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ESTIMATION OF LIMIT OF DETECTION (LOD), LIMIT OF QUANTIFICATION (LOQ) AND MACHINE STANDARDIZATION BY GAS CHROMATOGRAPHY

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Abstract

An experiment was conducted in the pesticide and environmental toxicology laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) in 2014 to establish Limit of detection (LOD) and limit of quantification (LOQ) along with machine standardization for method validation in chemical analysis by gas chromatography. This work compares three methods based on the International Conference of Harmonization (ICH) and EURACHEM guidelines, namely, signal-to-noise, blank determination, and linear regression, to estimate the LOD and LOQ for volatile organic compounds (VOCs) by experimental methodology using gas chromatography. Five VOCs, i.e. toluene, ethylbenzene, iso-propylbenzene, *n*-propylbenzene and vinylbenzene, were chosen for the experimental study. The results indicated that the estimated LODs and LOQs were not equivalent and varied by a factor of five to six for both LOD and LOQ for different tested methods. After conditioning of the GC machine, the baseline of chromatogram was set '0' position by generating straight line horizontally. The peak of DDT was produced at 12.34 minute. The retention time was same for all standard concentration. Moreover, the percent deviation of peak area was not varied more than 5%. Thus it could be remarked that the machine was standardized. It is therefore essential to have a clearly described procedure for estimating the LOD and LOQ during method validation to allow inter-laboratory comparisons.

Keywords: Gas Chromatography, LOD, LOQ, Standardization, VOCs

Introduction

The quality of an analytical method developed is always appraised in terms of suitability for its intended purpose, recovery, requirement for standardization, sensitivity, analyte stability, ease of analysis, skills technicians, time and cost required. Limit of detection (LOD) and limit of quantification (LOQ) are two important performance characteristics in method validation. In general, the LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test (NATA, 1998 and Sanagi *et.al.*, 2009).

Method validation enables analyst to demonstrate that a procedure is fit for purpose.

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For an analytical result to be fit for its intended purpose it must be sufficiently reliable. Any decision based on it can be taken with confidence. Thus the method of performance must be validated and the uncertainty on the result, at a given level of confidence needs to be estimated (Rambla *et al.*, 2009). Uncertainty should be evaluated and quoted in a way that must be recognized, internally consistent and easy to interpret. Most of the information required to evaluate uncertainty can be obtained during validation of the method (Hirsch, 1989).

Moreover, method validation is necessary in analytical laboratory to ensure that reliable analytical procedures those are used under defined conditions. It is internationally recognized as an essential part of a comprehensive quality assurance system in analytical chemistry (Swartz and Ira, 2012). In order to demonstrate that the method is suitable for its intended purpose, it must meet certain performance characteristics. During the post-approval commercial production stage of bulk drugs and pharmaceutical products, the official or in-house test methods that have resulted from the analytical method development and validation process cycle become indispensable for reliable monitoring of the integrity, purity, quality, strength and potency of the manufactured products (Shrivastava and Vipin, 2011).

There is often a need to transfer methodology from one laboratory to another and/or to include it in official compendia. Such exercises include the use of a method by large numbers of people, in various laboratories across the globe and on instruments manufactured by different manufacturers, thereby causing a greater probability of decreased reproducibility and reliability. These problems can be foreseen and avoided by thorough validation of the analytical method. The important the analytical methods include the characteristics such as applicability, specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and ruggedness (Chandran and Singh, 2007). LOD and LOQ are two fundamental elements of method validation that define the limitations of an analytical method (Bernal, 2014).

This study was designed to describe the comparison of the three methods to estimate LOD, LOQ and machine standardization based on practical approaches to a gas chromatographic method. Gas chromatography (GC) is chosen for the study because it is an important analytical tool which is available in almost major chemical analytical laboratory and it is suitable for almost any mixture of components that exhibit reasonable volatility. Considering the above facts, the present investigation was undertaken to determine the LOD and LOQ in GC-FID and to standardize the GC-ECD for chemical analysis.

Materials and Methods

The study was conducted in the pesticide and environmental toxicology laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) during July to September, 2014.

Methods of estimating LOD and LOQ.

Several possible conceptual methods regarding LOD and LOQ, followed a somewhat different principle. Depending on the principle, the values of LOD and LOQ

could vary greatly which make it difficult for comparative purposes. In this study, the three methods (namely, signal-to-noise, blank determination, and linear regression) based on International Conference of Harmonization (ICH, 1996) and EURACHEM, 1998 guidelines have been described.

