Gas Chromatography-Mass Spectrometry (GC/MS) Analysis of Phthalate Isolates in n-Hexane Extract of *Azadirachta indica A. Juss* (Neem) Leaves.

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Abstract: In this study, the bioactive components of the *Azadirachta indica* leaves have been evaluated using GC/MS. The data revealed presence of phthalates, which were resolved by the use of two complementary separation techniques namely: Thin Layer Chromatography and Urea and Thiourea Adduction respectively. The phthalates identified were **1.** Diisobutyl phthalate **2.** Dibutyl phthalate **3.** Ethylhexyl phthalate **4.** Heptylmethyl phthalate **5.** Mono(n-octyl) phthalate **6.** Mono(2-ethylhexyl) phthalate. Out of the 6 phthalates, 3 appear to be new compounds – namely, **1.** Ethylhexyl phthalate **2.** Heptylmethyl phthalate and **3.** Mono(n-octyl) phthalate. The bioassay of these phthalates show that 4 of them have antifungal activity: **1.** Diisobutyl phthalate **2.** Dibutyl phthalate **3.** Heptylmethyl phthalate **4.** Mono(2-ethylhexyl) phthalate. Mono(n-octyl) phthalate had no antifungal activity. Fungal activity of Ethylhexyl phthalate one was not tested. This study really gives a novel method of separating phthalates co-eluting at the same Retention time. Detail discussion on the separation and identification of these phthalates is presented in this study.

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Key words: *Azadirachta indica*, GC/MS, Urea and Thiourea adduction, Phthalate

1. INTRODUCTION

Thin Layer Chromatography, serves as one of the many analytical methods in providing a Chromatographic plant extract finger print (Wagner and Bladt, 1996). In current study, glass plate TLC was employed and the Rf values helped to locate the position of various fractions. TLC major contribution here helped us to untie the knot of phthalates in the system.

Urea and Thiourea Addution Technique was used for separation of n-alkanes, isoalkanes and cycloalkanes from
the n-Hexane extract of neem leaves. As a complementary technique to TLC, it pinpointed co-eluting peaks of the isolates as Urea adducts and Thiourea adducts respectively and at the same time confirmed separated phthalate in the non-adducts of Urea and Thiourea respectively.

The isolates of TLC, Urea and Thiourea adducts of the Neem Leaves extract were bioassayed against yeast fungus Candida albicans and zones of inhibition recorded.

Phthalates are naturally occurring bioactive components of plants. They have diverse properties from being plasticizers to toxic carcinogenic compounds. They are also known as curative drugs, antifungal (Ramasany *et al.*, 2010), antitumor (Hsouna *et al.*, 2011), antiretroviral (Syeda *et al.*, 2011), anticancer (Rahdary and Sobati, 2012), antidiabetic (Dorababu *et al.*, 2006), antimalarial (Udeinya *et al.*,2008), trypanocidal (Viviane *et al.*, 2011), just to mention but a few, for, Diisobutyl phthalate, Dibutyl phthalate and 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) phthalate.

Our reason in present study to hark reporting, is the novel technique of separating co-eluting phthalates. GC/MS is a very sensitive tool that diagnosed our phthalates.

GC/MS of the TLC 9 fractions and urea and Thiourea adducts and non adducts fractions were carried out using Agilent Technologies 7890A GC System. The detector was Agilent Technologies 5975C inert MSD with Triple-axis Detector, with (polysiloxanes) column 30m × 0.25m fused capillary silica tubing. Software adopted to handle mass spectra and chromatograms was National Institute of Standard and Technology MS. 2005 Library.

The temperature protocol for GC/MS detection was as follows: injection port temperature 200°C and Helium flow rate was 1ml/min. Oven temperature was programmed from 50°C with an increase of 8°C/min to 300°C and this temperature was held for 9 minutes. The ionization voltage was 70ev. The sample was injected in splitless mode and spectral scan range was set at 45-500(MHZ). The GC/MS characterized the isolates of the n-Hexane extract of *Azadirachta indica* leaves in TLC and Urea and Thiourea adduction fractions.

The fragmentation pattern of the mass spectra were compared (Head to tail) with those of the known compounds stored in the NIST Library. Total GC running time was 36mins.

