Comparison of Bioactive Sulfur Containing Compounds in Fresh Garlic and Garlic Products

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Abstract

Organosulfur compounds of garlic contribute to the bioactive properties of garlic. The processing condition of garlic is the important step to preserve the chemical compositions of garlic products. The objectives of this study were to determine alliin content in garlic products comparing with fresh garlic. Alliin content was determined using HPTLC densitometry on a pre-coated HPTLC silica gel 60 GF254 plate with a mixture of n-butanol, n-propanol, glacial acetic acid and water as the mobile phase. LC-MSMS was performed on an electrospray ion trap mass spectrometer. These results showed garlic powder in capsules and lyophilized garlic cloves still maintained alliin content and some organosulfur compounds. LC-MSMS has identified 7 organosulfur compounds including alliin, *S*-allylcysteine, γ-glutamyl-*S*-methylcysteine, γ-glutamyl-*S*-1-propenylcysteine, γ-glutamyl-*S*-2-propenylcysteine, γ-glutamyl-*S*-propenyl cysteine-*S*-oxide, and γ-glutamyl-*S*-propylcysteine-*S*-oxide. Fragmentation patterns of these organosulfur compounds showed similar product ions in garlic products comparing with fresh garlic. In summary, a drying process to prepare garlic products still maintained some organosulfur compounds as in fresh garlic.

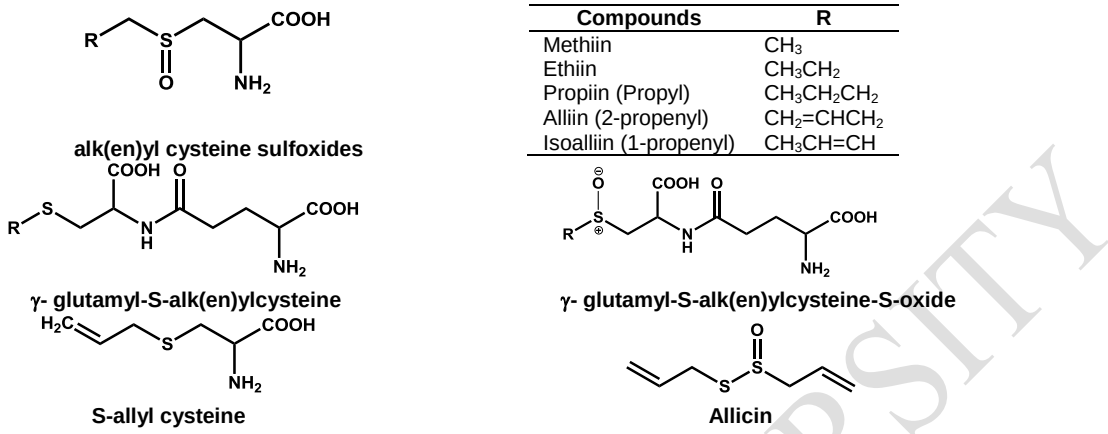
Keywords: *Allium sativum L., Alliin, garlic, organosulfur compounds*

1. Introduction

Garlic (*Allium sativum* L.) has been known as spice in oriental cuisine and as dietary supplement for several health benefits. Alliin is a major component found in garlic cloves which are used as antihyperlipidemic, antihypertensive, and cardiovascular protection (Gebreyohannes & Gebreyohannes, 2013). Garlic cloves contain bioactive organosulfur compounds. These organosulfur compounds can be categorized to S-alk(en)ylcysteine S-oxides (or alk(en)yl cysteine sulfoxides; ACSO), S-allylcysteine (SAC), thiosulfinates (TS), S-amino acids (i.e. cysteine and methionine), and nonvolatile γ-glutamylcysteine peptides (γ-GP) (Lanzotti, 2006) (Figure 1). These organosulfur compounds exhibit distinct characteristic odor. Thiosulfinates i.e. allicin, E-/Z-ajoene, diallyl disulfides and diallyl trisulfides are not naturally occurring compounds, but they are degradation products from the naturally cysteine sulfoxide, alliin. S-allyl cysteine sulfoxide is known as a chemical name of alliin. When garlic cloves are crushed or minced, alliin is reacted with allinase enzyme and forms allicin. Allicin is chemically unstable; therefore, alliin is used to determine the quality of garlic.

