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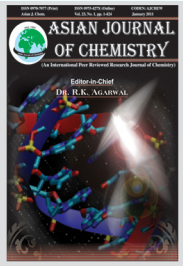

Spectroscopic and Chromatographic Analysis of Some Commercial Samples of Neem Oil

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Pesticides have been in use right from the time mankind started agricultural practice. Since, both bio pesticides and synthetic pesticides have become indispensable in the field agriculture and industry. It has become imperative to characterize and to test the quality of pesticides used today. The present work encompasses the qualitative and quantitative analysis of commercial samples of neem oil-a biopesticide using FTIR spectroscopy and HPLC technique. Commercial cold pressed, solvent extracted and mechanical pressed neem oil samples have been analyzed using HPLC and the content of azadirachtin, the active pesticide component in neem oil has been determined. FTIR spectra of some commercial neem oil samples and azadirachtin have been recorded in the range 4000-400 cm^{-1}. Samples of neem oil have been graded by comparing the internal standard ratio of the peaks corresponding to the specific modes of vibration of azadirachtin in the FTIR spectra.

Key Words: Neem oil, Azadirachtin, Qualitative and quantitative analysis, FTIR, HPLC.

INTRODUCTION

Modern advances in agriculture and industry have presented us with a bewildering array of pesticide products that are designed or naturally available to inhibit the undesired spread of pests. Pesticide use has increased 50-fold since 1950 and 2.5 million tons of industrial pesticides are now used every year. Chemical pesticides, once considered a boon for increasing the yield of food crops, have now become a bane owing to the numerous cases of pesticide poisoning. Alternatively, biopesticides are thus becoming increasingly important. These agents usually do not have toxic effects on animals and people and do not leave toxic or persistent chemical residues in the environment[1]. The added advantages of biological pesticides over conventional chemicals are their selectivity to targeted pest and lower toxicity to beneficial insects[2]. Agricultural entomologists, nematologists and pathologists all over the world[3] are now actively engaged in research into the use of plant derived products to fight and reduce the losses caused by agricultural pests and diseases.

Neem, *Azadirachta indica* A Juss (Meliaceae), the evergreen, multipurpose tropical tree has emerged as a suitable candidate for meeting the demand for environmentally benign pest control agents, safe medicinals, wood and non-wood products and has also proved to be a potential species for afforestation. Major attention has been paid in recent years to the use of neem based formulations in the control of pest infestation[4]. The main active ingredient, azadirachtin has been found to exhibit a variety of properties such as antifeedant and antiovicidal effects, disrupting the life cycle of different insects[5-7]. During the past few years it has been found that neem oil/extract based products are highly active against 200 insect species belonging to different orders. Since pest and disease management continues to challenge the agricultural community, it has become essential to test the quality of pesticides used today.

Neem oil, pressed from the fruits and seeds of neem is perhaps the most important of the commercially available products of neem. It comprises mainly triglycerides and large amount of triterpenoid compounds which are responsible for bitter taste. It is hydrophobic in nature. Neem oil contains steroids and a plethora of triterpenoids of which azadirachtin is the most well known and studied. In the present study, the quality analysis of azadirachtin and some commercial samples of neem oil have been carried out using FTIR spectrometry and HPLC.

EXPERIMENTAL

Azadirachtin A isolated and purified from neem seeds has been procured from Asthagiri Herbal Research Foundation, Chennai, India and it was used as such. The purity (95 %) was checked by HPLC. The FTIR spectrum of azadirachtin A has

been recorded in the region 4000-400 cm^{-1} by KBr pellet method at SAIF, IIT, Chennai, India. Fig. 1 represents the FTIR spectrum of azadirachtin A. High grade neem oil was procured from Fredrick Institute of Plant Protection and Toxicology, (FIPPAT), Padappai, Chennai and it has been used as the reference sample. Some leading commercial brands of neem oil were procured and their quality is been compared with the reference sample. FTIR spectra of samples of neem oil have been recorded in the range 4000-400 cm^{-1} at SAIF, IIT, Chennai. The FTIR spectra of reference and commercial samples A-D are presented in Fig. 2.