2. MATERIAL AND METHODS

500grams of fresh leaves of *Azadirachta indica A. Juss* were sourced from the National Research Institute for Chemical Technology, Zaria Kaduna State, Nigeria. Leaves were washed and dried at 30°C in an oven and ground to powder with an electric blender. Powdered leaves were extracted with n-Hexane for 4 days, filtered and dried to constant weight. Glass plate TLC was carried out with fraction 1 of Column Chromatography of the extract likewise Urea and Thiourea adduction was carried out. Fractions were bioassayed against Candida albicans and zones of inhibition recorded.

3. Results

Following results are obtained for Thin Layer Chromatography as given in Table 1.

Our Urea and Thiourea adduction results as shown by GC are presented in Table 2.

Table 1. The TLC Rf values of fractions 1-9 and GC Rt of the isolates of n-Hexane extract of *Azadirachta indica* leaves

S/N	TLC Fractions	Rf Value of TLC (cms)	GC Rt	Isolates
1.	F1	0.16	-	A
2.	F2	0.31	-	A
3.	F3	0.41	-	A
4.	F4	0.53	26.975	P1
5.	F5	0.59	26.975	P1
6.	F6	0.75	26.975	P1
7.	F7	0.92	26.981	P2
8.	F8	0.035	26.975	P3
9.	F9	Did not move	27.013	P4

Note: Unseparated fraction has GC Rt of a single peak.
A = n-alkanes
P = Phthalate
P1 = Ethylhexylphthalate
P2 = Mono(n-octyl) phthalate
P3 = Heptylmethyl phthalate
P4 = Mono (2-ethylhexyl) phthalate, also known as 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester
Rt = Retention Time
CC = Column Chromatography

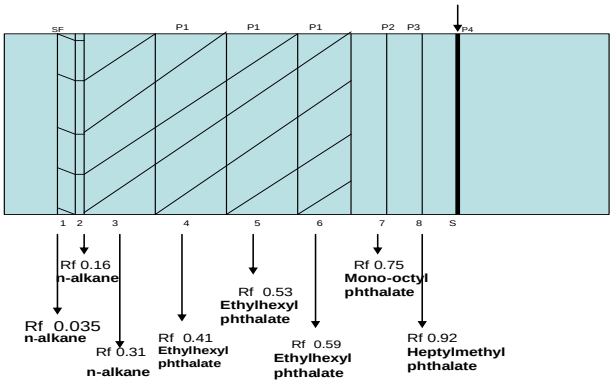
Table 2. Urea and Thiourea adduction of CC F1 as shown by GC

S/N	Fraction	GC Rt of Phthalate	Remarks
1.	Urea adduct	Only n-alkanes	---------
2.	Urea non-adduct	27.031 phthalate	Single peak overlapping phthalate
3.	Thiourea adduct	26.988 (3) phthalates.	Single peak of 3 overlapping phthalates
4.	Thiourea non-adduct	27.013 (3) phthalates	3 peaks of 3 separated phthalates**

=1.** Rt 19.537, Diisobutyl phthalate. **2.** Rt 20.713, Dibutyl phthalate. **3.** Rt 27.013, Mono(2-ethylhexyl) phthalate.
Note: Only = n –alkanes
CC = Column Chromatography
Rt = Retention time.
F1=Fraction 1

The RF values show the locations of properly separated fractions, due to our technique. The GC/MS of Column Chromatography fraction 1 came as a single peak with Rt 27.057. Here the phthalates were overlapping. Upon doing the Thin Layer Chromatography of the CC fraction 1 we saw them separate properly. Our glass plate TLC diagram, where the location of our separated phthalate were sited clearly is given in the plate 1:

Plate. 1. Glass Plate TLC Result Summary With Rf Values of Phthalates and n-Alkanes



Key:
P4 = 1,2-Benzenedicarboxylic acid, mono-(2-ethylhexyl) ester (High) – Known Isolated from other plants – very active
P3 = Heptylmethyl phthalate (Low) – New – Active
P2 = Mono(n-octyl) phthalate (Low) – New – Not Active
P1 = Ethylhexyl phthalate (Low) – New – Activity not tested yet

