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## Determination of Dimethyl Disulfide, Diallyl Disulfide, and Diallyl Trisulfide in Biopesticides Containing *Allium Sativum* Extract by Gas Chromatography

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### Abstract

**BACKGROUND:** Garlic (*Allium sativum*) extract has been allowed as commercial biopesticide material for pesticidal activity in the Environmentally-friendly Agriculture Promotion Act. Nine commercial biopesticides containing *A. sativum* extract have been marketed in Korea. However, the analytical method of the active substances in these materials has not been studied.

**METHODS AND RESULTS:** Cartridge clean-up method for the determination of dimethyl disulfide(DMDS), diallyl disulfide(DADS), and diallyl trisulfide(DATS) in biopesticides containing *A. sativum* extract was developed and validated by gas chromatography(GC). The clean-up method was optimized using hydrophilic lipophilic balance (HLB) solid phase extraction(SPE) cartridges for the bioactive sulfides in biopesticides containing *A. sativum* extract, and the eluate was analyzed to quantify the DMDS, DADS, and DATS using the GC. The developed method was validated, and the LOQ and recovery rates of DMDS,

DADS, and DATS were 0.226, 0.063, and 0.051 mg L<sup>-1</sup> and 80.6, 84.8, and 73.1%, respectively. From the nine commercial biopesticide samples, contents of DMDS, DADS, and DATS were analyzed using the developed method and results showed <LOQ, <LOQ-113.4, and <LOQ-2.3 mg L<sup>-1</sup>, respectively.

**CONCLUSION:** The developed method could be used in determining the quality of biopesticides for the manufacture of commercial biopesticides containing *A. sativum* extract.

**Key words:** *Allium sativum*, Biopesticide, Diallyl disulfide, Diallyl trisulfide, Dimethyl disulfide

### Introduction

Garlic (*Allium sativum*) is a species belonging to onion genus, *Allium*, and its close relatives include the onion, shallot, leek, and chive(Block, 2010). This has been used for culinary and medicinal purposes and widely distributed in Mediterranean region, Asia, Africa, and Europe(Simonetti, 1990; Ensminger, 1994). *A. sativum* contains polyphenols and sulfur compounds such as sulfides and cysteine derivatives.

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These components are recognized as antioxidant, antithrombotic, anticancer, antibacterial, antimicrobial, nematocidal, and insecticidal activity material (Al-Delaimy and Ali, 1970; Kamanna and Chandrasekhara, 1983; Horie *et al.*, 1992; Kyung, 2006; Nuttakaan *et al.*, 2006; Anwar *et al.*, 2009).

Alliin [*S*-(2-propenyl)-*L*-cysteinesulfoxide], a major component of *A. sativum*, is broken down into alliin (*S*-2-allyl-*L*-cysteinesulfoxide) and other allylthiosulfonates by alliinase (Lawson and Gardner, 2005). At least thirty five compounds containing sulfur in *A. sativum* have been identified (Yu *et al.*, 1989). In these sulfur-containing compounds, alliin, alliin, allylmethyl sulfide (AMS), allyl sulfide (AS), dimethyl disulfide (DMDS), dipropyl sulfide (DPS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) have been reported as active compounds for crop protection (Ankri and Mirelman, 1999; Dugravot *et al.*, 2003; Kim *et al.*, 2004; Ogita *et al.*, 2007; Pongsak and Parichat, 2008; Casella *et al.*, 2013).

*A. sativum* extract is considered as a commercial biopesticide material for pesticidal activity in the Environmentally-friendly Agriculture Promotion Act. Nine commercial biopesticides containing *A. sativum* extract have been marketed in Korea. However, these commercial biopesticides have been marketed without indicating the active substance and their contents for pesticidal activities. The analytical method of the active substances in these commercial biopesticides has not been studied.

Recently, Lee *et al.* (2013) and Lim *et al.* (2014) reported that insecticidal limonoid and matrine substances in commercial biopesticides containing neem and *Sophora flavescens* extract could be quantified by clean-up method using hydrophilic lipophilic balance (HLB) and ENVI-Carb solid phase extraction (SPE) cartridge, respectively. The study aimed to select the marker compounds on the basis of the characteristic and abundant constituents of *A. sativum* for crop protection, and to develop the SPE clean-up and gas chromatography (GC) methods for the quantification of selected marker compounds in biopesticides containing *A. sativum* extract. The study further aimed to determine the marker compounds contents in commercial biopesticides containing *A. sativum* extract using the developed method.

