INTRODUCTION

Population growth was increasing rapidly. It led to the birth of the industrialization era. In this era, the development of science and technology was more advanced. One impact of science and technology is the use of chemicals in many work processes. Currently, various chemicals are widely used in industries, such as food additives, pesticides, metals and compounds, as well as various organic chemicals including organic solvents [1]. One of the most widely used organic solvents in the industry is hexane.

Hexane is a non-polar solvent [2]. Hexane is a very good and cheap solvent. These compounds are found in glue, varnish, paint, and ink. Commercially, hexane is used to extract vegetable oil from various grains such as soybeans and cottonseed. These compounds are also widely used in the pharmaceutical and cosmetic industries [3].

Hexane is very volatile and will be metabolized in the body. The main metabolite of hexane is 2,5-hexanedione [3]. The mice that were exposed to hexane were expected to quantifying the 2,5-hexanedione level in the urine sample. The method was simple, selective, and sensitive for the determination of 2,5-hexanedione in the urine.

MATERIALS AND METHODS

Preparation of standard 2.5-hexanedione

Standard of 2, 5-hexanedione (Sigma-Aldrich), dichloromethane (Sigma-Aldrich), sodium sulphate anhydrous (EMSURE®), aquabidest, pooled urine.

Tools

The tools used in this study were a set of GC (Agilent Technologies®) with Flame Ionization Detector (FID), crosslinked methyl siloxane, centrifugation (Eppendorf AG®), analytical balance (Mettler Toledo®), vortex (Digisystem®), flask, test tube, micropipette 10-100 µl and 100-1000 µl (ACURA 825®), and glass tools commonly used in Analytical Laboratory. Data analysis and interpretation using Microsoft Excel 2013 64-bit software and online GC Software and Agilent Technologies GC offline.

Preparation of standard 2,5-hexanedione

Standard of 2,5-hexanedione was prepared by dissolved 5 mg standard with 10 ml dichloromethane. Then the standard solution was diluted to obtain the concentration of 0.1; 0.2; 0.4; 1.2; 2 µg/ml.
Preparation of pooled urine

Pooled urine prepared by mixing urine with aquabidest with a ratio of 20:80. The prepared urine is used as a matrix in the analysis by spike method.

Preparation of spiked urine

Standard of 5 mg 2,5-hexanedione was prepared, then added 20 ml of pooled urine. The solution was diluted to obtain the concentration of 0.1; 0.25; 0.5 and 2 μg/ml.

**GC condition**

The chromatographic system was optimized by injecting 2 μg/ml standard under the following conditions [12]: Columns: HP-5 (Crosslinked methyl sioxane) capillary columns, 30 m x 0.320 mm long, film thickness 0.25 μm, detector temperature 300 °C, injector temperature 250 °C, column temperature was programmed from 30 °C to 325 °C. The initial temperature of 30 °C was held for 3 min, gradually increased to 60 °C at a rate of 6 °C/min and held for 5 min. Then the temperature was increased to 90 °C at a rate of 15 °C/min. Helium gas flow rate: 2 ml/min with detector Flame Ionization Detection (FID) and the injection volume was 1 μl.

**System suitability test**

The system suitability test was performed by injecting a standard solution of 2 μg/ml under optimum conditions. Then determined the Retention Time, Tailing Factor (TF), Resolution (Rs), Number of Theoretical Plate (N), and High-Efficiency Theoretical Plate (HETP) from the standard solution 5 times [13]. From the system suitability test can be seen at fig. 1.

**Validation of analysis method**

Validation methods include linearity, accuracy, specificity, the limit of detection, and limit of quantification [14].

**Linearity**

The linearity was determined from the standard curve. Preparation of the standard curves with external standard method by preparing the standard of 2,5-hexanedione at concentration 0.1; 0.2; 0.4; 1.2; 2 μg/ml.

**Accuracy**

Accuracy was done by prepare the spike urine solution with 3 different concentrations (0.1, 0.5, and 2 μg/ml). Each concentration was triplicate. Then, determined the recovery. Recovery (%CV) should be between 80-120% [14].

**Precision**

The precision test was performed by prepare the spike urine solution with 3 different concentrations (0.25, 0.5 and 2 μg/ml). Each concentration was triplicated. The value of precision was expressed by the Relative Deviation Standard (RSD) response. The RSD should be ≤ 2.0% [14].