Initially, GC/MS analysis of CC fraction 1 shows a single peak at Rt 27.057, which is indicative of co-elution of the phthalates. The same result was observed for Urea non-adduct with Rt. 27.031. GC of Thiourea adduct shows a single peak of 26.988 and still mixture of 3 phthalates co-eluting. For Thiourea non-adduct GC gave 3 separated peaks of Rt. 19.537, 20.713 and Rt 27.013 respectively and so 3 different phthalates were sited. Phthalates which have long chain of C6, C7, C8 respectively could be Thiourea adducted. From mass spectral studies of Thiourea adduct, it is seen that C8 phthalates, which is in abundance, overshadowed, the properties of C7 and C6 phthalates, which we also observed in TLC separation and that is why adduction and TLC are complementary to each other. These phthalate with 3 different Rt are characterized as 3 different phthalates (names in Table 2). They are known compounds and from the intensity of their peaks,the peak at Rt. 27.013 has the highest concentration of phthalates which appeared in Thiourea non-adduct.

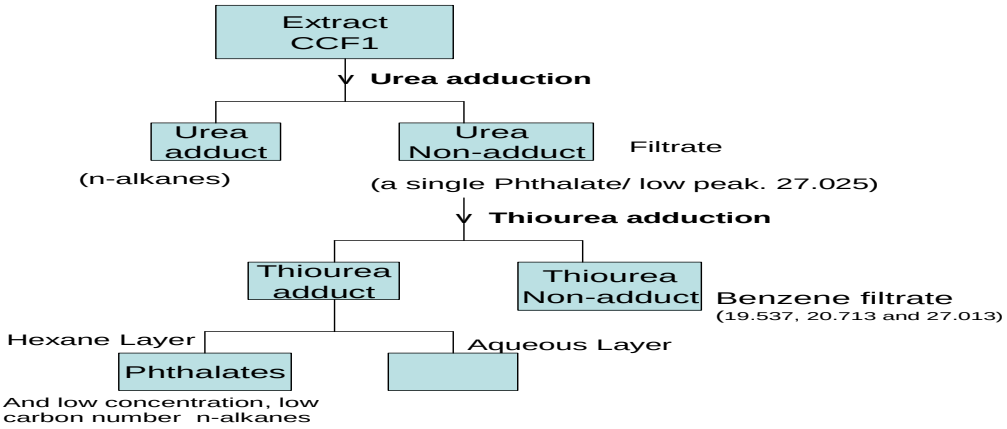
Urea and Thiourea Adduction

The GC/MS of CC F1 showed Rt 27.057 before adduction was performed. Turning towards non-adduct of Thiourea GC Rt is 27.013.

As impurity of other phthalates increases (i.e 3 out of 4 phthalates in number) the Rt is seen in the range of 26.981 to 27.013. We are speculating that the phthalate Rt to come out in the range, 26.975 to 27.057 when contamination of other phthalates occurs, the phthalate with higher concentration moves the Rt towards its own characteristics. Evidently before adduction, we had the CC F1 Rt 27.057 and its GC/MS has properties of 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester, because it is present in higher concentration and properties are similar to it. When we did Urea adduction, we found, Urea non-adduct GC/MS gave single peak at Rt 27.031, which indicates we have removed impurities and it is coming closer to 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester. But upon doing Thiourea adduction, we got separation of 2 peaks of the co-eluting phthalates for Thiourea adduct.

For Thiourea non-adduct Rt 27.013 gave us the actual Rt of 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester, and this means separation has purified it. For Thiourea adduct Rt 26.988 is a single peak of co-elution of phthalates (Evidence is TLC fraction 7 Rt 26.981). Rt 26.988 means it is co-eluting with two phthalates of Rt. 26.975.

