

VALIDATION AND ANALYSIS OF DIALLYL DISULFIDE AND DIALLYL TRISULFIDE IN GARLIC (*ALLIUM SATIVUM* L.) USING GAS CHROMATOGRAPHY

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ABSTRACT

Objective: The purpose of this research was to optimize and validate a method for measuring the levels of diallyl disulfide (DADS) and diallyl trisulfide (DATS) in garlic and single clove garlic.

Methods: The analysis was performed using gas chromatography (GC) equipped with an HP-1 column and a flame ionization detector. The initial column temperature was set at 140°C and increased at 1°C/min to 180°C. The injector and detector temperatures were set to 200°C, the carrier gas flow rate was 0.80 mL/min, and the injection volume was 1.0 µL. The optimized conditions of analysis were then validated which included selectivity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

Results: Using the validated assay and a concentration range of 0.5–20 µg/mL, the coefficient of correlation (r) for DADS was 0.9999 and the LOD and LOQ for DADS were 0.3063 µg/mL and 1.0210 µg/mL, respectively. Using the validated assay and a concentration range of 0.5–20 µg/mL, the coefficient of correlation for DATS was 0.9999 and the LOD and LOQ for DATS were 0.1986 µg/mL and 0.6621 µg/mL, respectively. The percentage of recovery was in the range of 98.05–101.76% and coefficient of variation ≤ 2%.

Conclusion: This GC method accurately measures the levels of DADS and DATS in garlic.

Keywords: Diallyl disulfide, Diallyl disulfide, Diallyl trisulfide, Diallyl trisulfide, Garlic, Gas chromatography, Flame ionization detector, Optimization, Validation.

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INTRODUCTION

In Indonesia, garlic is an important farming commodity because it has various benefits [1]. Garlic is seen as an important plant due to its benefits for use as a raw material for culinary purposes and its use in traditional and modern medicine [2]. Garlic has been demonstrated to have antioxidant, anticancer, and cardioprotective effects [3]. For instance, garlic has a natural antioxidant activity which can remove reactive oxygen species and lower low-density lipid peroxidation and lipoprotein oxidation [4]. Due to all effects for the health related to garlic consumption, this plant is considered as one of the important remedies to improve health [5].

Secondary metabolites contained in garlic are important for the beneficial properties of garlic. In particular, organosulfur compounds are important to the bioactivity of garlic, including its derivative products. Flavor, smell, and pharmacological activity of garlic are also related to its organosulfur compound [6].

During processing and digestion, organosulfides in garlic undergo complex chemical conversion reactions that result in the formation of compounds responsible for the unique flavor and smell of garlic [7]. Alliin is a sulfoxide derivative of cysteine that is the precursor to the organosulfur compound allyl sulfide and its derivatives [8]. The generation of organosulfides is initiated by conversion of alliin (S-allyl-L-cysteine-sulfoxide) to allicin. This occurs in response to the disruption of the cell structure such as when garlic is cut and crushed. Subsequently, because allicin is a very unstable compound, particularly at high temperatures, it rapidly converts to various oil-soluble sulfurs which contain organosulfides. The organosulfides formed from allicin include diallyl disulfide (DADS), diallyl trisulfide (DATS), methyl allyl trisulfide, and diallyl tetrasulfide (DATS) [9,10].

DADS and DATS are both high-performance liquid chromatography (HPLC) and gas chromatography (GC) which have been validated for use in separating DADS and DATS. Research that has been done using HPLC with column C18 at 30°C, with a mobile phase of acetonitrile-water. While in another study, analyzed using GC with column DB-5, hexane solvent, and initial temperature of 40°C [11,12].

Analysis methods often need to be modified based on the available equipment and materials in the laboratory. When changes are implemented, the method of modification results must be validated to assure the implementation of proper analysis method testing. In this research, analysis method of DADS and DATS was validated using GC with a flame ionization detector (FID). It is expected that this is a more selective analysis method than what is currently used.

MATERIALS AND METHODS

Materials

Diallyl disulfide (HHD); diallyl trisulfide (Rousechem); garlic cloves; single clove garlic (Total Fruit Market); aquadest (Ikaparmindo); hexane for analysis (Merck), acetone for analysis (Merck), acetophenone for analysis (Merck); hydrogen gas (UHP); nitrogen gas (UHP).