**Limit of quantification and limit of detection**

Limit of detection (LOD) and Limit of Quantification (LOQ) was obtained by the determination based on the standard deviation and slope. LOD and LOQ were calculated by the following formula [13]:

\[
LOD = \frac{3 \times SD}{\text{slope}} \\
LOQ = \frac{10 \times SD}{\text{slope}}
\]

Note:

SD = Standard deviation of standard curve intercept
LOD = Limit of Detection
LOQ = Limit of Quantification

**Specificity**

Specificity was determined by analyzing a standard solution 0.4 μg/ml and a spike urine 0.5 μg/ml. The specificity was determined by comparing the retention time of the standard 2,5-hexanedione chromatogram and the spike solution chromatogram and then determined the value of coefficient variation [15].

**Preparation of test solution**

Test solution was prepared by mixing 2.5 ml of 2,5-hexanedione standard solution with 2.5 ml dichloromethane, then vortex it. The solution was centrifuged at 3000 rpm for 10 min. Its organic phase was taken, then 500 mg of anhydrous sodium sulphate was added. It was centrifuged again at 3000 rpm for 10 min. Take the dichloromethane phase. Inject to GC-FID.

**RESULTS AND DISCUSSION**

**Preparation of pooled urine**

Pooled urine preparation needs to be done because the urine pooled will be used as a matrix for spike solutions. Pooled urine was made by mixing urine with aquabidest with a ratio of 20:80.

**Preparation of spiked urine**

Spike urine preparation was used for the analysis of validation parameters such as accuracy, precision, specificity and system suitability testing. Spike urine preparation was done by added the standard into the pooled urine to obtain the required various concentration for the analysis. Urine was spiked into several concentrations of 0.1; 0.25; 0.5 and 2 μg/ml.

**Optimization of GC condition**

The optimum condition of GC for 2,5-hexanedione was using column temperature programmed at 30 °C-325 °C and helium gas flow rate in column 2 ml/min. Used also O2 gas to heat the FID detector.

**System suitability test**

System suitability test was a series of experiments conducted to ensure that a method will produce acceptable accuracy and precision. The chromatogram of an analyte using the optimum condition can be seen at fig. 1.

The parameters used to determine the suitability of the system in this study include retention time (RT), theoretical plate (N), high-efficiency theoretical plate (HETP), tailing factor (TF) and resolution (Rs) in standard solution 5 times [13]. From the system suitability test, the CV, RT, N, HETP, and Rs values appropriated with the system suitability parameters. The result of the system suitability test can be seen in table 1.

**Validation of the analytical method**

Validation of the analytical method was used to ensure that the methods used corresponding with the requirements in use so that the results obtained are acceptable and reliable. In this research, the validation parameters used are linearity, accuracy, precision, the limit of detection, the limit of quantification, and specificity [16].

**Linearity**

Linearity test results obtained from the equation of 2.5-hexanedione calibration curve \( y = 4.0526x + 0.0787 \) with the value of correlation coefficient 0.99963. Analysis method was valid if linearity parameter was>0.99 [15].

**Accuracy**

The accuracy of an analytical procedure describes the closeness between the measured value and the value received either the convention value or the reference value, or the actual value [13]. The calculation of % recovery can be seen in table 2.
Fig. 1: Chromatogram of 2,5-hexanedione with concentration (a) 0.1 μg/ml (b) 0.2 μg/ml (c) 0.4 μg/ml (d) 1.2 μg/ml

### Table 1: System suitability test results

| Parameters | Results          |
|------------|------------------|
| % CV RT    | 0.013±0.001      |
| N          | 806±68.60        |
| HETP       | 0.00372±0.0008   |
| TF         | 3.20±0.54        |
| Rs         | 13.23±2.34       |

Number of experiments = 5. Note: CV: Coefficient variation, RT: Retention time, N: Number of Theoretical Plate, HETP: High-Efficiency Theoretical Plate, TF: Tailing factor, Rs: Resolution

### Table 2: Accuracy test results

| Theoretical concentration (μg/ml) | Recovery (μg/ml) | % Recovery | Average % recovery |
|----------------------------------|------------------|------------|--------------------|
| 0.1                              | 0.120            | 120        | 114.13±10.16       |
|                                  | 0.120            | 120        |                     |
|                                  | 0.102            | 102.4      |                     |
| 0.5                              | 0.580            | 116        | 114±0.721          |
|                                  | 0.600            | 120        |                     |
|                                  | 0.530            | 106        |                     |
| 2                                | 2.040            | 102        | 99.16±0.38         |
|                                  | 1.940            | 95         |                     |
|                                  | 2.010            | 100.5      |                     |

Note: Number of experiments = 3
Accuracy was obtained by calculating the recovery of urine spike solution. The recovery was obtained by calculating the difference between the concentrations of the results obtained with the blanks. The % recovery in the three concentrations shows that in all three concentration appropriated with the requirements of the accuracy parameter, % recovery requirement is 80-120% [15].

