

MARKER BASED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION FOR ANALYSIS OF SEED OIL FROM *ANNONA SQUAMOSA*

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ABSTRACT

Herbal medicines are widely used for disease treatment, even when their biologically active compounds are unknown. Although *Annona squamosa* (Custard apple) has high medicinal value, the precise methods to study the active components are not investigated. A simple, precise and accurate marker based high performance liquid chromatographic technique has been developed for quantitative estimation of linoleic acid from *Annona squamosa* oily seeds (ASO) extract. The chromatographic separation was performed on C-18 column Qualisil (150mm x 4.6mm, 5 μ m) as a stationary phase and mobile phase comprising of Acetonitrile: Methanol: THF: water containing 0.5% glacial acetic acid (36:32:31:1 v/v) at a flow rate of 0.5ml/minutes and analysis was screened with UV detector at 236nm. The proposed method was linear within the concentration range of 4-100 μ g/ml as well as accurate, precise and specific. Thus validated marker based HPLC method can be used for a routine quality control analysis and quantitation of linoleic acid from oil extracted from *Annona squamosa* seeds and its formulations.

KEY WORDS: *Annona squamosa* Seed oil, marker based HPLC analysis, Linoleic acid, validation.**INTRODUCTION**

Herbal medicines are increasingly drawing interest in disease treatment for their effectiveness, minimal side effects in clinical experience and relatively low cost. *Annona squamosa* Linn, commonly known as custard apple or Sitaphal (local name) is a multipurpose tree with edible fruits and medicinal properties. Traditionally, *Annona squamosa* is extensively used in the treatment of various diseases. The studies have reported that petroleum ether extract of the bark of *Annona squamosa* was effective for analgesic and anti-inflammatory activity.^[1] Acetogenin extracted from seeds of *Annona squamosa* showed to be effective as an anthelmintic agent.^[2] The ethanolic leaf extract of *Annona squamosa* also showed free radical scavenging activity.^[3] The leaf extract of *Annona squamosa* was studied in streptozotocin induced diabetic rat model and proved to be anti-hyperglycemic.^[4] Moreover, the bark extract of *Annona squamosa* was found to be anti-genotoxic when studied in DMBA induced genotoxicity in golden Syrian hamster.^[5] Although the medicinal uses of *Annona squamosa* are well accepted, the details of pharmacologically active components are poorly investigated.

Previously, HerbOshield vaginal gel containing hydroalcoholic extract of *Annona squamosa* seeds has been studied as a contraceptive agent using a rat as an animal model.^[6] *Annona squamosa* seed extract

containing annonaceous acetogenin compounds tested for its anti-tumor activity by *in vitro* MTT cytotoxicity assay and H22 hepatoma cells transplantation tumor model *in vivo*, showed significant antitumor activity.^[7] Antitumor activity of Annonaceous acetogenins was also reported, when studied using S180 and HepS xenografts bearing mice.^[8]

Annona squamosa seed oil (ASO) tested for its anti-tumor activity using H22 xenograft mice model, revealed that ASO suppresses the H22 solid tumor development due to unsaturated fatty acid content.^[9] The presence of oleic acid and triglyceride ester in *Annona squamosa* seed hexane extract showed *in vitro* anti head lice activity.^[10] Radical scavenging activity was observed with aqueous extract of *Annona squamosa* seeds.^[11] Topical application of *Annona squamosa* seeds extract was also found to be insecticidal agent.^[12] Herbal drugs or formulations containing herbal extracts are routinely analyzed by marker based HPLC method. Despite ASO exhibit wide medicinal importance, till today a simple, marker based HPLC method for its estimation or routine analysis is not reported.

Annona squamosa seed oil (ASO) was previously standardized by gas chromatography, and results depicted that ASO contains approximately 20- 25 % of linoleic acid.^[13] Mariod have reported that petroleum ether oily extract of *Annona squamosa* seeds showed

22.3% and 22.7% w/w of linoleic acid when extracted using Soxhlet extraction and cold extraction methods respectively.^[14] Linoleic acid, an omega-6 polyunsaturated fatty acid was therefore chosen as marker compound to develop HPLC analysis for routine analysis of ASO and its formulations. Linoleic acid primarily from vegetables and nuts (e.g. sunflower, safflower, soya, corn and walnuts) has medicinal importance and reduces chronic heart disease.^[15] The topical application of linoleic acid on acne microcomedones revealed that almost 25% reduction in the size of follicular casts and microcomedones.^[16] Moreover, the topical application of linoleate based oils massage enhanced the skin barrier function and improved the outcome in neonates with compromised barrier function.^[17]