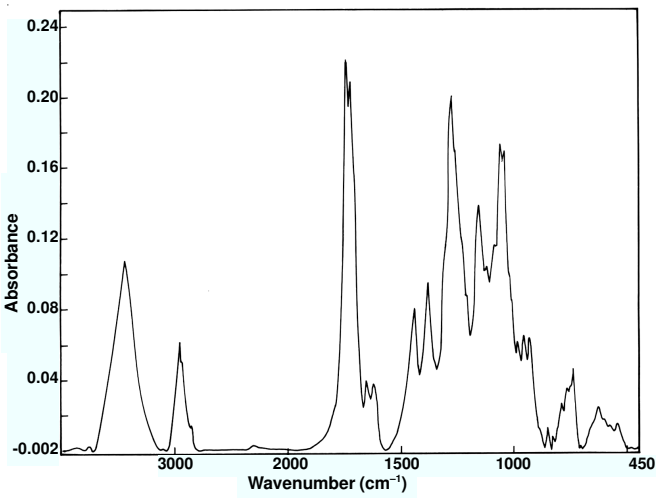

Fig. 1. FTIR spectrum of azadirachtin A

Azadirachtin A of 95 % purity extracted from neem seed kernels was procured from Asthagiri Herbal Research Foundation, Chennai, India and was used for HPLC analysis without further purification. Solution of azadirachtin A was prepared at the concentration of 1 mg/10 mL in methanol. An aliquot of 25 mL of this was utilized as standard for HPLC measurement[8]. Cold pressed, solvent extracted and mechanical pressed commercial neem oil samples have been collected in and around Chennai and 1 g of neem oil sample was extracted with 10 mL of methanol. 25 mL of the sample was injected to Shimadzu LC-A high performance liquid chromatograph. C_{18} columns (250 mm × 4.6 mm i.d.) were used with distilled water and acetonitrile in the ratio 50:50 as the mobile phase with a flow rate of 1.5 mL/min. The detector was Shimadzu SPD-6AV UV-visible spectrophotometer at the wavelength 215 nm adopting Winchrom software.

RESULTS AND DISCUSSION

Qualitative analysis of azadirachtin: Azadirachtin is a secondary metabolite present in the neem tree seeds. It is a chemical compound belonging to the family of liminoids. Its molecular formula is $C_{35}H_{44}O_{16}$. It is a highly oxidized tetranortriterpenoid which boasts a plethora of oxygen functionality, comprising enol, ether, acetal, hemiacetal and tetra-substituted oxirane as well as a variety of carboxylic esters. Additionally, both secondary and tertiary hydroxyl groups and tetrahydrofuran ether are present. Inspection of the molecular structure reveals 16 stereogenic centres, 7 of which are tetra substituted. Several types of azadirachtins are present in the neem oil ranging

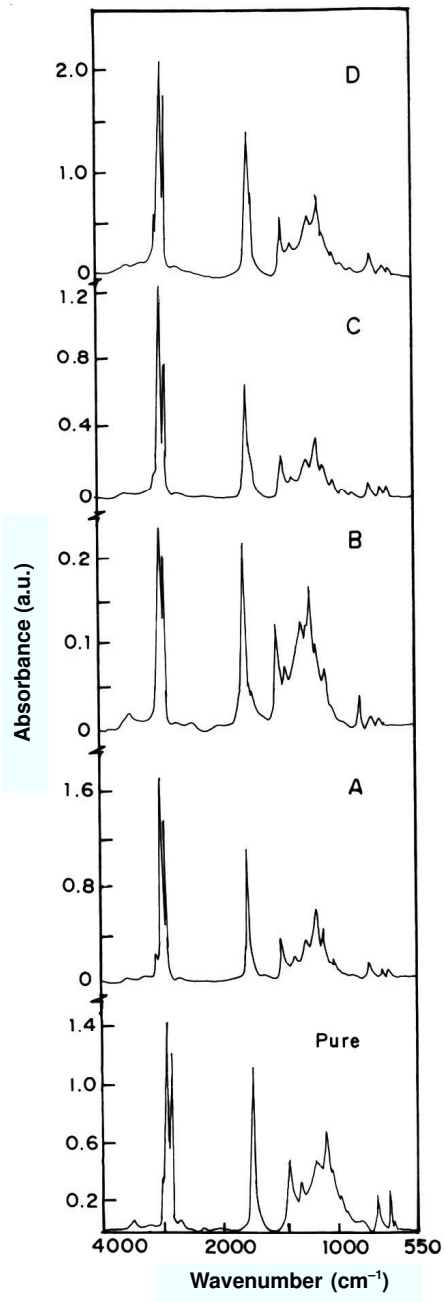

Fig. 2. FTIR Overlay spectra of reference and commercial samples of neem oil

from azadirachtin A-K. The type, which is of more significance, is azadirachtin A. The molecular structure of azadirachtin A is shown in Fig. 3.