Signal- to-noise

By using the signal-to-noise method (ICH, 1996), the peak-to-peak noise around the analyte retention time was measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio was estimated. The noise magnitude was measured either manually on the chromatogram printout or by auto-integrator of the instrument. A signal-to-noise ratio (S/N) of three: one (3:1) is generally accepted for estimating LOD and signal-to-noise ratio of ten: one (10:1) is used for estimating LOQ (Fig. 1). This method was commonly applied to analytical methods that exhibit baseline noise.

Blank determination

The blank determination was applied when the blank analysis gave results with a non-zero standard deviation. LOD was expressed as the analyte concentration corresponding to the sample blank value plus three standard deviations and LOQ was the analyte concentration corresponding to the sample blank value plus ten standard deviations as shown in the following equations:

$$LOD \cong \bar{X}b_1 + 3S_{b1} \text{ and } LOQ \cong \bar{X}b_1 + 10S_{b1}$$

Where, x_b is the mean concentration of the blank and s_b is the standard deviation of the blank.

Linear regression

For a linear calibration curve, it was assumed that the instrument response *y* is linearly related to the standard concentration *x* for a limited range of concentration. It could be expressed in a model such as:

y = a + bx (Where a is intercept and b is slope)

This model was used to compute the sensitivity *b* and the LOD and LOQ. Therefore, the LOD and LOQ could be expressed as:

$$LOD \cong 3 \text{ x } \frac{Sa}{b} \quad \text{and} \quad LOQ 10 \text{ x } \frac{Sa}{b}$$

Where s_a is the standard deviation of the response and b is the slope of the calibration curve. The standard deviation of the response can be estimated by the standard deviation of either y-residuals, S_{res} or y-intercepts, S_{Yo} of

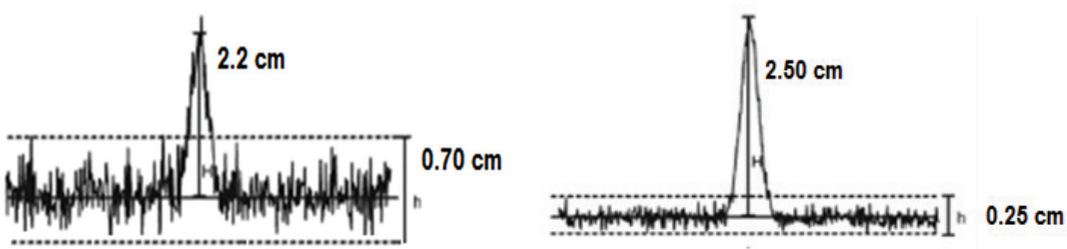

Fig. 1. Signal-to-noise of 3:1 (left) and 10:1 (right)

regression lines. This method was applied in all cases, and it was most applicable when the analysis method did not involve background noise. It used a range of low values close to zero for calibration curve, and with a more homogeneous distribution would result in a more relevant assessment.

Experimental

Standards and reagents

The reference standards for toluene, ethylbenzene, iso-propylbenzene, *n*-propylbenzene and vinylbenzene with purity higher than 98% were supplied by registered chemical importer of Bangladesh. Dichloromethane (DCM) was obtained from Merk, Germany (Fig. 2). A stock solution of 1000 µg/mL in DCM was prepared separately for the five test compounds. A mixture solution at a level of 10µg/mL was prepared from the stock solution above using the same solvent. A series of working standard solutions ranging from 0.2µg/mL to 4.0µg/mL were prepared from the mixture solution (10µg/mL) by dilution in DCM.