## Materials and Methods

### Chemicals and reagents

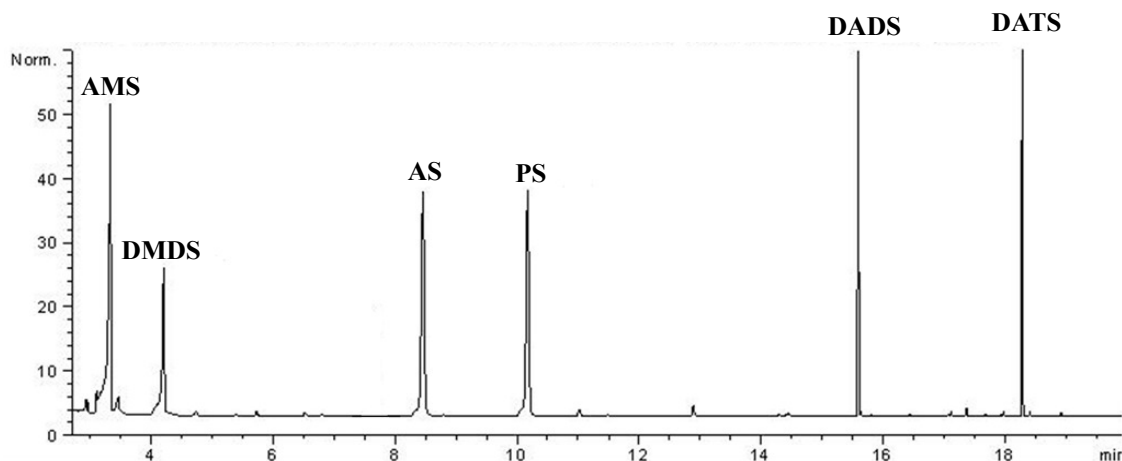
Alliin (99.5% purity), alliin (74.8% purity), allylmethyl sulfide (AMS, 94.3% purity), allyl sulfide (AS, 99.6% purity), dipropyl sulfide (DPS, 98.5% purity), dimethyl disulfide (DMDS, 99.4% purity), diallyl disulfide (DADS, 71% purity), and diallyl trisulfide (DATS, 95% purity) were purchased from CromaDex Irvine, California, USA. Acetone was obtained from Tedia, Ohio, USA. Analytical grade ethyl acetate, dichloromethane, ether, pentane, and hexane were purchased from Dae Jung Chemicals, Siheung, Korea. The anhydrous sodium sulfate, an analytical reagent grade was purchased from Merck India Ltd., Mumbai, India. ENVI-Carb solid phase extraction (SPE) cartridge (0.5 g, 6 mL, from Supelco, Philadelphia, USA) and hydrophilic and lipophilic balance (HLB) SPE cartridge (60 mg, 3 mL, from Waters, Milford, USA) were used for clean-up. Likewise, nine commercial biopesticides containing *A. sativum* extract were purchased from seven Korean local companies.

### Selection of marker compounds in biopesticides

In selecting the marker compounds in the biopesticides containing *A. sativum* extract, the materials' characteristics and abundant constituents for crop protection were considered in the study. Eight constituents characterized and studied in the *A. sativum* extract were alliin, alliin, allylmethyl sulfide (AMS), dimethyl disulfide (DMDS), allyl sulfide (AS), dipropyl sulfide (DPS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). Gas chromatography from Aligent, Santa Clara, USA with flame ionization detector (FID) was used in characterizing the eight marker compounds.

### Clean-up of marker compounds

To clean-up the marker compound in biopesticides containing *A. sativum* extract, liquid-liquid extraction, ENVI-Carb SPE cartridge, and HLB SPE cartridge were used. The liquid-liquid extraction was performed using a hexane. 5 mL diluted biopesticide (100 times), 85 mL distilled water, and 10 mL saturated sodium chloride (NaCl) placed in the separatory funnel and added with 60 mL hexane (20 mL × 3). After the organic solvent layer were filtered through the anhydrous sodium sulfate into a round-bottomed flask and evaporated using a evaporator (Rotavapor



**Fig. 1.** Chromatogram of allylmethyl sulfide(AMS), dimethyl disulfide(DMDS), allyl sulfide(AS), propyl sulfide(PS), diallyl disulfide(DADS), and diallyl trisulfide(DATS). AMS, allylmethyl sulfide; DMDS, dimethyl disulfide; AS, allyl sulfide; PS, propyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide.

