CHAPTER-III

PROXIMATE AND ULTIMATE ANALYSES OF SEEDS

Chapter-III

STUDIES ON OILS, FATS AND SUGARS

3.1 ISOLATION OF OILS :

The seed samples for the present study were collected and dried in the absence of sunlight. The seeds were made to powder. The oils from these powdered seeds were extracted completely by refluxing them with petroleum ether (60-80°) in a Soxhlet extractor for 8 to 10 hours in each case. The process was repeated in order to get the total oil extracted. The petroleum ether was distilled off and the remaining concentrate (oil) was first dried on hot plate and then was air dried at a temperature of 40±2°C. The seed oils were filtered through Whatman filter paper No. 1 to remove any foreign particles and the pure oils were preserved in cold properly. Official and tentative methods of AOCS were followed for the determination of physico-chemical characteristics of seed oils[1]. Various physico-chemical constants of these seed oils are given in Table No. **3.1**.

3.2 SAPONIFICATION OF THE OILS AND ISOLATION OF MIXED FATTY ACIDS :

The Oils obtained from different seeds were saponified in order to isolate mixed fatty acids. 20.0 gms of each seed oil was saponified with 7.5 gms potassium hydroxide dissolved in 25 ml of anhydrous ethanol by refluxing for 6 to 8 hours. Excess of ethanol was distilled off. The soap so obtained was dissolved in water and then extracted with ether. The ethereal extract was repeatedly washed with distilled water and dried over anhydrous sodium sulphate. Unsaponifiable matter was recovered from the ethereal extract by evaporating the ether. The aqueous soap solution

was warmed with dilute (4 N) sulphuric acid in an atmosphere of carbon-di-oxide for half an hour or so. The solution was cooled and was extracted several times with solvent ether. Removal of ether of the ethereal layer by evaporation, yielded mixture of free fatty acids. These were dried with anhydrous sodium sulphate and stored in well stoppered bottles in desiccators.

Table No. 3.1

Extraction data and Physico-Chemical Constants of the Seed Oils

Name of Seed	Oil Contents(%)	Colour of Oils	Specific Gravity	Refractive Index	Iodine Value (Hanus)	Saponification Value	Acid Value	Unsaponifiable Matter(%)
Annona Squamosa Linn	28.2%	Pale Yellow	0.9202	1.4834	67.6	187.8	10.2	1.52
Citrullus Vulgaris Var. fistulosus (stocks)	32.3%	Deep Yellow	0.9026	1.434	64.4	166.6	2.3	1.64
Brassica Oleracea Linn (Gongylodes-Group)	25.6%	Golden Yellow	0.9020	1.4752	62.8	128.08	2.2	1.60
Moringa Oleifera Lam	38.2%	Yellow	0.9036	1.4590	70.8	186.8	2.14	0.87

3.3 PREPARATION OF METHYL ESTERS OF FATTY ACIDS : [2]

Free fatty acid mixtures, thus obtained were mixed with methanol (Excellar) in the ratio 1:6 (w/v) and 1 ml. concentrated sulphuric acid and contents were refluxed under water condenser for 8 hours. Excess of methanol was distilled off. Methyl esters of mixed fatty acids were extracted with ether and repeatedly washed with water, aqueous solution of sodium carbonate and finally with distilled water. Ethereal layer consisting of esters was separated and dried over anhydrous sodium sulphate. Removal of ether of ethereal layer by evaporation yielded pure methyl esters of fatty acids.

3.4 PREPARATION OF POTASSIUM HYDROXAMATES OF METHYL ESTERS : [3,4]

Methanolic solution of hydroxyl amine hydrochloride and potassium hydroxide were mixed together (1:2-ratio) and precipitated potassium chloride obtained was filtered off. The resulting alkaline methanolic solution of hydroxyl amine was mixed with an equal amount of methyl ester of free fatty acids. This mixture was warmed for 10 minutes over a water bath. The mixture of potassium hydroxamates thus obtained was used for chromatographic studies.

3.5 DETERMINATION OF R_f VALUES AND IDENTIFICATION OF FREE FATTY ACIDS AND THEIR DERIVATIVES BY USING PAPER CHROMATOGRAPHY : [5-10]

Whatman filter paper No-1 (Chromatographic Grade) was impregnated with a 10% solution of paraffin oil in acetone and was dried in air. Solutions of authentic samples of fatty acids and their methyl esters prepared in chloroform and methanolic solutions of potassium hydroxamates were used for spotting the chromatograms. Paraffin impregnated filter papers were spotted with authentic samples of fatty acids and their derivatives and dried in air. The loaded chromatograms were irrigated with 85% glacial acetic acid by ascending run technique. The developed chromatograms were dried in air and then heated at 120°C for two ours in an electric oven.