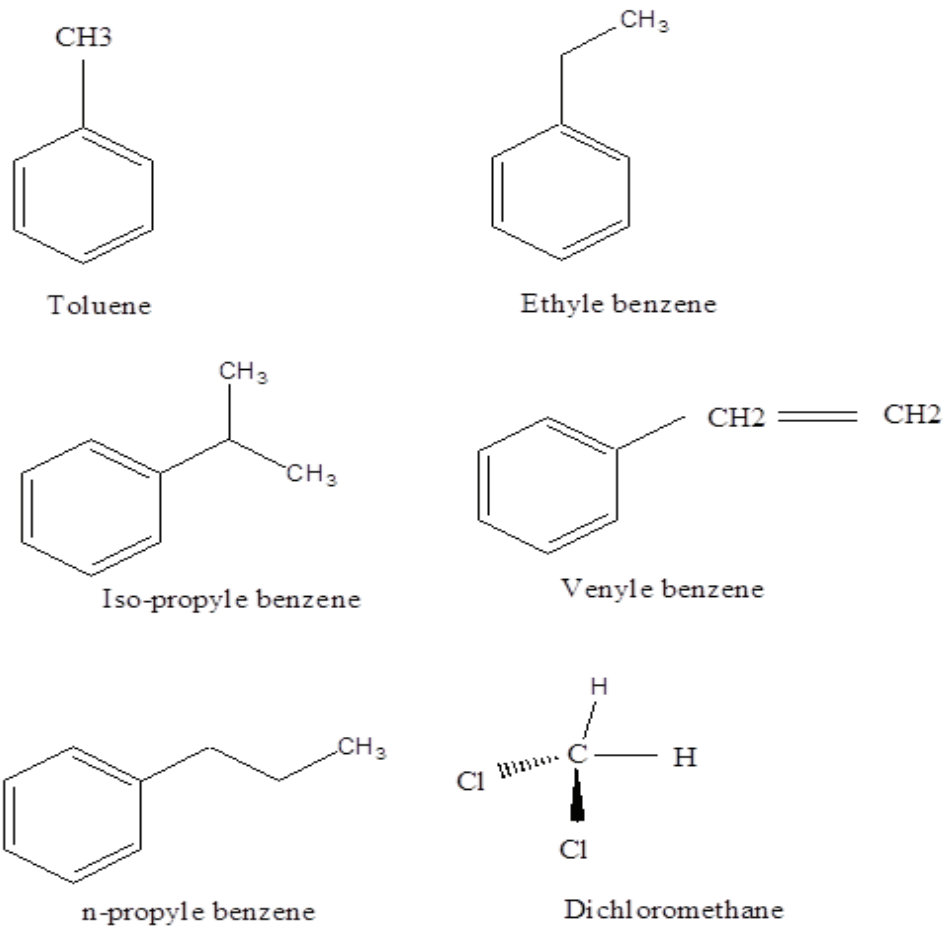

Fig. 2. Chemical structures of the standards and reagents

Instrument and chromatographic conditions

A Shidmazu (Kyoto, Japan) Model GC-17Agas chromatograph equipped with flame-ionization detector (FID) Electron Capture Detector (ECD), and a Shimadzu AOC-20i autosampler was used. A capillary column (30 m × 0.25 mm I.D., Varian) with 0.25 µm film thickness was used with helium as carrier gas at a rate of 8 ml/min. The initial oven temperature was held isothermically at 50°C for 5 min, increased to 110°C at 4°C min-1, then raised to 230°C at20°C min-1 and maintained at 230°C for 4 min. The injector was set at split mode (1:10), injector temperature was held constant at 220°C, and injection volume was maintained at 1.0 µL. The FID and ECD temperature was kept at 240°C. The chromatographic data were analyzed and processed using a Shimadzu Class-VP 4.3 acquisition program.

Procedure

For the purpose of comparison between different approaches, DCM was used as a blank sample. The levels of concentration for each compound and number of replicates and measurements performed by using the three methods are listed in Table 1.

In order to measure independently, each replicate was performed on a newly prepared standard solution and the replicates were carried out on different days to take into consideration of run effect. The run effects accounts for day-to-day variations in theanalytical system, such as batches of reagents, recalibration of instruments, and changes in the laboratory environment. For generation of calibration curve, the concentration levels were chosen in a range around LOD and LOQ to ensure the homoscedasticity, the independence of the area dispersion in relation to analyte quantity.

Data Analysis

Microsoft office excels (2016) spreadsheet was chosen to store, display raw data and to perform statistical analysis with the built-in statistical functions.

Results and Discussion

Under the instrumental operating conditions as described above, the Volatile Organic compounds (VOCs) were perfectly separated as shown in Fig 3. The elution order for the five compounds is toluene, ethylbenzene, iso-propylbenzene, n-propylbenzene and vinylbenzene.