R-124, Büchi, Flawil, Switzerland), the dried residue was re-dissolved into 2 mL hexane for analysis using the GC. One mL diluted biopesticide(100 times) with distilled water was loaded into an ENVI-Carb SPE cartridge, and eluted with 12 mL hexane(3 mL×4). Same process was applied to the method of the liquid-liquid extraction method. Finally, HLB SPE cartridge method was performed according to the modified method of Lee *et al.*(2013). Commercial biopesticides samples were diluted 100 times with distilled water. One mL diluted solution was loaded into HLB SPE cartridge which was successively activated with two mL acetone and one mL distilled water, and was eluted with 6 mL(2 mL×3) acetone after washing with 2 mL distilled water. Eluate was used for DMDS, DADS, and DATS analysis.

#### Gas chromatography(GC) analysis

GC conditions for analyzing DMDS, DADS, and DATS were: system, Agilent 6890 series with FID (Agilent, Santa Clara, USA); column, RTX-5(30 m×250 mm×250 mm, RESTEK, Pennsylvania, USA); carrier gas, He(99.999%, 3 mL min<sup>-1</sup>); inlet temperature, 23 0°C; column temperature, initial 40°C(8 min hold), 5°C min<sup>-1</sup>, 60°C, 15°C min<sup>-1</sup>, 230°C(hold 1 min); detector temperature, 300°C; and injection volume, 1 µL.

#### Validation methods

Sample preparation and analytical methods were validated in terms of linearity, limits of quantitation (LOQ), confirmatory, and precision. The linearity of the calibration curves in five replicates was injected at

0.5, 1, 2.5, 5, 10, and 25 mg L<sup>-1</sup> concentrations, respectively. The LOQ for DMDS, DADS, and DATS were considered as the concentration that produced a signal-to-noise ratio of 10. Confirmatory of three replicates was determined through the recovery assay results of samples spiked with all analytes at 6 mg L<sup>-1</sup>. Recovery rates were calculated by comparing the concentrations of the extracted compounds with those from the DMDS, DADS, and DATS calibration curves, respectively. The intermediate precision, expressed as relative standard deviation(RSD, %), was determined in five replicates at different levels for three days.

## Results and Discussion

#### Selection of marker compound

Eight sulfide substances, namely, alliin, alliin, AMS, DMDS, AS, PS, DADS, and DATS were studied as marker compounds in biopesticides containing *A. sativum* extract based on the material's characteristic and abundant constituents for crop protection. Generally, commercial biopesticides have been manufactured by mixing natural extracts and additive such as surfactant, expander, mineral, and solvent. FID has been used for the component analysis in matrix containing surfactant(Tacx and German, 1989; Fendlnger *et al.*, 1992; Yan *et al.*, 2012). As such, FID was used for analyzing the eight marker compounds. AMS, DMDS, AS, PS, DADS, and DATS showed good sensitivities and retention times of 3.3, 4.2, 8.4, 10.2, 15.6, and 18.3 min, respectively(Fig. 1). However, alliin and alliin were not detected under the same

**Table 1. Validation parameters**

| Materials | Linearity (r <sup>2</sup> ) | Recovery rate <sup>a</sup> (%) | LOQ (mg L <sup>-1</sup> ) | RSD (%)   |           |
|-----------|-----------------------------|--------------------------------|---------------------------|-----------|-----------|
|           |                             |                                |                           | Inter-day | Intra-day |
| AMS       | 0.9997                      | 4.8±3.6                        | 0.130                     | 75.3      | 4.3       |
| DMDS      | 0.9998                      | 80.6±3.5                       | 0.202                     | 2.7       | 1.9       |
| AS        | 1.0000                      | 58.6±3.0                       | 0.037                     | 5.2       | 3.6       |
| PS        | 1.0000                      | 47.4±5.2                       | 0.206                     | 11.0      | 9.8       |
| DADS      | 1.0000                      | 84.8±1.9                       | 0.048                     | 1.0       | 1.6       |
| DATS      | 0.9998                      | 73.1±1.8                       | 0.036                     | 0.8       | 2.2       |

<sup>a</sup>The data represent the mean values ±SD of three replicates.

LOQ, limit of quantitation; RSD, Relative Standard Deviation; AMS, allylmethyl sulfide; DMDS, dimethyl disulfide; AS, allyl sulfide; PS, propyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide.