Fatty acids and their hydroxamate derivatives were located and detected by copper acetate-rubeanic acid reagent. Methyl esters were converted on the developed paper chromatogram into potassium hydroxamates by spraying with hydroxylamine solution and then identified by further spraying with acidified ferric chloride solution. Red spots of ferric hydroxamates appeared against yellow background on the paper and were subsequently identified.

Unsaturated components were located and identified on the developed paper chromatogram by exposing it to iodine vapours. R_f values of various fatty acids, their methyl esters and corresponding hydroxamates were measured and the same are given in Table No. 3.2.

Table 3.2

R_f values of paper chromatographically resolved free fatty acids, methyl esters and hydroxamates at 30±1°C in 85% glacial acetic acid system

(Mean values of 5 determinations)

S.No.	Fatty Acids	R_f Values		
		Free Fatty Acids	Methyl esters of Fatty acids	Hydroxamates of Fatty acids
1	Lauric	0.14	0.18	0.20
2	Stearic	0.17	0.20	0.26
3	Palmitic	0.30	0.35	0.38
4	Oleic	0.34	0.37	0.47
5	Linoleic	0.48	0.50	0.54
6	Palmitoleic	0.54	0.53	0.58
7	Linolenic	0.73	0.68	0.74
8	Behenic acid	0.77	0.74	0.80

3.6 CELLULOSE THIN LAYER CHROMATOGRAPHY OF FATTY ACIDS AND THEIR DERIVATIVES : [11-16]

Thin layer chromatographic method was used for identification of fatty acids. 10.5 gms of cellulose was shaken thoroughly with 35.0 ml distilled water for 5 minutes. Slurry thus obtained was uniformly spread over the glass plates using a "Stahl type" TLC applicator adjusting the layer thickness to 0.350 mm. These TLC plates were dried overnight in air and were impregnated with 10% paraffin oil solution in acetone and dried

in air. These paraffin impregnated plates were spotted with authentic samples of free fatty acids and their derivatives, and dried in air. The loaded plates were irrigated in 85% glacial acetic acid solvent system. The developed TLC plates were air dried and finally stained with suitable staining reagents as described earlier. The R_f values of fatty acids and their derivatives from the developed TLC plates were measured and the same recorded in Table No. 3.3

Table No. 3.3

R_f values of thin layer chromatographically resolved fatty acids, their methyl esters and hydroxamates at 30±1°C in 85% glacial acetic acid system

(Mean Values of five determinations)

S.No.	Fatty Acid	R_f Values		
		Free Fatty Acids	Methyl esters of Fatty acids	Hydroxamates of Fatty acids
1	Lauric	0.24	0.33	0.26
2	Stearic	0.29	0.36	0.30
3	Palmitic	0.39	0.52	0.34
4	Oleic	0.46	0.64	0.42
5	Linoleic	0.66	0.68	0.55
6	Palmitoleic	0.72	0.75	0.63
7	Linolenic	0.84	0.84	0.76
8	Behenic acid	0.86	0.87	0.80

3.7 DETECTION OF FATTY ACIDS PRESENT IN THE OIL SAMPLES BY USING ARGENTATED THIN LAYER CHROMATOGRAPHY OF THEIR METHYL ESTERS AND HYDROXAMATE DERIVATIVES : [9,13,17]

A slurry of 12.5 gms silica gel-G (TLC-Grade) in 48.0 ml distilled water was prepared by shaking the contents for about five minutes. TLC plates of uniform thickness, (0.25 mm) with this slurry were prepared using Stahl type TLC applicator. These coated plates were dried in air and were impregnated with 12.0% silver nitrate solution. These argentated TLC plates were activated at 110°C for one and half hour in an electric oven. Spotting solutions of methyl esters and potassium hydroxamates were prepared in chloroform and glacial acetic acid respectively. Activated plates were spotted with respective solutions of methyl esters and hydroxamate derivatives and dried. The loaded plates were developed in petroleum ether, diethyl ether (9:1,v/v) solvent system. The separated spots of methyl esters and hydroxamates were detected by charring technique. Unsaturated esters or hydroxamates form π-complexes with silver nitrate. Stronger complexes are formed when number of double bonds in the ester or hydroxamates is greater and their migration becomes slow.