Chart: 1. Results of Urea and Thiourea Adduction

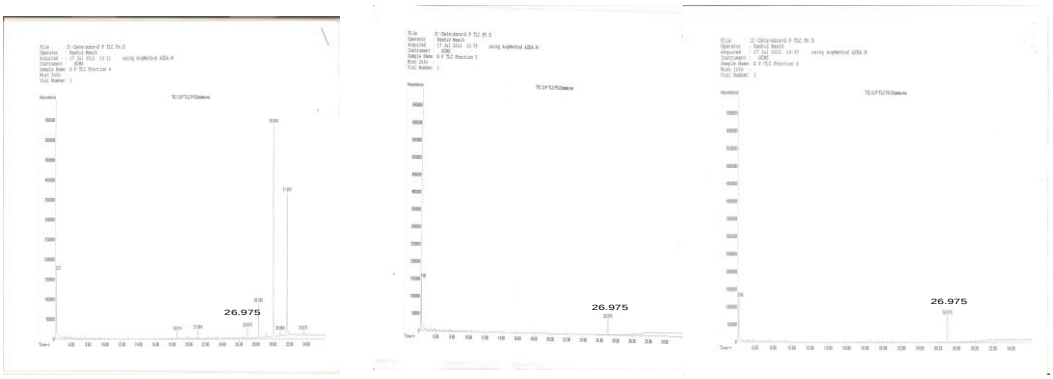


4. Discussion

Discussion of the difference in the mass spectras of 4 phthalates.

Figure 1:
TLC Chromatograms (4,5,6), fractions

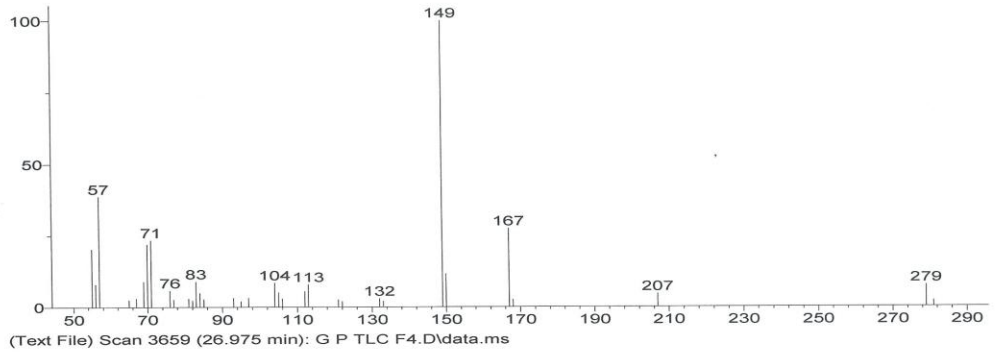
Fig 1. CHROMATOGRAMS OF THIN LAYER CHROMATOGRAPHY FRACTIONS 4,5,6



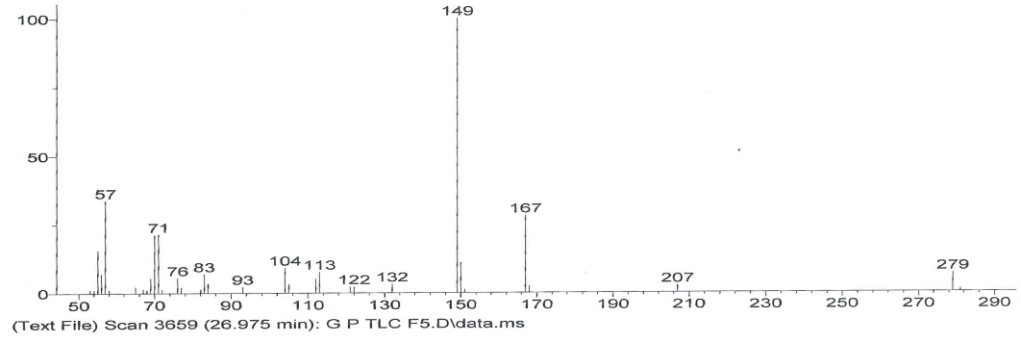
GPTLC- F4 Chromatogram **GPTLC – F5 Chromatogram** **GPTLC F6 Chromatogram**

Figure 2: Mass-Spectras of Thin Layer Chromatography fractions GPTLC fractions (4,5, and 6)