HPLC has been preferred analytical method for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for thorough screening.^[18] As HPLC is less time-consuming technique and does not require any special sample preparation it is widely accepted analytical method. Tarole *et al.* carried out HPLC analysis for determination of fatty acids from drying oils by lipase action. Enzymatic hydrolysis of the oil triacylglycerols was achieved by the action of *Candida rugosa* lipase. Fatty acids (linoleic, linolenic, palmitic, oleic, stearic, myristic acids) were extracted and further derivatized before injecting to HPLC system.^[19]

Till today to best of our knowledge there is no HPLC method reported for estimation of free ASO or its formulations. In the present study, an attempt has been made to develop and validate marker based HPLC method for ASO and its formulations in terms of linoleic acid ensuring high accuracy, precision, and recovery.

MATERIALS AND METHODS

Chemicals and Reagents

Annona squamosa seeds extract (ASO) was obtained from dried seeds by extracting (maceration) with petroleum ether. HPLC grade water, acetonitrile, methanol, THF (Tetrahydrofuran) were purchased from SD Fine Chem Limited, India. HPLC grade glacial acetic acid and Linoleic acid were obtained from Sigma-Aldrich, India.

Instruments and chromatographic conditions

HPLC analysis was performed on Agilent 1200 series liquid chromatograph comprising quaternary pump, UV-Visible detector, and Qualisil C18 Column (250mm x 4.6mm i.d.) with a particle size of 5 μ m. A manually operated rheodyne injector with 20 μ l sample loop was used for injecting samples into HPLC system. The HPLC system was equipped with EZChrom Elite processing software. The mobile phase consists of acetonitrile, methanol, THF and water containing 0.5% glacial acetic acid in the ratio 36:32:31:1 v/v. The mobile phase was set at a flow rate of 0.5ml/min, and the column

temperature was maintained at 40^oC. Detection wavelength for analyte was monitored at 236nm. The sample run time was 20 minutes. Linoleic acid was determined using area under curve (AUC) method.

Preparations of standards

Stock and working Standard Solution

Accurately 10 mg of linoleic acid was weighed and transferred to 10 ml conical flask. The solvents, methanol: THF (1:1 v/v) system was used to prepare a stock solution of linoleic acid. The volume was made up to give a concentration of the stock solution to 1000 μ g/ml. Working solutions (4, 8, 10, 20, 40, 80, 100 μ g/ml) were made from standard stock solution and diluted with mobile phase. Care was taken while analysis that all dilutions were made in amber color glassware to protect linoleic acid solutions from photo degradation.

Sample preparation

Extracted ASO was weighed accurately (50mg) and dissolved in a solvent system containing methanol: THF (1:1v/v) system (40ml). The solution was sonicated for 30 minutes and volume was made to 50 ml with methanol: THF (1:1 v/v) system in a volumetric flask. Further dilution of ASO solution was made using mobile phase to a final concentration of 50 μ g/ml. The analyte solutions were filtered through the 0.2 μ m filter before injecting (20 μ l) to HPLC system. Three injections were performed for each sample.

Validation of proposed method

The developed method was validated as per ICH guidelines for the linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantitation (LOQ).

Standard Calibration curve for Linoleic acid

The standard solutions of linoleic acid (4, 8, 10, 20, 40, 80, 100 μ g/ml) were injected (20 μ l) three times into the HPLC system. The area under curve (AUC) of each concentration was recorded. Linearity graph of mean AUC versus concentration (μ g/ml) was plotted, and regression coefficient (R^2) was calculated.

Precision

Intra-day precision was obtained by carrying out three replicates at three concentration levels (10, 30, 50 μ g/ml) of Linoleic acid. The % relative standard deviation (% RSD) of the three values was calculated. Inter-day precision was determined at three different concentrations 10, 30 and 50 μ g/ml of linoleic acid on three different days. Repeatability of the method was calculated by analyzing the 15 μ g/ml of concentration of linoleic acid on three different days. The percent relative standard deviation was calculated for each day.

Accuracy

Accuracy or recovery study was carried out at three different concentration levels (80%, 100%, and 120%) by adding known concentration of linoleic acid. The

recovered amount of linoleic acid, % RSD of recovery, % recovery of each concentration was calculated to determine accuracy.