Fatty acids of subjected seed oils and their methyl esters and hydroxamates were co-chromatographed and were identified by reversed phase techniques of paper, cellulose and Argentated Silica gel-G thin layer chromatography. The results of qualitative chromatography obtained are given in Table No. **3.4.**

Table No. 3.4

Fatty acids detected in various seed oils by paper, cellulose and argentated paper and TLC methods

S. No.	Fatty Acids	Annona Squamosa Linn	Citrullus Vulgaris var. fistulosus	Brassica Oleracea Linn	Moringa Oleifera Lam
1	Lauric (C, 12:0)	-	-	-	-
2	Myristic (C, 14:0)	-	-	-	+
3	Palmitic (C,16:0)	+	+	+	+
4	Palmitoleic (C, 16:1)	-	+	-	-
5	Stearic (C, 18:0)	+	+	+	+
6	Oleic (C, 18:1)	+	+	+	+
7	Linoleic (C, 18:2)	+	+	+	+
8	Linolenic (C, 18:3)	+	-	+	+
9	Arachidic (C, 20:0)	-	+	-	+
10	Behenic (C, 22:0)	-	-	+	+

3.8 QUANTITATIVE DETERMINATION OF FATTY ACIDS PRESENT IN DIFFERENT SEED OILS : [18-24]

The methyl esters of fatty acids of different seed oils were analysed for their fatty acid composition by Gas liquid chromatography. The apparatus used for this purpose was Perkin-Elmer Autosystem-XL equipped with flame ionisation detector (FID). The other details of instrument and experimental conditions are as below,

Column type, Packed column (10 feet) OB-1, means 100% methyl gum. (coated with 0.20 μm 10% DEGS)

Carrier gas : Helium at the rate 2.0 ml/sec

Injection temp : 280°C

Detector temp : 280°C

Programme start : 80°C

Temperature : 4°C/minute increased upto 240°C

Final temperature : 240°C

Amount inject : 1 to 2 μl

Detector : FID (flame ionisation detector)

The peaks were recorded and were identified by comparing their retention times with those of standard methyl esters analysed under the same condition. Relative percentage of the fatty acids were determined by comparing their peak areas (peak area = 1/2 x peak height x peak width). GLC chromatograms are given in Figures 3.1 to 3.4.

The percentage composition of various fatty acids in different seed oils under investigations are recorded in Table 3.5.

Two unidentified fatty acids (each 0.04%) in Annona Squamosa, one fatty acid (0.02%) in B. Oleracea and two fatty acids of 0.04% and 0.08% respectively in M. Oleifera have also been observed.

Table 3.5

Percentage Composition of Fatty acids present in different Seed Oils

S. No	Fatty Acids	Name of Plant Seeds			
		Annona Squamosa Linn	Citrullus Vulgaris var. fistulosus	Brassica Oleracea Linn	Moringa Oleifera Lam
A. Saturated Fatty Acids :					
1	Lauric Acid ($C_{12:0}$)	-	-	-	1.50
2	Myristic Acid ($C_{14:0}$)	-	-	-	6.20
3.	Pentadecanoic Acid ($C_{16:0}$)	-	-	-	1.15
4	Palmitic Acid ($C_{16:0}$)	4.84	19.65	6.21	24.56
5	Stearic Acid ($C_{18:0}$)	32.42	61.13	40.22	45.54
6	Arachidic ($C_{20:0}$)	-	0.28	-	0.11
7	Behenic Acid ($C_{22:0}$)	-	-	37.01	-
B. Mono unsaturated Fatty Acids :					
8	Palmitoleic Acid ($C_{16:1}$)	-	0.03	-	0.02
9	Oleic Acid ($C_{18:1}$)	1.01	18.85	2.90	14.10
10	9-Eicosenoic Acid (Gadoleic Acid) ($C_{20:1}$)	-	-	-	3.28
11	Erucic Acid ($C_{22:1}$)	0.09	-	0.07	-
C. Poly unsaturated Fatty Acids :					
12	Linoleic Acid ($C_{18:2}$)	7.98	0.04	11.51	2.85
13	Linolenic Acid ($C_{18:3}$)	53.58	0.02	2.02	0.19
Unsaturated Fatty Acid %		62.46	18.94	16.43	20.44
Saturated Fatty Acid %		37.26	81.06	83.44	79.06