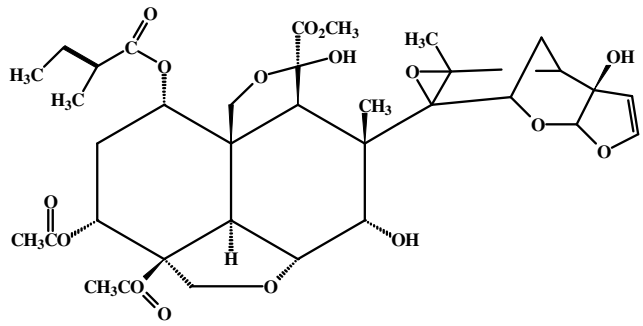

Fig. 3. Molecular structure of azadirachtin A

Azadirachtin has low mammalian toxicity and does not affect most beneficial organisms[9,10]. Unlike synthetic chemical insecticides, which are mostly contact neurotoxins, azadirachtin is a selective compound affecting the endocrine system of insects in addition to being an antifeedant[11]. Because of this selectivity and its rapid degradation[5,12], azadirachtin is considered to be less damaging than synthetic insecticides to the environment and to pose a much smaller threat to non-target organisms, including humans *via* food residues, surface and ground water contamination or accidental exposure[13-15]. Further, it is readily biodegradable and hence is perceived to be environmentally safe and ecologically acceptable[16-18]. Structural and quality analysis of azadirachtin has been carried out using different spectroscopic techniques[19-21]. The structure of azadirachtin A was elucidated by extensive analysis of NMR spectra[22,23] and X-ray crystallography of a derived product[24].

Vibrational band assignment: The aim of the vibrational analysis is to decide which of the vibrational modes in the molecule give rise to each of the observed bands at specific wave numbers in the FTIR spectra. Observing the position shape and intensity of the bands, the functional groups present in the molecule can be identified confirming the quality of the sample. A satisfactory vibrational spectral assignment of azadirachtin A has been made for the fundamental modes of vibrations. Vibrational frequencies of similar compounds like hemiacetals, acetals *etc*., have been taken into consideration for the assignment of fundamental vibrations of azadirachtin A.

The medium to strong intensity peak at 3437 cm^{-1} has been assigned to the O-H stretching vibrations of hemiacetal group. The CH_3 stretching vibration of esters results in weak bands at around 2950 cm^{-1} [25]. The medium weak band at 2956 cm^{-1} has been assigned to CH_3 stretching vibration. Similarly the C=O stretching vibrations of esters occur at 1750-1725 cm^{-1} [26]. In the FTIR spectrum of azadirachtin, the strong bands at 1735 and 1721 cm^{-1} are due to C=O stretching vibration of ester group. Acetates have a medium to strong band near 1375 cm^{-1}, due to the CH_3 symmetric deformation and medium to weak bands near 1430 cm^{-1} due to the asymmetric deformation of the same group[27]. In the IR spectrum of azadirachtin, the vibrations at 1379 and 1437 cm^{-1} are of medium to strong, medium to weak intensity, respectively and they are assigned to CH_3 symmetric and asymmetric deformation vibrations, respectively. The band due to the C-O-C asymmetric stretching vibration of esters occurs at 1275-1185 cm^{-1} and that due to the symmetric stretching vibration occurs at 1160-1050 cm^{-1} [28]. Both the bands are strong: in the present case the strong vibrations 1271 and 1154 cm^{-1} have been assigned to C-O-C asymmetric and symmetric stretching vibrations.