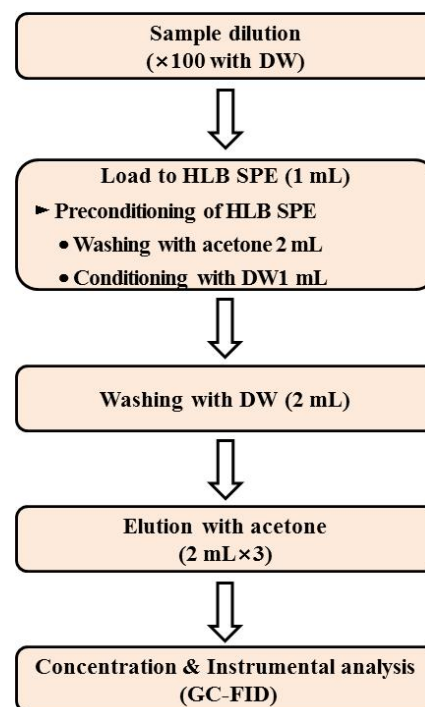
analytical condition.

### Clean-up of marker compound

Liquid-liquid extraction and SPE cartridge were used in the clean-up of the biopesticide containing *A. sativum* extract. The liquid-liquid extraction was used to clean the targeted material analysis in matrix such as soil, water and plant (Sabik and Jeannot, 1998; Wang *et al.*, 2008; Pirsahab *et al.*, 2013). The method was applied to clean-up for AMS, DMDS, AS, PS, DADS, and DATS analysis. These materials were spiked to distilled water, and liquid-liquid extraction was conducted using hexane. The recovery rate of the liquid-liquid extraction using hexane was higher than 93.8%. However, the 100 times diluted commercial biopesticides with distilled water were not divided in hexane layer. Ethyl acetate, dichloromethane, ether, and pentane also showed the same result as hexane. Hence, liquid-liquid extraction could not be applied to clean-up for six materials.

ENVI-Carb and HLB SPE cartridges have been used in cleaning the marker compounds in commercial biopesticides (Lee *et al.*, 2013; Lim *et al.*, 2014). ENVI-Carb cartridge, used for cleaning matrices in commercial biopesticides containing *Sophora flavescens* extract (Lim *et al.*, 2014), showed lower recovery than 44% in the six marker compounds studied (data not shown). The HLB SPE cartridge, used for cleaning the marker compound (azadirachtin A, azadirachtin B, deacetylsalannin, and salannin) in commercial biopesticides containing neem extract (Lee *et al.*, 2013), showed better recovery rates than >70% in DMDS, DADS and DATS (Table 1). However, the recovery rates of AMS, AS, and PS were lower than 60%. These results showed that DMDS, DADS and

DATS could be selected as marker compounds of commercial biopesticides containing *A. sativum* because of their good recovery rates, linearity using the HLB SPE cartridge and GC-FID analysis. Therefore, these results indicated that the HLB SPE method was an effective method for DMDS, DADS and DATS analysis in biopesticides containing *A. sativum* extract (Fig. 2).



**Fig. 2. Flow chart of dimethyl disulfide (DMDS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) analysis in biopesticides. DW, distilled water; HLB, lipophilic balance; SPE, Solid Phase Extraction; GC-FID, Gas Chromatography - Flame Ionization Detector.**

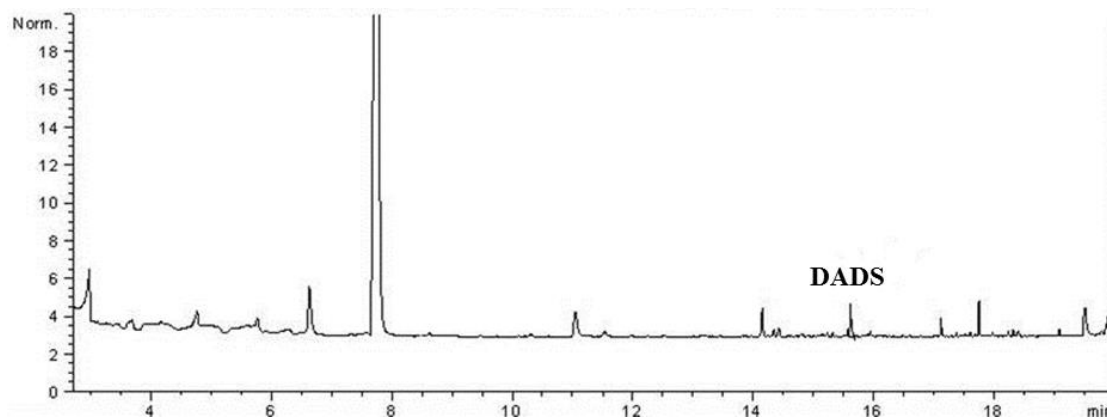


Fig. 3. Representative chromatogram of dimethyl disulfide(DMDS), diallyl disulfide(DADS), and diallyl trisulfide(DATS) in biopesticide samples. DADS, diallyl disulfide.