Signal- to- noise

The noise value was calculated based on the peak height of the blank (DCM) around the retention time of each analyte using auto-

Table 1. Concentrations of test compounds and number of replicates used for experimental application

Method	Levels (µg/mL), p	Replicates, n	Measurements, np
Signal-to-noise	0, 0.4	3	6
Blank determination	0, 0.4	3	6
Linear regression	0.2, 0.4, 0.6, 0.8,1.0	3	15

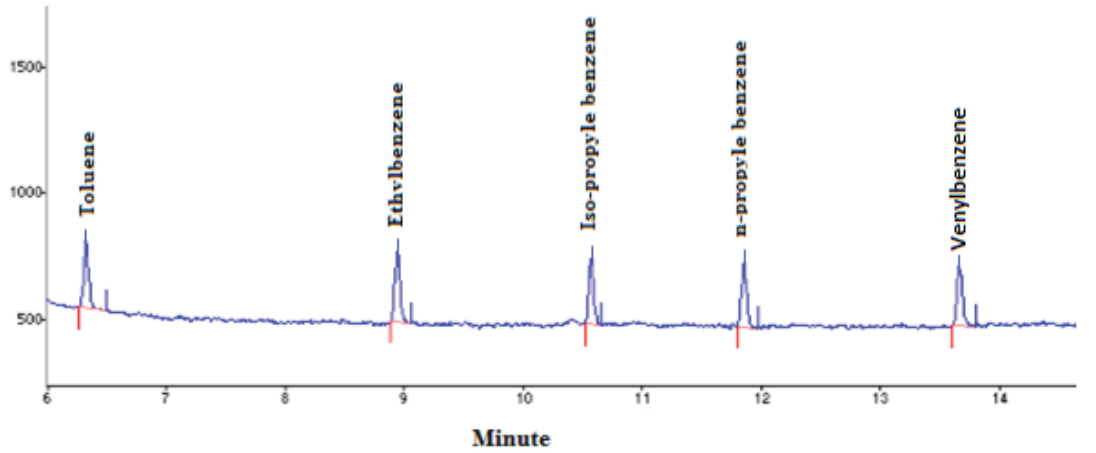

Fig. 3. GC-FID chromatogram of a 1.0 µg/mL standard solution of the five test compounds

integrator. LOD was estimated as three times noise value and LOQ was estimated as ten times noise value as shown in Table 2.

This method is widely used for instrument method such as gas chromatography as it is easy to implement. However, the stability of the instrument response on day-to-day basis will affect the results obtained. In this study, 3 independent numbers of blank together with the standard solution at the level of 0.4 µg/mL were analyzed separately on different days. Due to the run effect of the instrument, the relative standard deviations for the 6 measurements ranged from 20% to 45% for the five test compounds. This method depends heavily on individual analyst's interpretation of how to obtain the magnitude of noise whether by manual measurement or using auto-integrator of the instrument. Therefore, the values obtained are difficult for comparison between different analysts or laboratories.

Blank determination

Blank determination was carried out by analysis of 3 independent sample blanks, the mean concentration and the standard

Table 2. Data obtained for each test compound based on signal-to-noise method

Compound	Mean peak height value (n)			
	Blank	% RSD	S/N = 3	S/N = 10
Toluene	16	41	49	163
Ethylbenzene	15	45	44	149
iso-Propylbenzene	15	38	45	151
n-Propylbenzene	14	25	41	142
Vinylbenzene	13	20	38	132

RSD = Relative standard deviation, S/N = Signal to noise ratio, n (number of peak hight) = 3

Table 3. The mean concentration and standard deviation of blank obtained using blank determination method

Compound	Blank (n)		LOD (µg/mL)	LOQ (µg/mL)
	Mean conc. $\bar{x}_b$, µg/mL	Standard deviation ($S_{b1)}$		
Toluene	0.054	0.022	0.12	0.28
Ethylbenzene	0.052	0.021	0.12	0.26
iso-Propylbenzene	0.056	0.018	0.11	0.23
n-Propylbenzene	0.051	0.013	0.09	0.18
Vinylbenzene	0.050	0.015	0.10	0.20

Note: n (number of blank) = 3

deviations of the blank results were calculated (Table 3).

The blank determination method as described in EURACHEM guide, LOD is estimated as 3 times more than the blank value as it assumes that a signal more than 3 times above the standard deviation of the sample blank value is likely to have arisen from the measured. For this approach, it needed a sample blank for each sample matrix has analyzed, and the estimated LOD and LOQ varied for different sample matrices. However, getting a true sample blank could be difficult and in certain situation, reagent blank was used as a blank. The LOD and LOQ estimated by using reagent blank did not take into consideration of matrix interference, and the estimated values can be smaller than that using true sample blank.