In the case of acetals, the C-O stretching vibration band is spilt into three, 1190-1140, 1195-1125 and 1100-1060 cm^{-1}. A fourth band at 1060-1035 is due to C-O-C-O-C symmetric vibration[29]. The present compound has series of strong bands due to C-O-C-O-C vibrations at 1154, 1116, 1080 and 1055 cm^{-1}. In addition, acetals have a characteristic, strong band in the region 1115-1105 cm^{-1} due to a C-H deformation vibration that being perturbed by the neighboring C-O groups. This band is present at 1116 cm^{-1} as a medium strong band. Cyclohexane derivatives have C-C skeletal vibrations in the range 1015-950 cm^{-1} producing weak intensity bands[30]. The structure of azadirachtin has produced two medium to weak intensity bands at 977 and 949 cm^{-1} due to cyclohexane ring vibrations. The ring vibrations of epoxides group are in the range 865-750 cm^{-1}. The bands will be of medium intensity[31]. The weak intense vibrations at 840, 736 cm^{-1} are designed to the ring vibrations of epoxide group. Thus a satisfactory vibrational spectral assignment has been carried out and the results are given in Table-1.

TABLE-1
INFRARED VIBRATIONAL BAND ASSIGNMENT OF AZADIRACHTIN A

Frequency (cm^{-1})	Assignment
3437 (ms)	O-H stretching
2956 (mw)	C-H stretching
1735 (vs)	C=O stretching
1721 (vs)	C=O stretching
1437 (mw)	CH_3 asymmetric deformation
1379 (ms)	CH_3 symmetric stretching
1271 (vs)	C-O-C asymmetric stretching
1154 (s)	C-O-C symmetric stretching/C-O stretching
1116 (ms)	C-O-C-O-C stretching/C-H deformation
1080 (s)	C-O-C-O-C stretching
1055 (vs)	C-O-C-O-C stretching
1041 (s)	C-H deformation
977 (mw)	C-C skeletal vibration
949 (mw)	C-C skeletal vibration
840 (w)	Epoxide ring vibration
736 (w)	Epoxide ring vibration

Qualitative analysis of neem oil: Neem oil is derived by pressing the seed kernels of the neem tree. It is very bitter with a pungent smell. It is an excellent moisturizing oil and contains various compounds that have insecticidal and medicinal properties. It also contains vitamin E, other essential amino acids and some percentages of fatty acids. Since neem oil has a broad spectrum of usage as pesticide, cosmetic and medicine, it is indispensable to check the quality of commercially available neem oil samples[32-34]. In the present work, comparison of some commercial samples of neem oil has been carried out using FTIR spectroscopy.

FTIR Spectral analysis: As neem oil has various biologically active compounds present in it, the FTIR spectrum of neem oil in Fig. 2 has peaks corresponding to these compounds. Among them the vibrations corresponding to azadirachtin A, the main active ingredient have been chosen for the quality analysis. The internal standard ratio among the peaks 2925, 1746, 1327, 1163, 1118, 1098, 1030 and 722 cm^{-1} of the neem oil have been found for the reference neem oil and the commercial samples A-D. The Tables 2-6 project the internal standards of some specific modes of vibration of reference neem oil and commercial samples. Comparing the internal standards of the spectra of commercial neem oil samples with that of the reference sample, the correlation coefficients are determined and tabulated in Table-7.

From the Table-7, it is clear that the commercial samples have the main active ingredient azadirachtin A and the intensity ratio parameters corresponding to the peaks of azadirachtin A of the samples get 98 % correlated with that of the reference sample. *i.e*., the quality of azadirachtin A present in the

TABLE-2
INTERNAL STANDARDS OF SOME SPECIFIC MODES OF VIBRATION OF REFERENCE NEEM OIL

Frequency (cm^{-1})	2925	1746	1377	1163	1118	1098	1030	722
2925	1.0000	0.7660	0.3607	0.4685	0.3038	0.2880	0.1772	0.1709
1746	1.3055	1.0000	0.4709	0.6115	0.3966	0.3760	0.2314	0.2231
1377	2.7722	2.1236	1.0000	1.2986	0.8423	0.7985	0.4913	0.4737
1163	2.1347	1.6352	0.7700	1.0000	0.6486	0.6148	0.3783	0.3648
1118	3.2913	2.5212	1.1873	1.5418	1.0000	0.9480	0.5833	0.5625
1098	3.4720	2.6596	1.2524	1.6265	1.0549	1.0000	0.6153	0.5933
1030	5.6424	4.3222	2.0354	2.6432	1.7143	1.6251	1.0000	0.9642
722	5.8517	4.4825	2.1108	2.7412	1.7779	1.6854	1.0371	1.0000