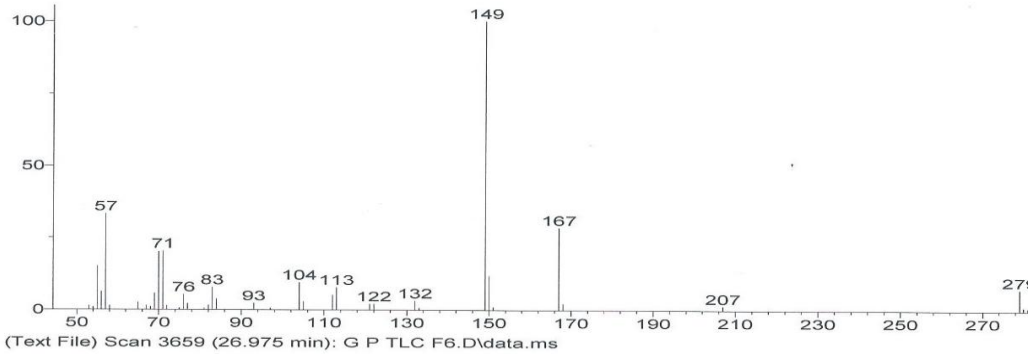
Spectra 4



Spectra 5



Spectra 6



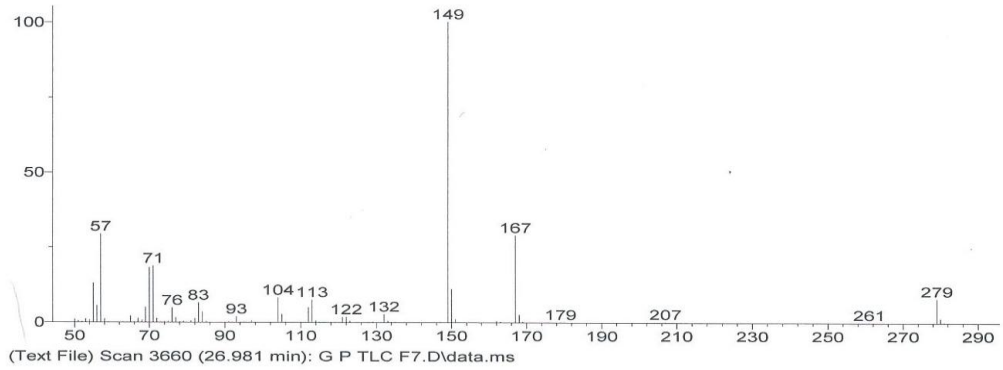
From the GC/MS results, it is obvious, TLC F4, F5 and F6 have chromatogram peaks at Rt 26.975- this means the compound is the same and have similar mass spectras.

Interpretation of mass spectra of 207 and 278. 207 is indicative of alkyl group of hexyl chain, cracking at the 5th carbon atom of the alkyl chain leaving a molecular ion of 207 from this logical discussion the compound is 1,2-Benzenedicarboxylic acid, ethylhexyl ester with a molecular mass of 278.