Chromatographic conditions

Shimadzu GC model GC-17A equipped with an FID, capillary column (length 30 m, inner diameter 0.32 mm, 0.25 µm thickness film with stationary phase HP-1), GC Solution Class data processor; gas nitrogen carrier (UHP) and hydrogen (UHP).

Methods

Determination of optimum analysis conditions

Two milliliters of acetophenone were added to 1 mL of a 20 ppm diallyl disulfide and diallyl trisulfide standard mixture solvent and

then vortexed until the mixture was homogenous. The solutions were injected to GC with 1.0 μL injection volume.

The determination of the optimum analysis conditions was conducted by evaluating different temperature programs with initial temperatures of 130°C, 140°C, and 150°C. The rate of temperature increase from the initial temperature was 1°C/min to 180°C. The flow rate variations were 0.8, 1.0, and 1.2 mL/min. The injector and detector temperatures were set at 200°C. Retention time, tailing factor, the number of theoretical plate, height of packing equivalent to a theoretical plate (HETP), and resolution of each condition were recorded.

System suitability test

Two milliliters of acetophenone were added to 1 mL of a 20 ppm diallyl disulfide and diallyl trisulfide standard mixture solvent and then vortexed until the mixture was homogenous. The solutions were injected to GC with 1.0 μL injection volume.

The system suitability test was conducted 6 times and then the results were recorded and used to calculate the coefficient of variation (% CV). To pass the system suitability test, the CV was set at $\leq 2\%$. The parameters which could be seen were retention time (t_r), area, separation between two adjacent peaks (R), the number of theoretical plate (N), HETP, and tailing factor (T_p).

Validation methods

The assay was validated based on assay selectivity, linearity, limit of detection (LOD) and limit of quantity (LOQ), accuracy, and precision parameter.

Selectivity test

Two milliliters of acetophenone were added to 1 mL of a 20 ppm diallyl disulfide and diallyl trisulfide standard mixture solvent and then vortexed until the mixture was homogenous. The solutions were injected to the GC at a 1.0 μL injection volume. Matrix blank solution was injected at the same volume. The chromatogram was evaluated for whether there was a disturbance of the retention time of DADS and DATS from the matrix component.

Preparation of calibration curve and linearity test

Diallyl disulfide and diallyl trisulfide standard mixture solvent with a 20 ppm concentration were transferred to 1 mL volumetric flasks at 25 μL , 50 μL , 125 μL , 250 μL , 0.5 mL, and 1 mL per flask. The solutions were diluted with acetone solvent until the limitation mark and homogenized to obtain the concentrations of 0.5 ppm, 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, and 20 ppm.

For each solution, 0.2 mL acetophenone was added and shaken until the mixture was homogenous. The solutions were injected to the GC for 1.0 μL with selected analysis conditions. A calibration curve was made with the peak area as the Y-axis and the injected concentration as the X-axis. Then, the linear regression equation and correlation coefficient were calculated.

LOD and LOQ Test

LOD and LOQ were determined using the linear regression line equation from the calibration curve. LOD and LOQ were calculated based on the standard deviation of the blank by measuring the blank response several times and then calculating the standard blank deviation. The standard blank deviation (S_b) was the same as the standard residual deviation (S_y/x).

Accuracy and precision test

In these tests, the addition method was used by adding the number of analytes with a certain known concentration into observed samples. For the recovery test, 250 L of the sample was aliquoted into a 1 mL volumetric flask in replicates of four. For three of the flasks, the standard solution was added at three different concentrations (80%, 100%, and 120%). The remaining flask was used as the blank control.

The accuracy and precision tests were conducted by injecting 1.0 μL solution for each concentration at the selected analysis condition. The precision and recovery tests were conducted with six repetitions for each concentration. The peak area was recorded, and the recovery test was calculated for the accuracy test. The standard deviation and the CV were calculated for precision test. The test solution was stated to meet the accuracy test if the percentage of recovery test (% recovery) was between 98 and 102% and to meet the precision test if the CV was no more than 2.0%.

Preparation of garlic sample

Garlic was peeled and crushed and then 10 g was placed into a 100 mL Erlenmeyer. The Erlenmeyer flask opening was covered with plastic and then kept at for 30 min. After that, 30 mL aquadest was added to the flask and then the flask was covered again. The Erlenmeyer flask was put into a pan containing boiling water for 10 min and let allowed to cool to room temperature. The solution was shaken at 150 rpm for 12 h and then stored at room temperature for 6 days. The organic layer was removed and then stored at -20°C to separate the remaining water by freezing it. The solvent in the extraction was evaporated using a rotary evaporator at 40°C to produce yellowish oil and strong smell.