The organosulfur compounds possess some biological activities. Volatile oil, essential oil, water and ethanol extracts of garlic cloves showed antibacterial and antifungal activities in *in vitro* studies (Jaber & Al-Mossawi, 2007). This medicinal property was applied to use in topical preparation. Allicin is attributed to antibacterial activity but its highly reactive property may not show antibacterial *in vivo*. Ajoene and diallyl trisulfides also exhibited antibacterial and antifungal activities (Gebreyohannes & Gebreyohannes, 2013). Garlic lowered blood cholesterol and plasma lipids, decreased blood glucose and blood pressure (Koch & Lawson, 1996; Gebhardt, Beck, & Wagner, 1994; Gebhardt, 1995; Ohaeri, 2001; Benavides et al., 2007). In addition, garlic inhibited platelet aggregation *in vitro* and *in vivo* studies and ajoene was responsible for this activity (Fukao, Yoshida, Tazawa, & Hada, 2007). Garlic also showed potential effect to prevent some cancers especially esophagus, stomach and colon cancer (Galeone et al, 2006). This activity may be due to antioxidant effect of allicin. SAC has been interested as it produced antioxidant activity, anti-carcinogenic activity and anti-hepatopathic activity also. Therefore, different methods have been used to preserve active components in garlic. Garlic products in dietary supplements can

be powder in capsule, oil and dehydrated or lyophilized garlic. The analytical methods to determine active components in garlic are important to estimate the nutraceutical value of garlic.



Compounds	R
Methiin	CH_3
Ethiin	CH_3CH_2
Propiin (Propyl)	$CH_3CH_2CH_2$
Alliin (2-propenyl)	$CH_2=CHCH_2$
Isoalliin (1-propenyl)	$CH_3CH=CH$

Figure 1 Chemical structures of some organsulfur compounds in garlic cloves

Analytical methods to determine organosulfur compounds in garlic can be classified as direct and indirect methods. Direct methods determine ACSO before enzymatic degradation while indirect methods determine various compounds forming after enzymatic conversion. Thin layer chromatography (TLC) is used for identification (Ministry of Public Health, 2016; WHO 1998) and high performance thin layer chromatography (HPTLC) is used for identification and quantification of alliin (Siddiqui, Mothana, & Alam, 2016). HPLC with reversed phase column and ultraviolet detector (UV) or with photodiode array detector (PDA) was used for determination of SAC and ACSO (Arnault et al., 2003). Due to less UV absorbance, the samples are needed to derivatize with some reagents to obtain high sensitivity (Kubec & Dadakova, 2009). Gas chromatography (GC) is useful to analyze some volatile compounds and diallyl di- and tri sulfides (Dewi, Kusnadi, & Shih, 2016). However, some organosulfur compounds are unstable under high temperature. This study focused on determination of alliin and identified some organosulfur compounds using LC-MSMS.

Thai Herbal Pharmacopoeia described alliin as a major component that can be identified using thin layer chromatography (TLC). However, it does not specify the criteria for alliin content. Thai herbal pharmacopoeia suggests oral doses of garlic for 2-4 g three times a day as carminative, expectorant or antihyperlipidemic (Ministry of Public Health, 2016). In WHO monograph recommends the average daily dose: fresh garlic 2-5 g, dried powder 0.4-1.2 g, oil 2-5 mg and extract 300-1000 mg (as solid material) which corresponds to 4-12 mg of alliin (WHO,1998). This study presented the determination of alliin content in fresh garlic and garlic products. Other organosulfur compounds were identified using LC-MSMS technique.

2. Objectives

This study aimed to determine alliin content in garlic products compared with fresh garlic. Some organosulfur compounds were also identified using LC-MSMS.

3. Materials and methods

Materials

Alliin was purchased from Sigma-Aldrich (USA). Analytical grade n-butanol was purchased from Fischer, n-propanol from Unilab, absolute ethanol and ninhydrin from Univar, glacial acetic acid and formic acid from Qrec. Methanol and acetonitrile (HPLC grade) were purchased from Burdick&Jackson Honeywell (Korea). Fresh garlic was bought from a local market. Garlic capsules were bought from two commercial products and lyophilized garlic was obtained from Preservefood Speciality Co. Ltd.

Sample preparation

Fresh and lyophilized garlic samples (1 g, each) were crushed in a mortar and for capsule products, the capsule shell was removed, and the powder (1 g, each) was added into 80% methanol. The samples were warmed in a water bath (70 ^{o}C) for 15 minutes. Then they were filtered through Whatman No.1 filter paper and the solution was tested with HPTLC and LC-MS/MS.