Linear Regression

Linear ordinary least squares regression parameters were calculated based on the analysis of 3 replicates of test compounds at five different concentration levels. The data obtained were used to compute the two coefficient of the calibration curve and also to perform a lack offit test, which was used to verify that the selected calibration domain was actually linear. The standard deviation of the blank was estimated by using both standard deviation of regression residual

Table 4. Parameters of linear ordinary least-squares regression for the five test compounds at five different levels of concentration (n)

Compound	Correlation coefficient, r	Slope, b	Y-intercept, a	Y-intercept standard deviation, s_{yo}	Residual standard deviation, s_{res}
Toluene	0.992	798	18	16	91
Ethylbenzene	0.990	804	18	18	102
iso-Propylbenzene	0.994	804	15	13	78
n-Propylbenzene	0.992	787	36	15	89
Vinylbenzene	0.995	802	17	13	73

Note: n (number of concentration) = 5

Table 5. Results of the statistical evaluation of the linear regression curve

Compound	Regression test		Lack of fit test	
	Observed value, F_{obs} (1, 24, 0.5%)	Critical value, F_{crit} 1, 24, 0.5%)	Observed value, F_{obs} (5, 24, 0.5%)	Critical value, F_{crit} (5, 24, 0.5%)
Toluene	7923.943		0.714	
Ethylbenzene	6175.352		0.157	
iso-Propylbenzene	10677.743	3.993	0.223	2.361
n-Propylbenzene	7827.221		0.286	
Styrine	12309.553		0.360	

(s_{res}) and y-intercept (s_{yo}) as shown in Table 4 and results of the statistical evaluation of the linear regression curve is shown in Table 5. The calculation in the table showed that the test for regression was significant while the *F* observed value for each analyte was much higher than the critical value of 3.993, which is corresponded to F (1, 69, 5%). This meant that the instrumental response was significantly correlated to the analyte concentration. When the lack-of-fit test was performed, the Fisher variable associated to the test for the error of model was smaller than the critical value of 2.361. It could be concluded that the error of model was not significant at the risk level of 5% and the proposed linearity domain could be accepted.

The linear regression method could help to solve the problem of difficulty in obtaining matrix blank for other methods. This was because calibration curve was prepared by sample addition method. From this study, the results showed that the y-intercept standard deviation and y-residual standard deviation varied greatly. The values of y-intercept standard deviation were much lower than those of y-residual standard deviation for the five analytes. These results were in agreement with the study reported by Vial and Jardy, 1997.

The values of LOD and LOQ obtained by this method can vary depending on the number of concentration levels, range of concentration used, number of measurement and data heteroscedasticity. The ordinary least-square regression is used in this case with the assumption that the data obtained are homoscedastic, if not; the weighted least-square regression is preferred.

Comparison of LOD and LOQ by different methods

Based on the experimental results, the LOD and LOQ were estimated for the different methods as summarized in Table 6 and Table 7. For LOD, the values obtained by signal-to-noise and blank determination methods were close to each other. The linear regression method by using s_{res} showed largest values of LOD and by using s_{yo} gave lowest values of LOD for all the analytes. For LOQ, similar trend S_{y0} observed for linear regression method as in LOD.

However, the signal-to-noise method presented about two times values of LOQ as compared to blank determination method. The LOQ values obtained by blank determination were comparable to linear regression method using sy_o. From these findings, it seemed that not all

Table 6. Summary of estimated LOD by different methods

Compound	Estimated LOD, µg/mL			
	Signal to noise	Blank determination	Linear regression	
			s_{y0}	s_{res}
Toluene	0.16	0.12	0.06	0.34
Ethylbenzene	0.16	0.12	0.07	0.38
iso-Propylbenzene	0.17	0.11	0.05	0.29
n-Propylbenzene	0.15	0.09	0.06	0.34
Vinylbenzene	0.15	0.10	0.05	0.27

S_{y0} = Y intercept, S_{res} = Regression residual

Table 7. Summary of estimated LOQ by different methods

Compound	Estimated LOD, µg/mL			
	Signal to noise	Blank determination	Linear regression	
			s_{y0}	s_{res}
Toluene	0.54	0.28	0.20	1.15
Ethylbenzene	0.52	0.26	0.22	1.27
iso-Propylbenzene	0.56	0.23	0.17	0.97
n-Propylbenzene	0.51	0.18	0.20	1.13
Vinylbenzene	0.50	0.20	0.16	0.91

S_{y0} = Y intercept, S_{res} = Regression residual

the methods used to estimate LOD and LOQ in this study were equivalent. The differences between the smallest and the largest s_{y0} values estimated by different methods could vary by a factor of five to six for both LOD and LOQ.