Qualitative and quantitative analysis of diallyl disulfide and diallyl trisulfide

The garlic oil sample was diluted with 500 μL acetone and then 100 μL acetophenone was added. The solution was filtered through a polyvinylidene fluoride 0.45 μm filter. One microliter of the sample solution was injected to the GC.

Qualitative analysis

The retention times of the diallyl disulfide and diallyl trisulfide in the garlic sample and the standard were recorded. The diallyl disulfide and diallyl trisulfide in the garlic sample were identified based on comparison with the retention times of the standard.

Quantitative analysis

The obtained peak area was recorded and the levels were calculated based on linear regression equation.

RESULTS AND DISCUSSION

Analysis condition optimization

The run conditions were optimized to obtain conditions that would result in relatively short retention times and good separation. The initial column temperature and carrier gas flow rates were optimized. In this study, the injector and detector temperatures were set at 200°C . The selections of initial column temperature and carrier gas flow rate were conducted with three different variations. The initial column temperatures evaluated were 130°C , 140°C , and 150°C . The carrier gas flow rates evaluated were 0.8, 1.0, and 1.2 mL/min. Each test condition was conducted twice (Duplo). The optimum parameter was assessed as the one that resulted had a short retention time, big peak area, big number of theoretical plates, small column efficiency (HETP), small tailing factor (T_p), and good peak separation.

After optimization was done, it could be seen that the higher the column, the shorter the retention time. However, the selected initial column temperature was 140°C and the flow rate was 0.80 mL/min because the obtained speed was appropriate, the separation was also good and not too long. An initial temperature of 150°C was not selected because chromatogram peak of diallyl disulfide was too close to the chromatogram peak of the solvent which would interfere with accurate calculation of the diallyl disulfide peak area. The selected flow rate was also not too short because chromatogram peak of solvent was close to retention time of diallyl disulfide that might disturb. Therefore, the optimum condition for diallyl disulfide and diallyl trisulfide analysis was an initial temperature of 140°C and a flow rate of 0.80 mL/min. The chromatograms with two different peaks, area of diallyl disulfide

was 288,670 $\mu\text{V}/\text{s}$ and 298,789 $\mu\text{V}/\text{s}$, while for diallyl trisulfide were 67,905 $\mu\text{V}/\text{s}$ and 69,087 $\mu\text{V}/\text{s}$. Retention times on diallyl disulfide were 5.963 min and 5.947 min, while for diallyl trisulfide were 12.270 min and 12.267 min (Fig. 1). The number of theoretical plates (N) on diallyl disulfide was 32,731.479 and 19,858.093, while for diallyl trisulfide was 47,069.792 and 41,774.894. HETP values for diallyl disulfide were 0.0916 and 0.1511, while for diallyl trisulfide were 0.0637 and 0.0718. The tailing factors for diallyl disulfide were 0.626 and 0.690, while for diallyl trisulfide were 0.748 and 0.733. The resolutions were 33.32 and 30.996. In a previous study conducted by Lim *et al.* (2014), the retention times of diallyl disulfide were 15.6 and 18.3 min. Here, the retention times of diallyl disulfide were < 6 min and the retention time of diallyl trisulfide was < 13 min. These results demonstrate that the conditions used here resulted in retention times that were improved over previous studies without compromising the quality of separation. These results show that the selected analysis condition was optimum and the proper analysis condition to be used.

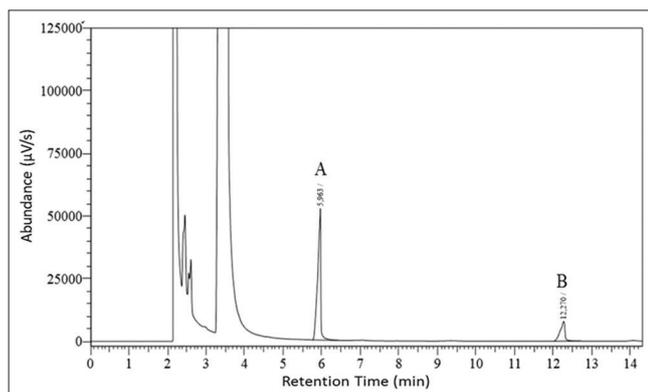


Fig. 1: Chromatogram of the standard mixture solution diallyl disulfide (A) and diallyl trisulfide (B) at an initial column temperature of 140°C and the flow rate of 0.80 mL/min