Figure 3: Chromatogram and Spectra of GPTLC fraction 7

Chromatogram 7

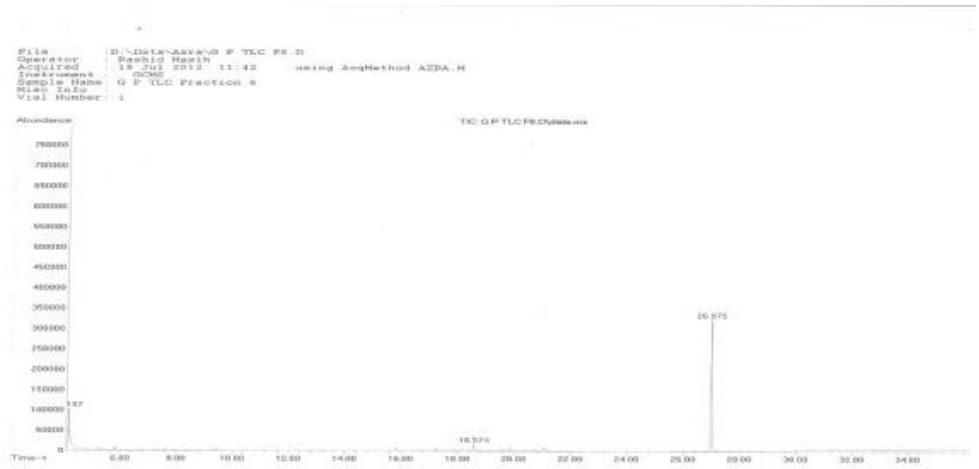
Spectra 7



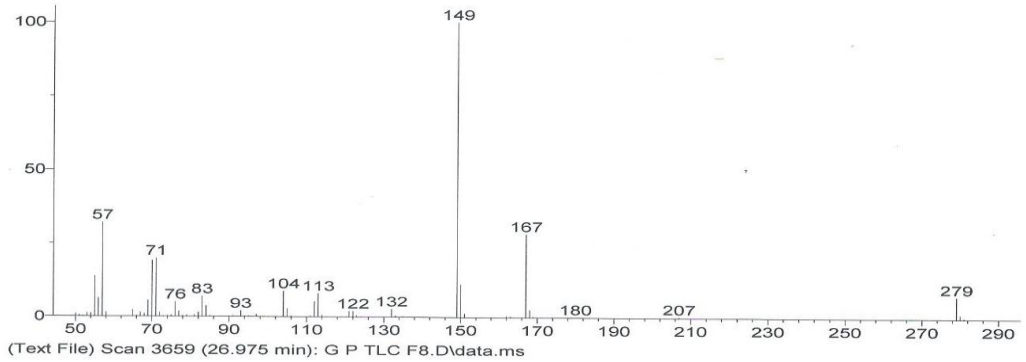
TLC fraction 7 (fig.3), has Rt 26.981 in chromatogram has a single peak which has properly separated. In the Mass Spectra, fragments show 180, 207, 261, 279. The distinct fragmentation 261 is due to OH group release from the compound. 207 shows 5 carbon breaking off from the 8 carbon chain. Since its an octyl, it can also break at 7 carbons giving us 179/180 molecular ion. From this evaluation the compound is 1,2-Benzenedicarboxylic acid, mono(n-octyl) ester which has 278 as its molecular mass.