TABLE-3
INTERNAL STANDARDS OF SOME SPECIFIC MODES OF VIBRATION OF NEEM OIL A

Frequency (cm^{-1})	2925	1746	1377	1163	1118	1098	1030	722
2925	1.0000	0.6442	0.1346	0.3590	0.1737	0.1538	0.0769	0.0705
1746	1.5523	1.0000	0.2089	0.5572	0.2696	0.2388	0.1194	0.1094
1377	7.4295	4.7861	1.0000	2.6670	1.2902	1.1427	0.5714	0.5238
1163	2.7858	1.7946	0.3750	1.0000	0.4838	0.4285	0.2142	0.1964
1118	5.7585	3.7096	0.7751	2.0671	1.0000	0.8857	0.4429	0.4060
1098	6.5015	4.1883	0.8751	2.3338	1.1290	1.0000	0.5000	0.4584
1030	13.0030	8.3766	1.7502	4.6677	2.2581	2.0000	1.0000	0.9167
722	14.1841	9.1375	1.9092	5.0917	2.4632	2.1817	1.0908	1.0000

TABLE-4
INTERNAL STANDARDS OF SOME SPECIFIC MODES OF VIBRATION OF NEEM OIL B

Frequency (cm^{-1})	2925	1746	1377	1163	1118	1098	1030	722
2925	1.0000	0.9191	0.3547	0.6834	0.4256	0.3740	0.2901	0.1870
1746	1.0880	1.0000	0.3859	0.7436	0.4630	0.4069	0.3157	0.2035
1377	2.8191	2.5910	1.0000	1.9267	1.1998	1.0544	0.8180	0.5272
1163	1.4632	1.3448	0.5190	1.0000	0.6227	0.5472	0.4245	0.2736
1118	2.3498	2.1596	0.8335	1.6059	1.0000	0.8788	0.6818	0.4394
1098	2.6738	2.4574	0.9484	1.8274	1.1379	1.0000	0.7758	0.5000
1030	3.4465	3.1676	1.2225	2.3555	1.4668	1.2890	1.0000	0.6445
722	5.3475	4.9148	1.8969	3.6547	2.2758	2.0000	1.5516	1.0000

TABLE-5
INTERNAL STANDARDS OF SOME SPECIFIC MODES OF VIBRATION OF NEEM OIL C

Frequency (cm^{-1})	2925	1746	1377	1163	1118	1098	1030	722
2925	1.0000	0.5548	0.1198	0.2900	0.1324	0.1198	0.0757	0.0693
1746	1.8023	1.0000	0.2160	0.5227	0.2386	0.2159	0.1364	0.1250
1377	8.3447	4.6300	1.0000	2.4200	1.1047	0.9997	0.6313	0.5787
1163	3.4483	1.9132	0.4132	1.0000	0.4565	0.4131	0.2609	0.2391
1118	7.5542	4.1913	0.9053	2.1907	1.0000	0.9050	0.5715	0.5238
1098	8.3476	4.6315	1.0003	2.4208	1.1050	1.0000	0.6315	0.5789
1030	13.2178	7.3337	1.5840	3.8332	1.7497	1.5834	1.0000	0.9166
722	14.4208	8.0012	1.7281	4.1820	1.9090	1.7275	1.0910	1.0000

TABLE-6
INTERNAL STANDARDS OF SOME SPECIFIC MODES OF VIBRATION OF NEEM OIL D

Frequency (cm^{-1})	2925	1746	1377	1163	1118	1098	1030	722
2925	1.0000	0.6577	0.1611	0.3892	0.1946	0.1745	0.0973	0.0973
1746	1.5204	1.0000	0.2449	0.5918	0.2959	0.2653	0.1479	0.1479
1377	6.2092	4.0840	1.0000	2.4169	1.2083	1.0834	0.6039	0.6039
1163	2.5691	1.6898	0.4138	1.0000	0.4999	0.4483	0.2498	0.2498
1118	5.1388	3.3800	0.8276	2.0002	1.0000	0.8967	0.4998	0.4998
1098	5.7310	3.7695	0.9230	2.2307	1.1152	1.0000	0.5574	0.5574
1030	10.2826	6.7633	1.6560	4.0024	2.0010	1.7942	1.0000	1.0000
722	10.2826	6.7633	1.6560	4.0024	2.0010	1.7942	1.0000	1.0000