Fig. 3.1 GLC CURVE OF METHYL ESTERS OF FATTY ACIDS OF SEED OILS OF ANNONA SQUAMOSA LINN

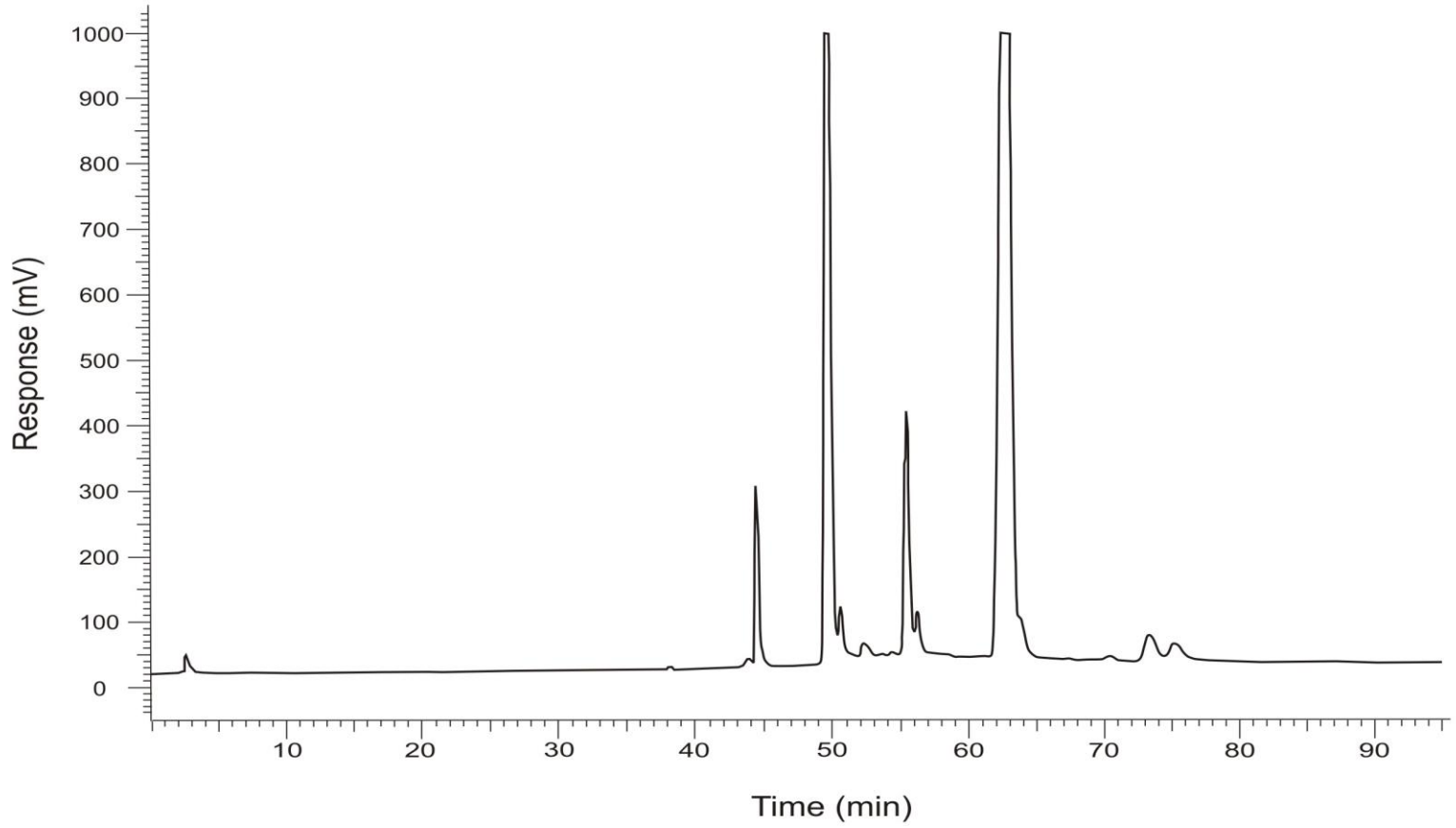


Fig. 3.2 GLC CURVE OF METHYL ESTERS OF FATTY ACIDS OF SEED OILS OF CITRULLUS VULGARIS VAR. FISTULOSUS (STOCKS)