Specificity

The specificity of method was determined by observing interference of other ingredients present in the ASO extract. Specificity of the developed method was determined by analyzing 15µg/ml of linoleic acid. The dilution of ASO was made in such manner that, final dilution contains linoleic acid 15µg/ml. The retention time of standard linoleic acid and linoleic acid from ASO were compared. The analysis was carried out in triplicate (n=3). The results obtained were compared with the results of those obtained with standard linoleic acid. Specificity of method was confirmed by injecting analyte containing high concentration of ASO and linoleic acid and retention time was observed.

Detection and quantitation limit

Limit of detection (LOD) is the lowest quantity of a substance that can be distinguished from the absence of that substance within a stated confidence level. Limit of quantitation (LOQ) is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met.^[20] Limit of detection and limit of quantitation were calculated from linearity using formula, $LOD=3.3\sigma/S$ and $LOQ=10\sigma/S$, where σ = standard deviation of responses and S = slope of the calibration curve.

Sample preparation and analysis of ASO based pharmaceutical products

Linoleic acid from in house developed lotion containing 10% ASO was determined using HPLC marker based analysis technique. Accurately 100mg of ASO lotion weighed and extracted using 5ml HPLC grade methanol. Stock solution was prepared using Methanol: THF (1:1). Working solution (1000µg/ml) was made from stock solution and diluted with mobile phase [Acetonitrile: Methanol: THF: water (0.5% glacial acetic acid) 36:32:31:1 v/v]. The working solution was filtered through 0.2µ filter membrane and analyzed by HPLC. The area under curve (AUC) was analyzed for quantification of linoleic acid from ASO lotion.

RESULTS AND DISCUSSION

When the active constituents of the herbal drug are unknown, marker-based methods need to be developed for analytical methods and standardization of drugs.^[21] Oil extracted from *Annona squamosa* seeds is used in the ayurvedic medicinal system. Though active constituents in ASO are unknown, an attempt has been made to analyze *Annona squamosa* seed extract oil using marker based HPLC method. As linoleic acid was prominently detected in ASO, it was used as a marker. The analytical method developed for detection of linoleic acid was validated for parameters like accuracy, precision, specificity, detection limit, quantitation limit, linearity and range according to ICH guidelines.^[22]

Given the complex composition of plant products, a critical aspect for the accurate identification and reliable quantification of the analytes is the optimization of the separation conditions. To determine marker compound linoleic acid, HPLC chromatographic conditions were optimized (Table 1).

Table 1: Optimized chromatographic conditions and system suitability parameters for proposed HPLC method for linoleic acid.

Instrument	Agilent 1200 series liquid chromatograph equipped with quaternary pump
Column	Qualisil C18 Column (250mm x 4.6mm i.d., 5µm particle size)
Detector	UV-visible
Diluents	Methanol:THF (1:1 v/v)
Mobile Phase	Acetonitrile: Methanol: THF: water (0.5% glacial acetic acid) (36:32:31:1 v/v)
Detection wavelength	236 nm
Flow rate	0.5 ml/min
Run time	20 minutes
Column temperature	40°C

Reversed phase C-18 column Qualisil (250mm x 4.6mm i.d., 5µm particle size) was found to be suitable considering the selectivity, symmetry, resolution and number of theoretical plates of the chromatographic peak of linoleic acid in ASO. The mobile phase was optimized with different ratios of acetonitrile: methanol: water based on peak resolution and retention time. Mobile phase consisting of different concentrations of acetonitrile, methanol, THF and water were tried. The mobile phase containing acetonitrile: methanol: THF: water (36:32:31:1 v/v) gave the well-resolved peak at retention time of 6.35 minutes but peak tailing was observed. The addition of 0.5% of glacial acetic acid to water in the mobile phase showed absence of tailing effect (Figure 1A).

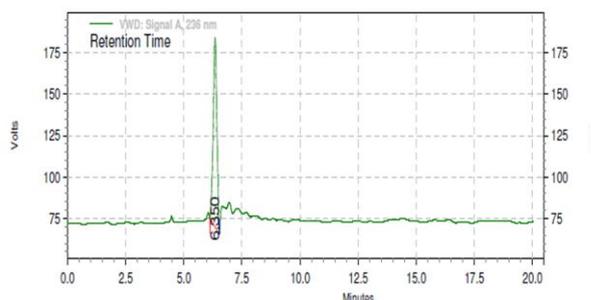


Figure 1(A): HPLC Chromatogram of linoleic acid.