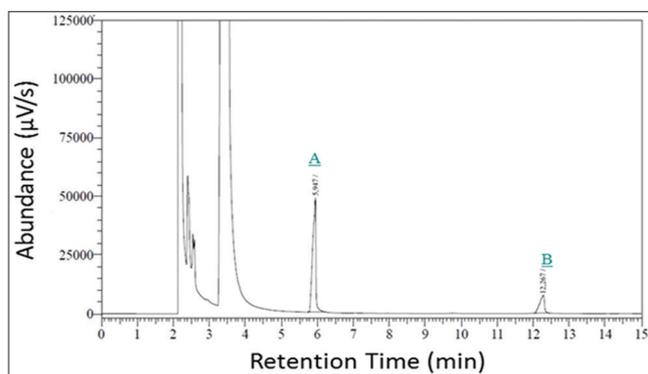


Fig. 2: Chromatogram of standard mixture solution diallyl disulfide (A) and diallyl trisulfide (B)

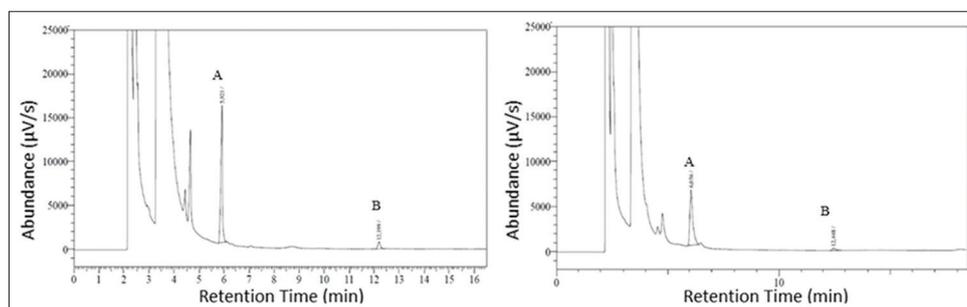


Fig. 3: Chromatograms of single clove garlic sample (1) and clove garlic sample (2) DADS (A) dan DATS (B)

System suitability test

System suitability test was conducted 6 times. The CV values for diallyl disulfide and diallyl trisulfide were 1.64% and 0.45%, respectively. The result shows that analysis method met system suitability test requirement which was repetition value or $CV < 2\%$. These data show that the operational system and operating parameters were suitable for the intended purpose of accurately separating diallyl disulfide and diallyl trisulfide.

Validation methods

Selectivity test

The selectivity test was used to see the disturbance of another chromatograph peak around retention times from diallyl sulfide and diallyl trisulfide compound. For the selectivity test, used blank sample or solvent without any diallyl sulfide and diallyl trisulfide compound was injected. The chromatograph of the blank sample showed that there was no another peak around the retention times of diallyl sulfide and diallyl trisulfide. Thus, it is concluded that this method was selective.

Calibration curve and linearity test

Based on the result of the linear regression calculation of the calibration curve, the calibration curve line equation for DADS was $y = 13068.97x - 3373.62$ and for DATS was $y = 3194.39x - 307.22$. The linearity test of DADS standard had a correlation coefficient (r) of 0.9999, and linearity test of DATS with standard for correlation coefficient (r) was 0.9999. The results could be stated as valid because they met the linearity criteria by obtaining correlation coefficient (r) close to 1 or $r \geq 0.9990$.

LOD and LOQ test

Based on the results of linear regression equation, the LOD and LOQ were calculated from each compound statistically. For diallyl disulfide, the LOD value was 0.3063 $\mu\text{g}/\text{mL}$ and the LOQ was 1.0210 $\mu\text{g}/\text{mL}$. For diallyl trisulfide, the LOD was 0.1986 $\mu\text{g}/\text{mL}$ and the LOQ was 0.6621 $\mu\text{g}/\text{mL}$.

Accuracy and precision test

Accuracy shows the degree of closeness of analysis results and the real analyte levels. Accuracy is expressed as the percent recovery of the analyte added. Accurate criterion is given if percentage recovery (%UPK) is between 98 and 102%. For both diallyl disulfide and diallyl trisulfide, the result of each concentrate with six replicas gave percentage recovery values between 98.05 and 101.76% which shows that they met the accurate criterion.