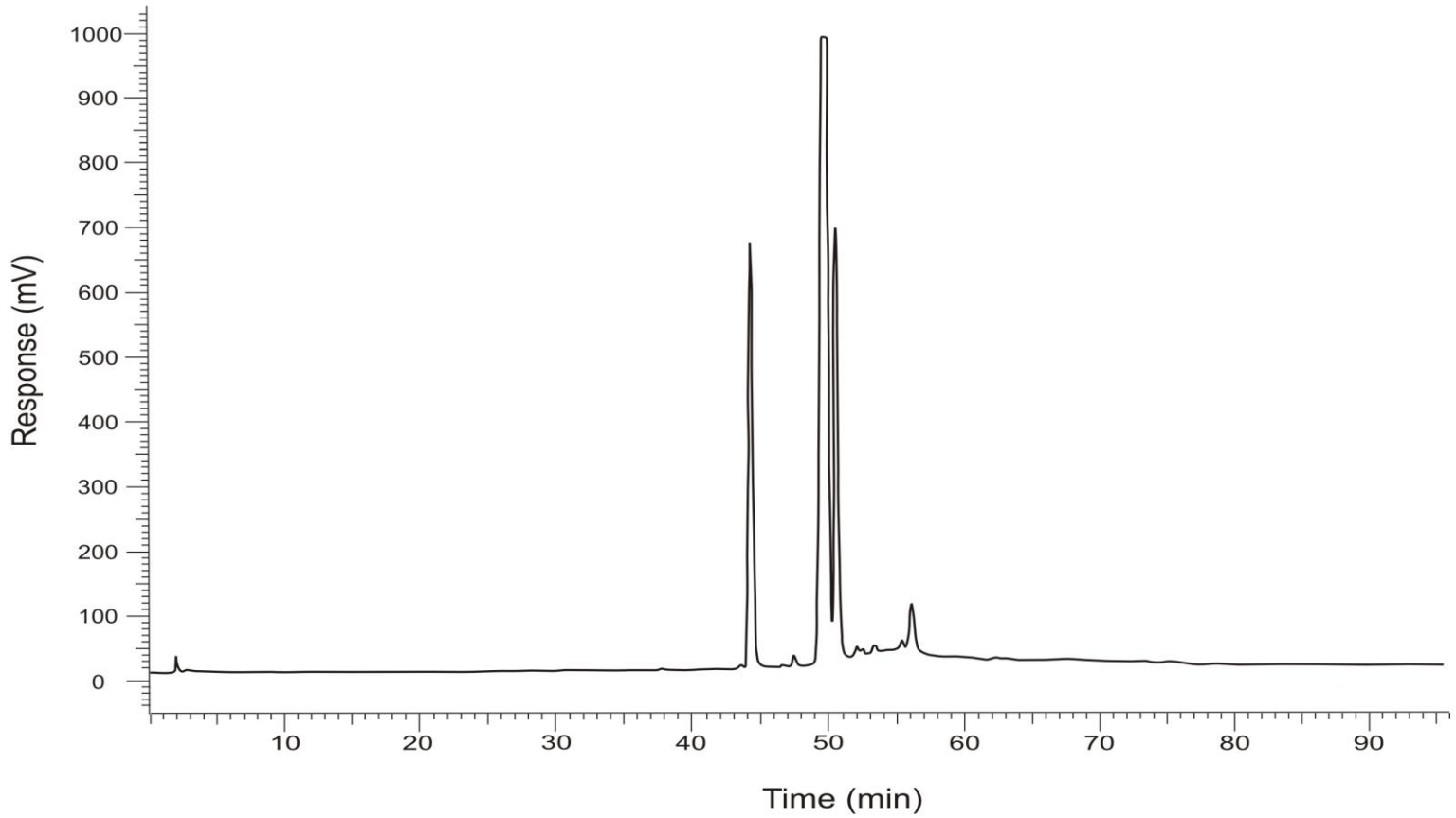


Fig. 3.3 GLC CURVE OF METHYL ESTERS OF FATTY ACIDS OF SEED OILS OF BRASSICA OLERACEA LINN (GONGYLODES GROUP)