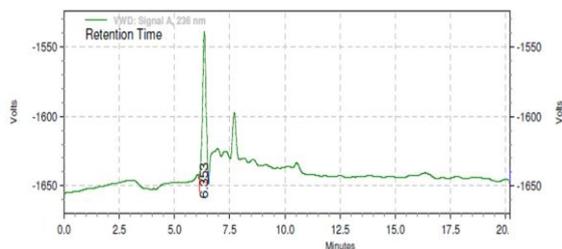


Figure 1(B): HPLC Chromatogram of oil extracted from *Annona squamosa* seeds.

The use of an acidic mobile phase improves separation performance, providing better peak shape and enhanced resolution. The optimized mobile phase consisted of acetonitrile: methanol: THF: water (0.5% glacial acetic acid) (36:32:31:1 v/v). The flow rate of mobile phase was maintained at 0.5ml/min thus having low solvent consumption making it attractive for routine use. The retention time for linoleic acid was found to be 6.350 minutes (Figure 1A), and linoleic acid peak in ASO eluting at retention time of 6.353 minutes was thus used as marker (Figure 1B). The marker linoleic acid showed maximum UV absorbance at 236nm and was well resolved in run time of 20 minutes.

The area under curve (AUC) of linoleic acid peak was linear with respect to the concentrations over the range of 4-100 µg/ml (Figure 2). The slope and intercept value for linear curve for linoleic acid was found to be $y = 50.248x$ and correlation coefficient (R^2) = 0.9998 suggesting excellent correlation exists between AUC and the concentration of drug within the said concentration range and thus the method can be said to be linear.

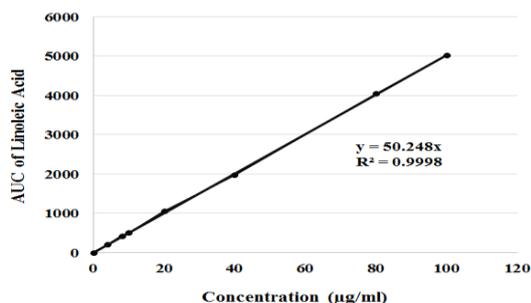


Figure 2: Calibration curve of Linoleic acid.

The precision of an analytical method describes the closeness of individual measures of an analyte when the analytical procedure is applied repeatedly to multiple dilutions of a single volume. Intra-day precision carried out on same day at three concentration levels (n=3), % RSD values at each concentration level was less than 2.

Table 2: Results of precision study (intra-day) for linoleic acid.

Concentration (µg/ml)	Mean AUC	%RSD [#]
10	503.333	1.05546
30	1513.67	0.18944
50	2507.67	0.06778

Acceptance criteria %RSD < 2.0

Table 3: Results of precision study (inter-day) for linoleic acid.

Day	Concentration(µg/ml)	Mean AUC	%RSD [#]
1	10	505.667	0.33613
	30	1511.33	0.20454
	50	2504.33	0.16731
2	10	502.333	0.49657
	30	1510.67	0.11251
	50	2503.67	0.10483
3	10	501	0.16297
	30	1512	0.216
	50	2506.33	0.10472

Acceptance criteria %RSD < 2.0

Table 4: Results of precision study (repeatability) for linoleic acid.

Days	Concentration (µg/ml)	Mean AUC	% RSD [#]
1	15	743.67	0.4437
2	15	748.33	0.3333
3	15	744.33	0.3351

Acceptance criteria %RSD < 2.0

The observed precision at three concentration levels for three different days and % RSD values at each concentration level less than 2 indicate that the method is precise for HPLC estimation of Linoleic acid. Repeatability carried out on three different days (Table 4) shows % RSD values less than 2. The correlation coefficient (R^2) was found to be 1. The data showed that the method is precise for estimation of linoleic acid from oil extracted from seeds of *Annona squamosa*. Limit of detection (LOD) for *Annona squamosa* was confirmed to be 0.1638 µg/ml and limit of quantitation (LOQ) was found to be 0.4964 µg/ml. LOD and LOQ values obtained revealed that method is sensitive for quality control of linoleic acid from oil extracted from seeds of *Annona squamosa*.^[20]

Table 5: Results of detection limit and quantitation limit of linoleic acid.

Limit of Detection (LOD)	0.1638
Limit of Quantitation (LOQ)	0.4964

Table 6: Accuracy study data for linoleic acid.