TABLE-7
CORRELATION COEFFICIENT OF DIFFERENT SAMPLES OF NEEM OIL WITH RESPECT TO THE REFERENCE SAMPLE

	Correlation coefficient			
	Sample A	Sample B	Sample C	Sample D
Pure	0.979	0.927	0.981	0.98

commercial neem oil samples is comparable with that of the pure neem oil.

Quantitative analysis of neem oil: Neem seed oil, a possible precursor to new botanical insecticides, varies widely with respect to concentrations of the liminoid azadirachtin, the active insect growth regulator. Isman *et al.*[35], have studied that from 72-90 % of the variation in bioactivity of the neem oil can be accounted for by variation in azadirachtin content. The azadirachtin content of neem oil varies from 300 ppm to over 2000 ppm depending on the quality of the neem seeds crushed and the methods of extraction of neem oil from neem seed kernels.

Methods of extraction: There are three main processes for extracting the neem oil from the seed kernels.

Mechanical press method: The one used since antiquity is the mechanical press method. Neem seed kernels are placed into a tub and either a screw or some form of press is used to squeeze the kernels under press until the oil is pressed out and collected.

Solvent extraction method: It is one of the most used methods of extracting neem oil. It uses a solvent preferably a petroleum/alcohol solvent for processing oil. It ensures maximum extraction of oil. The solvent usually used is hexane and this type of neem oil is of a lower quality as compared to the cold pressed oil. It is mostly used for soap manufacturing.

Cold pressed method: This method of extracting oil from neem seed kernels is most widely used by leading manufacturers though it is more expensive than the other methods. It is the best method for obtaining quality neem oil with a majority of the active compounds intact. In cold pressing the oil is lighter in colour with milder odour and can be rich in azadirachtin (> 2000 ppm)[36]. As azadirachtin is heat sensitive, cold pressing technology for need seeds is preferable method to produce quality neem oil.

HPLC analysis of neem oil: High performance liquid chromatography has been wonderful technique for the quantitative determination of major triterpenoids and liminoids in neem oil[35,37,38]. Deota *et al.*[39], have made the estimation and isolation of azadirachtin A from neem seed kernels using HPLC. In the present investigation, the quantitative determination of azadirachtin A in the commercial samples of neem oil has been carried out using reversed phase HPLC. Commercial samples of neem oil obtained by three main extraction methods namely, cold pressed oil, solvent extraction oil and mechanical pressed oil have been analyzed.

Fig. 4 gives the chromatogram of standard azadirachtin A having the retention time of 8.8 min. Figs. 5-7 give the HPLC of cold pressed, solvent extracted and mechanical pressed commercial neem oil samples (samples A-L). Azadirachtin A was identified in almost all the samples at around 8.8 min. The peak in neem oil samples at 13 min is due to nimbin. Knowing the area/height of the peak from the chromatogram,