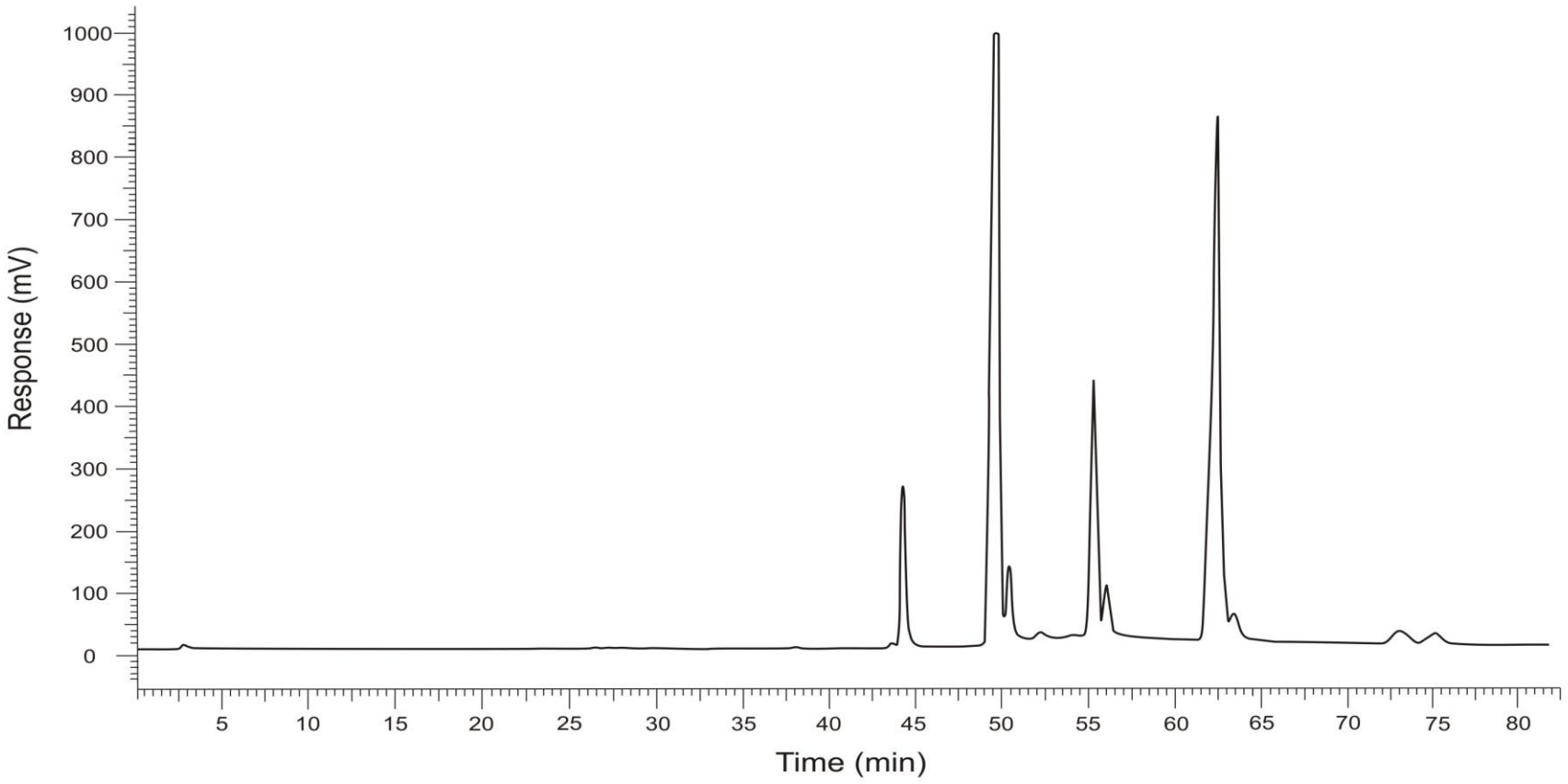
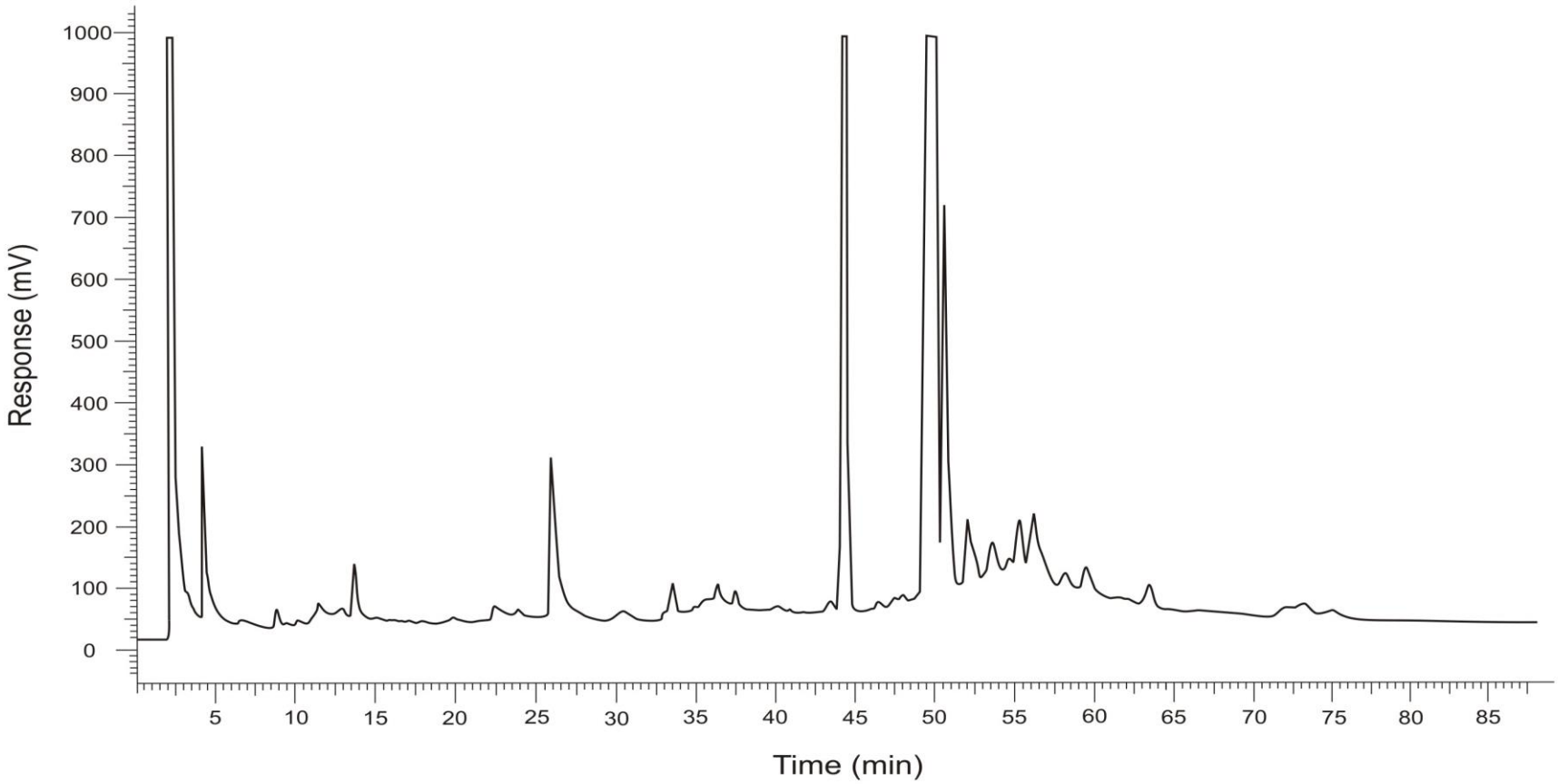