Precision shows the degree of precision suitability between individual test results measured using individual result distribution from average, measured as relative standard deviation or variation coefficient. Precise criterion is given if the method gives relative standard deviation or variation coefficient no more than 2%. The results gave coefficient values of 0.58–1.50%. It shows that the analysis method met the criterion of accurate and precise.

Preparation of garlic sample

Sample preparation was done using the extracting process on two samples: Single clove garlic and clove garlic. The extraction was conducted twice for each sample and the obtained single clove garlic

Table 1: Results of determination diallyl disulfide in single clove garlic and clove garlic sample

Type of sample	Weight of extract (g)	Areas ($\mu\text{V/s}$)	Measurable concentration ($\mu\text{g/mL}$)	Concentration (%)	Average (%)	
Single clove garlic	0.00981	73,725	5.8993	0.0301	0.0296	
		73,559	5.8866	0.0300		
	0.01011	73,402	5.8746	0.0290		
73,598		5.8896	0.0291			
Clove garlic	0.00912	56,595	4.5886	0.0252		0.0248
		56,423	4.5754	0.0251		
	0.00926	56,008	4.5437	0.0245		
		56,184	4.5571	0.0246		

Analysis was conducted using an initial column temperature of 140°C increasing at 1 °C/min to 180°C. The injector and detector temperatures were set at 200°C, and the flow rate was 0.80 mL/min

Table 2: Result of determination diallyl trisulfide in single clove garlic and clove garlic sample

Type of sample	Weight of extract (g)	Areas ($\mu\text{V/s}$)	Measurable concentration ($\mu\text{g/mL}$)	Concentration (%)	Average (%)	
Single clove garlic	0.00981	5623	1.8564	0.0095	0.0093	
		5646	1.8636	0.0095		
	0.01011	5558	1.8361	0.0091		
5533		1.8282	0.0090			
Clove garlic	0.00912	4818	1.6044	0.0088		0.0087
		4824	1.6063	0.0088		
	0.00926	4875	1.6222	0.0087		
		4813	1.6028	0.0086		

Analysis was conducted using an initial column temperature of 140°C increasing at 1°C/min to 180°C. The injector and detector temperatures were set at 200°C, and the flow rate was 0.80 mL/min

extracts were 0.00981 g and 0.01011 g, while for clove garlic were 0.00912 g and 0.00926 g.

Qualitative and quantitative analysis of diallyl disulfide and diallyl trisulfide

Qualitative analysis

The extraction samples diluted with solvent were analyzed so the results show that in single clove garlic and clove garlic sample, there were diallyl disulfide and diallyl trisulfide. The results are shown by the match of standard solution chromatogram and analyzed samples. On single clove garlic sample, there was diallyl disulfide compound with 5.921 min retention time and diallyl trisulfide with 12.199 retention time. On clove garlic sample, there was diallyl disulfide compound with 6.056 min retention time and diallyl trisulfide with 12.448 retention time (Fig. 2-3).

Quantitative analysis

Based on the results from the linear regression equation, the single clove garlic sample contained 0.0296% DADS and 0.0093% DATS. While clove garlic sample contained 0.0248% DADS and 0.0087% DATS shows that diallyl disulfide and diallyl trisulfide were more in single clove garlic than clove garlic (Tables 1 and 2). The results show the quite small level compared to the previous research. There were some factors which might cause them, including the different number of samples and extraction methods.

CONCLUSION

Based on the analysis method validation criteria, the method used met all criteria so that analysis method is valid. The results of the validation had correlation coefficient value (r) of 0.9999 in the range of 0.5–2.0 $\mu\text{g/mL}$ concentrations. The LOD and LOQ values for DADS were 0.3063 $\mu\text{g/mL}$ and 1.0210 $\mu\text{g/mL}$, respectively, while for DATS were 0.1986 $\mu\text{g/mL}$ and 0.6621 $\mu\text{g/mL}$, respectively. Accuracy values with % UPK were between 98.05 and 101.76% and precision was $CV \leq 2\%$. On single clove garlic sample, the average level of diallyl disulfide was 0.0296% and diallyl trisulfide was 0.0093%. On clove garlic sample, the average level of diallyl disulfide was 0.0248% and diallyl trisulfide was 0.0087%.

CONFLICTS OF INTEREST

All authors have none to declare.

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