### Validation methods

Calibration curves of DMDS, DADS and DATS showed peak area of analytes standard at 0.5-25 mg L<sup>-1</sup>, respectively. The coefficient of correlation of all calibration curves was higher than 0.9998. The LOQs of DMDS, DADS and DATS were 0.202 mg L<sup>-1</sup>, 0.048 mg L<sup>-1</sup> and 0.036 mg L<sup>-1</sup>, respectively (Table 1). Confirmatory test of the analytical method was determined based on the recovery rates of the spiked sample without *A. sativum extract*. Recovery rates of DMDS, DADS and DATS were 80.6%, 84.8% and 73.1% using the established method, respectively (Table 1). The inter- and intra-day precision methods were determined by the recovery rates of DMDS, DADS and DATS in three days. The method was effective since the RSD percentages ranged from 0.8% - 2.7% and below 15, the normal percent value (Table 1). Results indicated that the experimental method (with clean-up) and instrumental analysis were

suitable for analyzing the DMDS, DADS and DATS contents in biopesticides.

### Substance levels in commercial biopesticides

The developed method for DMDS, DADS and DATS analysis was applied to commercial biopesticides containing *A. sativum extract*. Fig. 3 presents the representative chromatogram of the DMDS, DADS and DATS in biopesticide samples. DMDS, DADS and DATS in all samples were detected at <LOQ, <LOQ-113.4 mg L<sup>-1</sup> and <LOQ-2.3 mg L<sup>-1</sup>, respectively (Table 2).

Recently, Pongsak and Parichat (2008) reported that the minimum inhibitory concentration (MIC) of DADS and DATS for antimicrobial activities were 8-24 mg L<sup>-1</sup> and 2-12 mg L<sup>-1</sup>, respectively. Also, the DMDS lethal concentrations causing 50% mortality (LC<sub>50</sub>) in *Dinarmus basalis*, *Callosobruchus maculatus*, and *Periplaneta americana* were 0.31 mL L<sup>-1</sup>, 0.65 mL L<sup>-1</sup>, and 1.01 mL L<sup>-1</sup> in air, respectively (Dugravot *et al.*,

Table 2. Contents of dimethyl disulfide (DMDS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) in commercial biopesticides containing *A. sativum extract*

| Samples | DMDS (mg L <sup>-1</sup> ) | DADS (mg L <sup>-1</sup> ) | DATS (mg L <sup>-1</sup> ) | Total (mg L <sup>-1</sup> ) |
|---------|----------------------------|----------------------------|----------------------------|-----------------------------|
| 1       | < LOQ                      | 113.4                      | < LOQ                      | 113.4                       |
| 2       | < LOQ                      | 82.0                       | < LOQ                      | 82.0                        |
| 3       | < LOQ                      | 90.7                       | < LOQ                      | 90.7                        |
| 4       | < LOQ                      | 106.9                      | < LOQ                      | 106.9                       |
| 5       | < LOQ                      | 86.4                       | < LOQ                      | 86.4                        |
| 6       | < LOQ                      | 97.5                       | < LOQ                      | 97.5                        |
| 7       | < LOQ                      | 61.3                       | < LOQ                      | 61.3                        |
| 8       | < LOQ                      | < LOQ                      | < LOQ                      | < LOQ                       |
| 9       | < LOQ                      | 67.9                       | 2.3                        | 70.2                        |

LOQ, limit of quantitation; DMDS, dimethyl disulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide.

2003).

The DADS excluding DMDS and DATS concentrations of the majority of commercial biopesticides studied was good for crop protection. However, commercial biopesticides were generally used after 100-1000 times dilution with water. If commercial biopesticides were practically scattered for crop protection, the DADS concentration should be lower than 100 times and lower than MIC for antimicrobial activity. Hence, the quality control of biopesticides should be conducted to determine their efficiency levels for crop protection.

### Conclusion

Three from eight marker compounds in biopesticides containing *A. sativum* extract studied, based on their characteristics and abundant constituents for crop protection, were selected. HLB SPE cartridge clean-up method for determining DMDS, DADS and DATS contents in biopesticides containing *A. sativum* extract was developed and validated by GC-FID. Contents of three marker compounds in commercial biopesticides studied were lower than the level required for crop protection. Findings showed that using the developed method in the manufacture of biopesticides containing *A. sativum* could determine the quality of biopesticides.

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