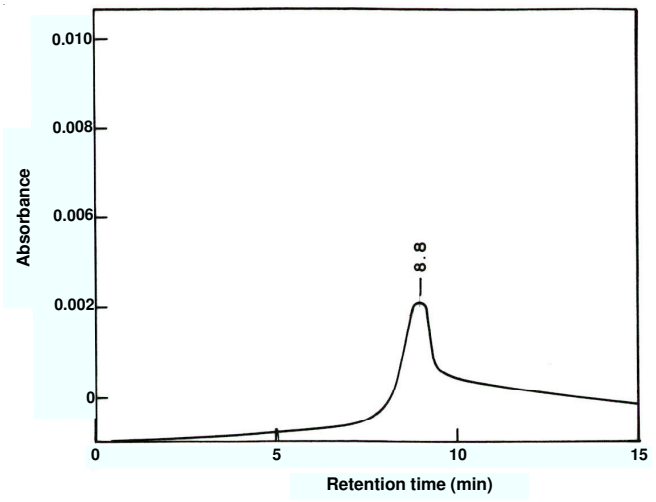

Fig. 4. HPLC of azadirachtin A

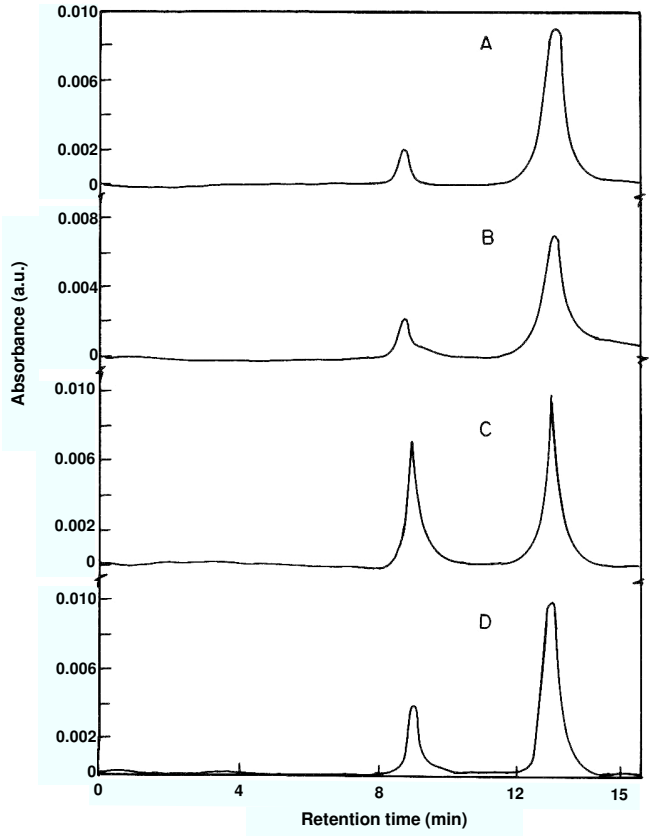

Fig. 5. HPLC of cold pressed neem oil

the azadirachtin A content in the sample has been found using the relation

Content of azadirachtin A in the sample

$$= \frac{\text{Area/height of peak in sample}}{\text{Area/height of peak in standard}} \times$$

$$\frac{\text{Mass of standard}}{\text{Mass of sample}} \times \text{Percentage of purity of the standard}$$

Table-8 presents the retention time, area/peak height of the samples and hence the content of azadirachtin in ppm in the commercial samples of neem oil. It is observed that the cold pressed neem oil samples had around 800 ppm of