Fig. 3.4 GLC CURVE OF METHYL ESTERS OF FATTY ACIDS OF SEED OILS OF MORINGA OLEIFERA LAM



3.9 STUDY OF SUGARS :

Stahl[25], Bourne et al.[26], Pridham[27] and Joshi and Shrivastava[28] have described various chromatographic separation methods of Sugars. Paper chromatography is the slowest method of separating sugars, while gas chromatography resolves sugars into its components within half an hour. In present investigations of Sugars, paper and cellulose thin layer chromatographic techniques have been used for their characterisation and separation.

Isolation of Sugars :

Various defatted seed meals were exhausted with 80% ethanol in cold, the extracts obtained were decolourised and deionised successively by passing through a column loaded with DOWEX-50 (H^+) and then through AMBERLITE-IRA 40 resin. The transparent liquids so obtained were evaporated to syrupy mass and were desiccated into vacuum. Each syrupy mass / liquid so obtained was dissolved in 30% iso-propanol. These solutions were used for chromatographic work.

3.10 PAPER CHROMATOGRAPHIC RESOLUTION OF AUTHENTIC SUGAR SAMPLES AND THEIR R_f VALUES IN DIFFERENT SOLVENT SYSTEMS : [27,28]

Authentic sugars solutions in iso-propanol were spotted on chromatographic paper (Whatman No. 1) and dried in hot air. These loaded chromatograms were equilibrated with solvent vapours and were developed by multiple (x 5) run technique. The solvent systems used were,

(i) n-Butanol, pyridine, water (6:4:3, v/v)
(ii) Tert-Butanol, pyridine, water (6:4:3, v/v)
(iii) Benzene, Butanol, pyridine, water (1:5:3:3, v/v)
(iv) n-Butanol, acetic acid, water (4:1:1.6, v/v)

The developed chromatograms were dried in air and stained with Orcinol-HCl, Aniline phthalate rand p-Anisidine phosphoric acid reagents. R_f values of different Sugars were measured and the same are given in Table No. 3.6.

Table No. 3.6

R_f values of Paper Chromatographically resolved authentic samples of Sugars in various solvent systems at 30 ± 2°C

S. No.	Sugars	R_f Values in Solvent Systems			
		n-Butanol : pyridine : water (6:4:3)	Tert-Butanol : Pyridine : Water (6:4:3)	Benzene : Butanol : Pyridine : water (1:5:3:3)	n-Butonal : Acetic acid: water (4:1:1.6)
1	Rhamnose	0.93	0.90	0.94	0.86
2	Fucose	0.86	0.845	0.87	0.80
3	Xylose	0.81	0.83	0.85	0.76
4	Arabinose	0.77	0.79	0.80	0.70
5	Fructose	0.73	0.77	0.76	0.69
6	Glucose	0.70	0.74	0.72	0.62
7	Galactose	0.64	0.67	0.63	0.58
8	Sucrose	0.56	0.59	0.57	0.47
9	Maltose	0.53	0.56	0.48	0.37
10	Cellobiose	0.48	0.47	0.44	0.36
11	Lactose	0.43	0.45	0.38	0.33
12	Raffinose	0.35	0.38	0.34	0.27
13	Stachyose	0.29	0.31	0.21	0.19
Mean values of 5 determinations					

