma V, Upadhyay N, Gill S, Sihag M. Flax, and flaxseed oil: an ancient medicine & modern functional food. J. Food Sci. Technol. 2014 Jan; 51(9):1633-53.
4. Ivanov S, Rashevskaya T, Makhonina M. Flaxseed additive application in dairy products production. Procedia Food Sci. 2011; 1:275-80.
5. Singh KK, Mridula D, Rehal J, Barnwal P. Flaxseed: a potential source of food, feed, and fiber. Crit. Rev. Food Sci. Nutr. 2011;Mar;51(3):210-22.
6. Touré A, Xueming X. Flaxseed lignans: source, biosynthesis, metabolism, antioxidant activity, bio-active components, and health benefits. Compr. Rev. Food Sci. Food Saf.. 2010 Apr;9(3):261-9.
7. Stawarska A, Jelińska M, Czaja J, Pacześniak E, Bobrowska-Korczak B. Oils' Impact on Comprehensive Fatty Acid Analysis and Their Metabolites in Rats. Nutrients.2020 Apr; 12(5), 1232. https://doi.org/10.3390/nu12051232
8. Cunnane SC, Ganguli S, Menard C, Liede AC, Hamadeh MJ, Chen ZY, Wolever TMS, Jenkins DJA.High α–linolenic acid flaxseed (Linum usitatissimum): some nutritional properties in humans. Br J Nutr.1993 MAr;69(2):443–453
9. Pellizzon MA, Billheimer JT, Bloedon LT, Szapary PO, Rader DJ. Flaxseed reduces plasma cholesterol levels in Hypercholesterolemic mouse models. J Am Coll Nutr. 2007 Jun ; 26(1):66–75.
10. Athira M, Nickerson MT, Ghosh S. Oxidative stability of flaxseed oil: Effect of hydrophilic, hydrophobic and intermediate polarity antioxidant. Food Chem.2018 Nov;266,524-533, ISSN 0308-8146,https://doi.org/10.1016/j.foodchem.2018.05.117.
11. Lan Y, Ohm JB, Chen B, Rao J. Physicochemical properties, and aroma profiles of flaxseed proteins extracted from whole flaxseed and flaxseed meal. Food Hydrocoll. 2020 Jul;1; 104:105731.
12. Pali V, Mehta N. Evaluation of oil content and fatty acid compositions of flax (Linum usitatissimum L.) varieties of India. J. Agric. Sc. 2014 Aug;6(9):198.
13. Magalhães BE, De Andrade SD, De Jesus Silva IM, Minho LA, Gomes MA, Silva Almeida JR, dos Santos WN. Determination of the phenolic composition of oilseed whole flours by HPLC-DAD with evaluation using chemometric analyses. Microchemical Journal. 2020 Jun; 155:104683.
14. Mukker JK, Kotlyarova V, Singh RS, Alcorn J. HPLC method with fluorescence detection for the quantitative determination of flaxseed lignans. J. Chromatogr. B . 2010 Nov;878(30):3076-82.
15. Rupani B, Kodam K, Gadre R, Najafpour GD. Lipase-mediated hydrolysis of flax seed oil for selective enrichment of α-linolenic acid . Eur J Lipid Sci Technol.2012 Nov;114(11):1246-53.
16. Hosu A, Cimpoiu C. Evaluation of various biological activities of natural compounds by TLC/HPTLC.J. Liq. Chromatogr. Relat. Technol.. 2020:1-4.
17. Chakraborty A, Bhattacharjee A, Dasgupta P, Manna D, Chun WO, Mukhopadhyay G. Simple Method for Standardization and Quantification of LA in Solanum nigrum Berries by HPTLC. J Chromatogr Sep Tech. 2016 Dec;7:6. doi: 10.4172/2155-9872.1000341
18. Andronie L, Pop ID, Sobolu R, Diaconeasa Z, Truţă A, Hegeduş C, Rotaru A. Characterization of flax and hemp using spectrometric methods. Applied Sciences. 2021 Sep 8;11(18):8341.
19. Herchi W, Arráez-Román D, Boukhchina S, Kallel H, Segura-Carretero A, Fernández-Gutierrez A. A review of the methods used in the determination of flaxseed components. Afr. J. Biotechnol. 2012 Jan;11(4):724-31
20. Indian Pharmacopoeia Commission. Indian Pharmacopoeia. Ministry of Health & Family Welfare Government of India. 2014;2(7): 100, 102,109.
21. ICH, (Q2B), Harmonized Tripartite Guideline, Validation of Analytical Procedures: Methodology, Text and Methodology, IFPMA. Geneva; 1996. p. 1-8.
22. ICH, Guidance on Analytical Method Validation, In: Proceeding of the International Convention on Quality for the Pharmaceutical Industry. Toronto, Canada; 2002
23. Gruia A, Raba DN, Dumbrava D, Moldovan C, Bordean D, Mateescu C. Fatty acids composition and oil characteristics of linseed (Linum Usitatissimum L.) from Romania. J. Agro aliment. Processes Technol. 2012;18(2):136-40.
24. Herchi W, Ammar KB, Bouali I, Abdallah IB, Guetet A, Boukhchina S. Heating effects on physicochemical characteristics and antioxidant activity of flaxseed hull oil (Linum usitatissimum L). Food Sci. Technol. 2016 Feb; 5;36:97-102. https://doi.org/10.1590/1678-457X.0109
25. Jain A, Parashar AK, Nema RK, Narsinghani T. High-performance thin layer chromatography (HPTLC): A modern analytical tool for chemical analysis. Current Research in Pharmaceutical Sciences. 2014.Mar; 30:8-14.
26. Guarrasi V, Mangione MR, Sanfratello V, Martorana V, Bulone D. Quantification of underivatized fatty acids from vegetable oils by HPLC with UV detection. J Chromatogr Sci.2010 Sep 1;48(8):663-8.
27. Carter JF. Potential of flaxseeds and flaxseed oil in baked goods and other products in human nutrition. Cereal Foods World. 1993;38: 754–759