Recovery level (%)	% Recovery	%RSD [#]
80	98.84	0.1646
100	99.13	0.1031
120	98.89	0.1200

Acceptance criteria % RSD < 2.0

The accuracy or recovery of an analytical method is the closeness of the test results obtained by that method to the true value.^[23] The percent recovery of marker linoleic acid was found to be 98.84%, 99.13% and 98.89 % at three levels of recoveries viz. 80%, 100% and 120% respectively (Table 06). The % RSD values at each level were found to be less than 2, proving that the method is accurate.

The mean AUC of working solution of linoleic acid was found to be 745.33, whereas that of ASO working solution (15 µg/ml of linoleic acid) showed mean AUC of 737.00. The Figure 3A indicates spiked higher concentration of linoleic acid to higher concentration of ASO. At higher concentration of ASO and spiked linoleic acid elution of linoleic acid was observed at 6.350 minutes, confirming the presence of linoleic acid. Various studies on GC-MS analysis of ASO state that it contains 20- 38% of linoleic acids.^[13b,24] The present developed HPLC analytical method elucidates that ASO extract consists of 22 % v/v of linoleic acid.

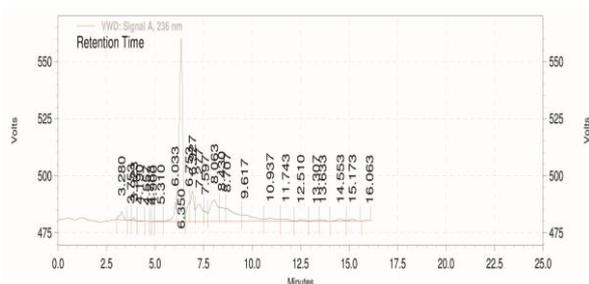


Figure 3(A): HPLC chromatograph of spiked higher concentrations of linoleic acid with ASO.

Table 7: Assay of ASO lotion containing 10% ASO.

Amount of ASO lotion weighed	Concentration of ASO lotion injected	Calculated concentration of linoleic acid (µg/ml)	% RSD [#]	% Recovery of linoleic acid
100mg	1000µg/ml	21.42	0.1927	97.38

Acceptance criteria %RSD < 2.0

The ASO lotion contains 97.38% of linoleic acid. Results revealed that developed marker based HPLC analytical method was simple and specific for quantitation of ASO formulated in ASO lotion (Figure 3B). The % RSD of the determinations was 0.2757, 0.2931 and 0.1927 for linoleic acid, ASO and ASO lotion respectively. As there was no interference of other constituents present in *Annona squamosa* oily extract (ASO) and ASO lotion on retention time and Area under curve (AUC), the method was specific for the analysis of ASO containing Linoleic acid. Thus the high repeatability in the retention time was obtained for Linoleic acid standard solution and extracted oil at different concentrations.

CONCLUSION

Thus the developed marker based HPLC method for the quantification of linoleic acid from oil extracted from *Annona squamosa* seeds has various advantages like low retention time, good peak symmetry. The developed method is simple, sensitive, accurate, precise and specific. The advantage of the method is that neither sample preparation requires any special technique like derivatization of fatty acids nor does detection system require sophisticated instruments but not at the expense of accuracy and reliability. These attributes contribute to the reliability of the method. The developed method can be used for routine analysis of ASO and herbal formulations containing ASO.

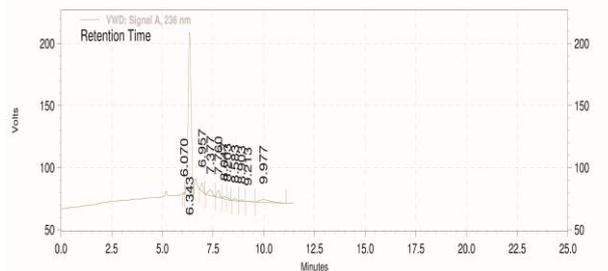


Figure 3(B): HPLC chromatograph of linoleic acid from ASO lotion.

As developed HPLC analytical method does not require special sample preparation technique nor time-consuming and is less expensive when compared with GC-MS analysis thus could be effectively used as an alternative. Thus, developed HPLC method is simple, less time-consuming, cost effective and reliable method of analysis over reported GC-MS techniques for routine analysis of ASO.

The results of drug content in ASO lotion are depicted in table 07.

Conflict of Interest

There are no conflicts of interest.

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