Reliability of the LOD and LOQ estimates

It is difficult to compare the degree of reliability of the LOD and LOQ estimates. In order to check predetermined LOD and LOQ values obtained by statistical or empirical approach, the laboratory could obtain test material with known quantities of analyte at the level of the estimated LOD and LOQ limit, or fortified true blank sample at the level of estimated LOD and LOQ levels, and determine the precision as expressed in the relative standard deviation (RSD). The RSD of 10% is generally acceptable for LOQ and 33% for LOD (Thomsen *et al.,* 2003). The LOD and LOQ values obtained using different methods can vary significantly based on this experimental study using GC method. In the absence of a uniform definition and guideline for LOD and LOQ determinations, during method validation, the exact procedure for determination of LOD and LOQ must be clearly stated in the documented method so that the estimated LOD and LOQ values could be used for comparison by other analysts or laboratories.

Machine Standardization

Gas Chromatograph (GC-17A) with ECD model Rtx-CL pesticide was conditioned to obtain the baseline of the chromatogram at zero level constantly. The machine was run

for one hour by adjusting column temperature 50^0C, oven temperature 70^0C, Pressure 100 kPa, purge flow 3 ml/Min and make up pressure 75 kPa (Sharma, 2015). Thus, the baseline of chromatogram was set '0' position by generating straight line horizontally.

The standard solution of o,p'-DDT was prepared at concentration of 1.0, 2.5, 7.5, 10.0 ppm. The working standard solution was injected thorough auto-injector at 1µL per injection setting above mentioned instrumental parameter on GC. The chromatogram of solvent and standard solution shown in Fig.4. The solvent (n-hexane) and standard solution of DDT shows peak at retention time 1.41and 12.34 minute respectively.

The standard solution of DDT at concentration of 0.10, 0.25, 0.50, 1.0 ppm were analyzed on GC as a single run. The chromatograms were produced at same retention time (12.34 minute out of 14 minutes' program). The area developed by different concentrations of DDT was shown in table 8.

The standardization data (Table 8) shows that the retention time was same for all standard concentration. Moreover, the percent deviation of peak area (considering each of the concentrations area) were not varied more than 5%. Percent deviation considering 0.1 ppm area was varied 1.81% to 4.51 %. Percent deviation Considering 0.25 ppm, 0.50 ppm and 1.0 ppm ranged from 0.18%

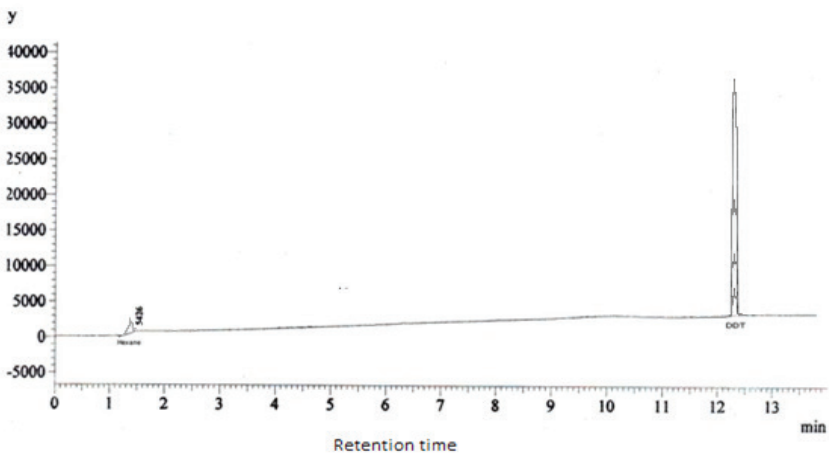

Fig. 4. Chromatogram of standard solution DDT

Table 8. Standardization of GC based on chromatogram area and retention time

Standard Conc. (ppm)	Retention times (min)	Area Produced	% deviation Considering 0.1 ppm area	% deviation Considering 0.25 ppm area	% deviation Considering 0.50 ppm area	% deviation Considering 1.0 ppm area
0.10	12.34	2975	0.00	4.33	1.78	4.16
0.25	12.34	7773	4.51	0.00	2.65	0.17
0.50	12.34	15144	1.81	2.60	0.00	2.42
1.00	12.34	31040	4.34	0.18	2.48	0.00

to 4.33%, 1.78% to 2.65% and 0.17% to 4.16%, respectively. Ong (2002) reported similar investigation on chromatogram area in Comprehensive Two-Dimensional Gas Chromatography. At least 5% variation of the peak area might be considered in gas chromatography. Thus it may be ensured that the GC machine is perfect and properly standardized for chemical analysis.

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