3.11 CELLULOSE THIN LAYER CHROMATOGRAPHY OF AUTHENTIC SUGAR SAMPLES : [25, 29, 30]

Stahl type TLC applicator was used for preparation of thin layer chromatographic plates with 0.35 mm thickness of chromatographic grade cellulose. These plates were activated by drying in air for an overnight. Various authentic sugar samples were spotted on these plates and again dried. These thin layer chromatographic plates were developed with the following solvent systems,

(i) n-Butanol, pyridine, water (6:4:3:3, v/v)

(ii) Benzene, Butanol, pyridine, water (1:5:3:3, v/v)

(iii) n-Butanol, acetic acid, water (4:1:1.6, v/v)

The above developed chromatograms were dried in air and were stained with following reagents,

(i) p-Anisidine - o - phosphoric acid

(ii) Orcinol-hydrochloric acid reagents

The R_f values of various sugar samples obtained are given in Table No. 3.7.

The alcoholic isolates of sugars of different seeds were co-chromatographed with authentic sugar samples using paper and thin layer chromatographic techniques. Various separated sugar components were identified by comparison of their R_f values with those of authentic samples. The results of qualitative paper and thin layer chromatographic separation of sugars are reported in Table No. 3.8.

Table No. 3.6

R_f values of authentic Sugar samples separated by cellulose thin layer chromatography at 30 ± 2°C

S. No.	Sugars	R_f Values in Solvent Systems		
		n-Butanol, pyridine, water (6:4:3)	Benzene, Butanol, Pyridine, water (1:5:3:3)	n-Butonal, Acetic acid, water (4:1:1.6)
1	Rhamnose	0.94	0.84	0.91
2	Fucose	0.92	0.82	0.87
3	Xylose	0.90	0.80	0.86
4	Arbinose	0.88	0.78	0.83
5	Fructose	0.86	0.76	0.80
6	Glucose	0.84	0.73	0.78
7	Galactose	0.81	0.70	0.77
8	Sucrose	0.78	0.66	0.74
9	Maltose	0.70	0.57	0.68
10	Cellobiose	0.64	0.54	0.64
11	Lactose	0.60	0.50	0.58
12	Raffinose	0.53	0.38	0.42
13	Stachyose	0.36	0.22	0.31
Mean values of 5 determinations				

Table No. 3.8

Qualitative paper and thin layer chromatographic identification of sugars in various sugar isolates

S. No.	Name of Seeds	Sugars Detected
1	Annona Squamosa Linn	Glucose, Fructose, Galactose, Rhamnose, Sucrose, Raffinose and Stachyose
2	Citrullus Vulgaris Var. fistulosus (Stocks)	Glucose, Fructose, Rhamnose, Sucrose, Cellobiose
3	Brassica Oleraca Linn (Gongylodes Group)	Glucose, Fructose, Sucrose, Raffinose and Stachyose
4	Moringa Oleifera Lam.	Glucose, Fructose, Galactose, Rhamnose, Collobiose, Raffinose

3.12 QUANTITATIVE ANALYSIS OF SUGARS IN VARIOUS SEEDS : [31]

Paper and thin layer chromatographic methods were used for the quantitation of sugars present in sugar isolates of various seeds. During the present investigation, chromatographic methods in association with extractive spectrophotometric determination of p-Anisidine-o-phosphoric acid colours of sugars were used.

The deionised sugar isolates of the seeds were chromatographed and developed by multiple ascending run (x 5) method in (a) n-butanol, pyridine, water (6:4:3) and (b) Benzene, n-Butanol, pyridine, water (1:5:3:3) solvent systems. The sugars were identified by staining the developed chromatograms with p-anisidine-o-phosphoric acid reagent. The areas containing the sugars were circumscribed with a pencil, and the circles of paper were cut into blood sugar tubes graduated 1 to 5, 10, 15 and 25 ml. p-anisidine colours of sugars were extracted in 5.0 ml of 0.5N-HCl acid solution in 80% ethanol and their colour intensities were measured by Systronix make spectrophotometer Model-169 at 410 nm. The concentrations of individual components were then determined and expressed in terms of glucose. The percentage of Sugars in various seeds are reported in Table No. 3.9.

Table No. 3.9

Percentage of Sugars Expressed as Glucose

S.No.	Name of Seed	Glucose	Fructose	Galactose	Rhamnose	Sucrose	Cellobiose	Raffinose	Stachyose
1	Annona Squamosa Linn	38.9	25.4	8.6	6.4	11.8	-	7.0	1.2
2	Citrullus Vulgaris Var fistulosus (Stocks)	36.7	27.2	-	9.8	18.4	5.57	-	-
3	Brassica Oleraca Linn (Gongylodes Group)	34.4	26.7	-	-	15.8	-	7.4	13.8
4	Moringa Oleifera Lam.	27.4	17.8	13.4	7.59	-	26.4	6.4	-

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