Figure 4: Chromatogram and Spectra of GPTLC fraction 8

Chromatogram 8



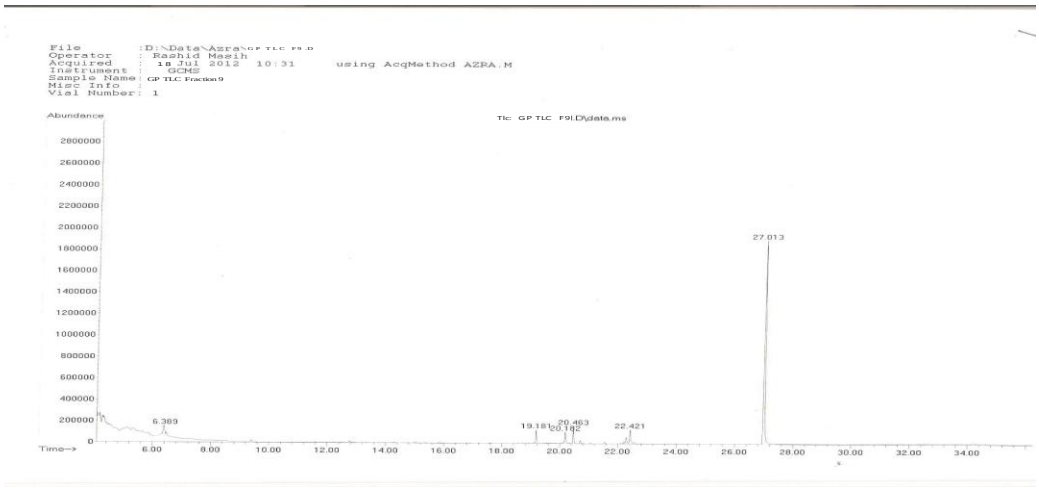
Spectra 8



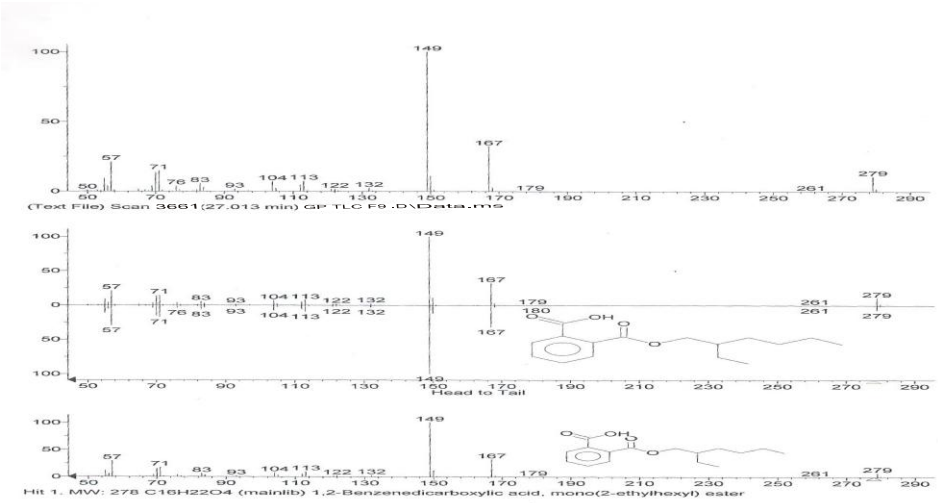
Looking at the chromatogram of TLC F8, Rt is 26.975 and a single peak. Even though this seems to be the same as TLC F4, F5 and F6 but on the basis of MS it is different. The MS shows fragments as 180, 207 both indicative of break of 5 or 7 carbons from the chain of a heptyl. Our compound is 1,2-Benzenedicarboxylic acid, heptylmethyl ester whose molecular mass is 278.

Figure 5: Chromatogram and Spectra of GPTLC fraction 9

Chromatogram 9



Spectra 9



TLC F9 has a clean single peak at Rt 27.013 in the chromatogram. Mass spectra has fragmentation 180, 261, 279.

Looking at the chain cracking pattern is on C5 so if it cracks the ethyl will go with 7 carbon moiety leaving behind 180 molecular ion. It can not crack on C4 and this peak is identified as 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester, with molecular mass of 278.

Further to our discussion, we have a table to summarize the differences of phthalates.

Table: 3. Mass Spectrum Differences for Phthalates

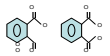
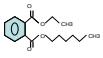
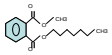
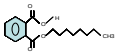
S/N	Compound	Molecular weight	Characteristic Peaks m/z 149, Base Peak, m/z 167 Daughter	m/z 207 m/z 279	m/z 180 207 279	m/z 180 207 261 279
1.	Diisobutyl Phthalate	278				
2.	Dibutyl Phthalate	278	Same Same			
3.	Ethylhexyl Phthalate	278	Same Same			
4.	Heptylmeth yl Phthalate	278	Same Same			
5.	Mono-octyl Phthalate	278	Same Same			
6.	Mono-(2-ethylhexyl) Phthalate	278	Same Same			

Table:3. Mass Spectrum Differences for Phthalates Cont'd

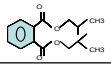
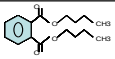
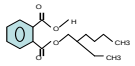
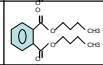
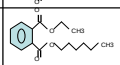
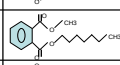
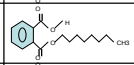
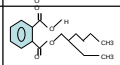
m/z 180, 261, 279	m/z 205, 223, 279	m/z 205, 223, 279
D S		
		
		

Table: 4. Phthalates in fraction 1 (CC separated)

S/N	RETENTION TIME	MOLECULAR FORMULAR	MOLECULAR WEIGHT	STRUCTURE	NAME	UREA / THIO UREA ADDUCTION	GLASS PLATE TLC 9 FRACTIONS	ACTIVITY
1	19.537	C16H22O4	278		Diisobutyl Phthalate	Non Thiourea adducted (Benzene)	N/A	Activity KNOWN
2	20.713	C16H2204	278		Dibutyl Phthalate	Non Thiourea adducted (Benzene)	N/A	Activity KNOWN
3	26.975	C16H2204	278		Ethylhexyl phthalate	Thiourea adducted (n-Hexane)	Fractions 4,5, 6	Activity not tested yet NEW COMPD.
4	26.975	C16H2204	278		Heptylmethyl phthalate	ThioUadducted (n-Hexane)	8	Active NEW COMPD.
5	26.981	C16H2204	278		Mono-octyl phthalate	ThioUadducted(n-Hexane)	7	Not Active NEW COMPD.
6	27.013	C16H2204	278		Mono(2-ethylhexyl) phthalate	Non Thio U adducted (Benzene)	9	Very Active KNOWN

Out of these compounds sixth compound is (91%). Others (1st – 5th)about 9% (5th>>3rd and 4th). 1st and 2nd very low

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