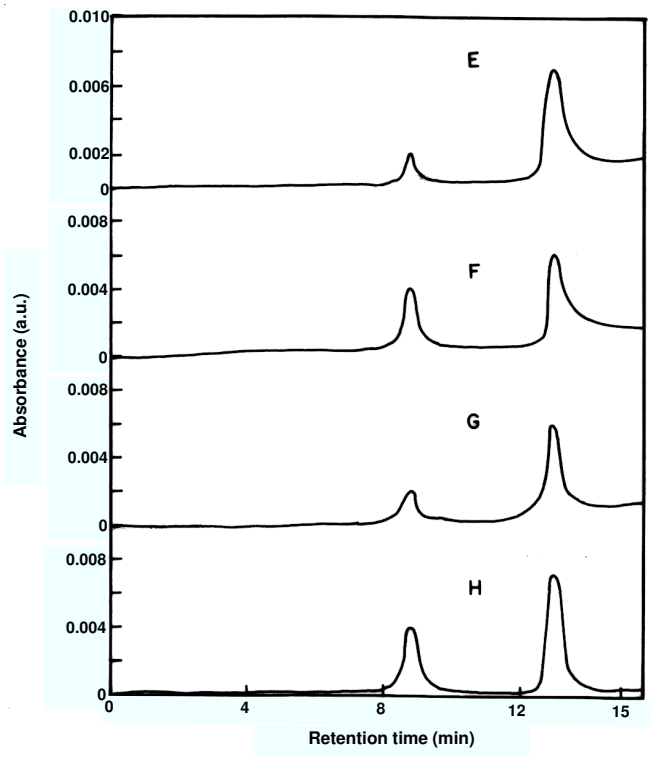

Fig. 6. HPLC of solvent extracted neem oil

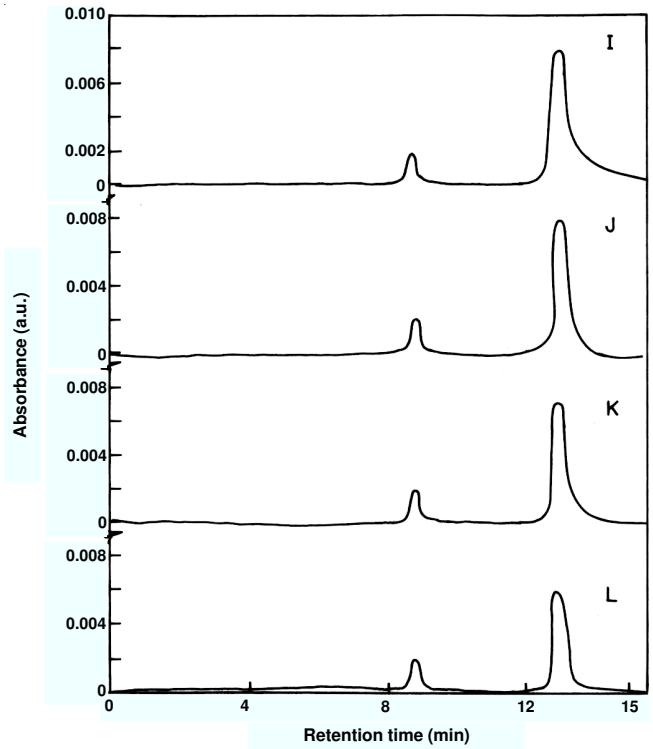

Fig. 7. HPLC of mechanical pressed neem oil

azadirachtin A, solvent extracted samples around 700 ppm and the mechanically pressed commercial samples at around 680 ppm *i.e.*, azadirachtin A content varies from 600-800 ppm in the commercial samples analyzed. Considering at ppm level, the difference among the azadirachtin A values in the neem oil samples obtained by three types of extraction procedures is marginal. Irrespective of the different extraction methods, all the commercial neem oil samples have lower value of azadirachtin A.

TABLE-8
HPLC ANALYSIS OF NEEM OIL

Methods of extraction	Sample No.	Retention time	Area peak height	Content of azadirachtin A (ppm)
–	Standard azadirachtin A	8.8	0.299	–
Cold pressed	A	8.7	0.239	759
	B	8.7	0.250	794
	C	8.71	0.249	791
	D	8.74	0.254	807
Solvent extracted	E	8.73	0.224	712
	F	8.75	0.229	728
	G	8.71	0.226	718
	H	8.71	0.223	708
Mechanical pressed	I	8.73	0.216	686
	J	8.74	0.212	674
	K	8.76	0.216	686
	L	8.71	0.211	670

Conclusion

Over the past 20 years remarkable advancements have been made in the science of "safe" pesticides. Organic or natural pesticides have received the most acclaim and certainly have the endorsement of environmentalists. But a great deal of progress has been made towards developing safer synthetic pesticides. There are risks and benefits in both types of pest control, natural and synthetic; both continue to evolve and both have a future. The potential use of FTIR, UV-visible and chromatographic techniques in quality analysis of materials has been exploited in this study. Qualitative analysis of some commercial samples of neem oil-a biopesticide, using FTIR spectroscopy and HPLC technique has been carried out. FTIR spectra of neem oil samples and azadirachtin, the active pesticidal ingredient in it have been recorded in the range 4000-400 cm^{-1}. A satisfactory vibrational frequency assignment of azadirachtin confirming its structure and quality has been carried out. Neem oils have been graded by comparing the internal standard ratio of the peaks corresponding to the specific modes of vibration of azadirachtin in the FTIR spectra. Commercial cold pressed, solvent extracted and mechanical pressed neem oil samples have been analyzed using HPLC and the content of azadirachtin in them has been determined. It is seen that all the commercial samples chosen were of poor quality having azadirachtin A around 600-800 ppm compared to 2000 ppm in fine quality cold pressed oil.

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