### Precision

Precision was a measure of the repetition of a homogenous sample measurement series. This test can be performed by preparing three different concentrations of the target analytical concentration [13]. The results of calculation %CV of the test of the precision can be seen in table 3.

| Theoretical concentration (µg/ml) | Measurable (µg/ml) | Average         | %CV  |
|----------------------------------|-------------------|-----------------|------|
| 0.25                             | 0.33              | 0.33+0.0082     | 2.47 |
|                                  | 0.34              |                 |      |
|                                  | 0.32              |                 |      |
| 0.50                             | 0.58              | 0.57+0.0294     | 5.16 |
|                                  | 0.60              |                 |      |
|                                  | 0.53              |                 |      |
| 2.00                             | 2.04              | 2.00+0.033      | 1.65 |
|                                  | 1.96              |                 |      |
|                                  | 2.01              |                 |      |

Number of experiments = 3

The calculation was performed on the concentration obtained from spiked urine. In the test, urine blanks are also measured to know whether there was 2,5-hexanedione content in it or not. Based on the research, the urine blank does not contain 2,5-hexanedione. Based on the results of the research, the % of the coefficient of variation obtained ranged from 1.65 to 5.16%. The criteria for receiving precision depends on the concentration of the analyte. For unit 1 µg/ml (ppm), % CV should not be more than 11% [13]. Based on these criteria, this study was appropriate with the precision requirement.

#### Limit of quantification and limits of detection

Limit of Detection (LOD) indicates the smallest number of analyte in the sample that can give a significant response, while the Limit of Quantification (LOQ) indicates the smallest number of analyte in the sample that can appropriate with the accuracy and precision criteria [14]. Based on the calculation results, the limit of detection was 0.054 µg/ml and the limit of quantification was 0.18 µg/ml. Result of LOD and LOQ can be seen in table 4.

| Concentration (µg/ml) | Width Area (y) | Averages         | LOD  | LOQ  |
|-----------------------|----------------|------------------|------|------|
| 0.1                   | 0.33           | 0.33+0.010       | 0.054| 0.18 |
|                       | 0.32           |                  |      |      |
|                       | 0.34           |                  |      |      |
| 0.2                   | 0.34           | 0.34+0.010       |      |      |
|                       | 0.33           |                  |      |      |
|                       | 0.35           |                  |      |      |
| 0.4                   | 0.32           | 0.32+0.005       |      |      |
|                       | 0.33           |                  |      |      |
|                       | 0.32           |                  |      |      |
| 1.2                   | 0.58           | 0.58+0.010       |      |      |
|                       | 0.57           |                  |      |      |
|                       | 0.59           |                  |      |      |
| 2                     | 0.60           | 0.60+0.015       |      |      |
|                       | 0.62           |                  |      |      |
|                       | 0.59           |                  |      |      |

Number of experiments = 3

#### Specificity

Specificity tests were performed to know the appropriateness of an analyte in the presence of other components in the sample matrix, such as impurities, product degradation, and matrix components. The results of the specificity test can be seen in table 5.

| Concentration | Retention time (min) |
|--------------|----------------------|
| Standard (0.4 µg/ml) | 9.798               |
| Standard (0.4 µg/ml) | 9.801               |
| Standard (0.4 µg/ml) | 9.807               |
| Spike (0.5 µg/ml)   | 9.803               |
| Spike (0.5 µg/ml)   | 9.801               |
| Spike (0.5 µg/ml)   | 9.801               |
| Average            | 9.801               |
| SD                 | 0.002               |
| % CV               | 0.027               |

Number of experiments = 3

Table 5 shows % CV of the standard and spike solution was 0.027%. It shows that there was no significant change. Therefore, this method can be used as a specific analysis for 2,5-hexanedione.

The proposed method gives a simple and sensitive method for the determination of 2,5-hexanedione. The previous methods need the...
derivation process. Maestri et al. [17] had used dansyl hydrazine; 1,3-diacetyl benzene (1,3-DAB) to react with 2,5-hexanediene before analyzed using high-performance liquid chromatography with fluorescence detection. Gori et al. [9] has developed the analytical method for the determination of 2,5-hexanediene with acid hydrolysis and derivatization step using 2,4-dinitrophenylhydrazine at 70 °C for 20 min. Moreover, the detection limit from this study was 0.054 μg/ml, was more sensitive than the previous study from Fedtke and Bolt [12] that was 0.12 μg/ml.

CONCLUSION

The optimum condition for the analytical method of 2,5-hexanediene by GC (Gas Chromatography) was carried out by using HP-5 (crosslinked methyl siloxane) capillary columns, 30 m x 0.320 mm long, film thickness 0.25 μm, detector temperature was 300 °C, injector temperature 250 °C, column temperature was programmed from 30 °C to 325 °C. The helium gas flow rate was 2 ml/min, and the injection volume was 1 μL.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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