Alliin determination

The alliin was determined by HPTLC densitometry and performed on HPTLC silica gel 60 GF254 plate (Merck). Standard alliin and samples were applied on the plate using Linomat V (Camag). Standard alliin was prepared in the concentration of 1 mg/mL in methanol and diluted to 0.25 mg/mL (250 ng/µL). The standard solution was spot 1-5 µL (250-1,250 ng/spot, n=3). The sample solution each 3 µL (n=2) was spot on the plate and the band length was 4.5 mm. The plate was developed in the mixture of n-butanol : n-propanol : water : glacial acetic acid (3 : 1 : 1 : 1) (THP 2016). The developing distance was 8 cm and developing time was about 1 hour. The plates were visualized under 254 and 366 nm (TLC visualizer, Camag). After that the plate was sprayed with ethanolic ninhydrin TS and detected at the wavelength of 497 nm. Eluted bands were determined the peak area using TLC scan (Camag). The scanning speed was 20 mm/sec and slit dimension was 4.00 x 0.30 mm. The content of alliin in fresh garlic and garlic products was calculated based on a standard curve of alliin. Statistical analysis was evaluated using IBM SPSS with ANOVA and posthoc analysis.

Organosulfur compounds determination

Liquid chromatography was performed on an Ultimate 3000, Dionex coupling with UV-visible detector and autosampler. Data analysis was carried out using Chromelon software. Separation was performed on a Poroshell 120 C18 (2.1 x 150 mm, 4 µm) at the temperature of 25 ^{o}C. The injection volume was 3 µL and the flow rate was 0.3 mL/min. The mobile phase composition was 5% acetonitrile with 0.2%formic acid (A) and 95%acetonitrile with 0.2%formic acid (B). The step gradient separation was 0-10 min, 0-2%B; 10-16 min, 2-5%B; 16-30 min 5-15%B; 30-32 min, 15-40%B; 32-39 min, 40-80%B; 39-40 min, 80%B; 40-45 min, 80-0%B; 42-45 min, 0%B, modified from the method by Dewi et al., 2017. The detection wavelength was monitored at 195 nm. ESI MS was performed on a Bruker Amazon SL mass spectrometer using Hystar and Trap control software. ESI MS was equipped with quadrupole ion trap. Capillary voltage was set at 4,500 V, nebulizer gas was set at 2 bars, and drying gas temperature was 220 ^{o}C with a flow rate of 7.0 L/min. MS evaluation was performed in both positive and negative modes and scanned at the mass range of m/z 70-900 amu. MSMS fragmentation was performed with MRM in positive mode and the precursor ions of 162, 178, 264, 291, 305 and 307.

4. Results

HPTLC results showed standard alliin was eluted at R_f 0.29±0.00 in this mobile phase system. The color of the eluted band was purplish brown. The eluted bands from each samples showed similar color and R_f values. At R_f 0.03 light orange, R_f 0.09-0.1 orange, R_f 0.19 orange-brown and some bands showed overlap of light blue-purple, R_f 0.29 purplish-brown, R_f 0.37-0.38 purplish-brown, R_f 0.43, R_f 0.5, and R_f 0.54 light to pale purple. The band that corresponded to alliin was not observed under 254 and 366, but can be detected after being spray with ethanolic ninhydrin TS. The eluted bands were similar to that of THP monograph, but slightly different in R_f values. HPTLC chromatograms are showed in Figure 2.

HPTLC scan evaluated the peak area from the band density. This method showed acceptable accuracy in the range of 750, 1,000 and 1,250 ng/spot with %recovery of 107.07±2.28, 101.76±1.07, and 94.35±0.78, respectively). Intraday precision for standard and samples were expressed as %RSD of 0.49-2.10 and 0.78-2.38, respectively. The correlation coefficient (R^2) was 0.9969 with a linear equation of y=8.7079+13099. The average R_f value of standard alliin was 0.29±0.00 with %RSD of 1.22. The content of alliin was calculated based on the standard curve of alliin (y = 12.154x+9579.3, R^2 =0.9553). The alliin contents of two garlic capsule samples were significantly different from fresh garlic. Garlic capsule 1 showed the highest alliin content while lyophilized garlic showed slightly higher alliin content comparing with fresh garlic (Table 1).

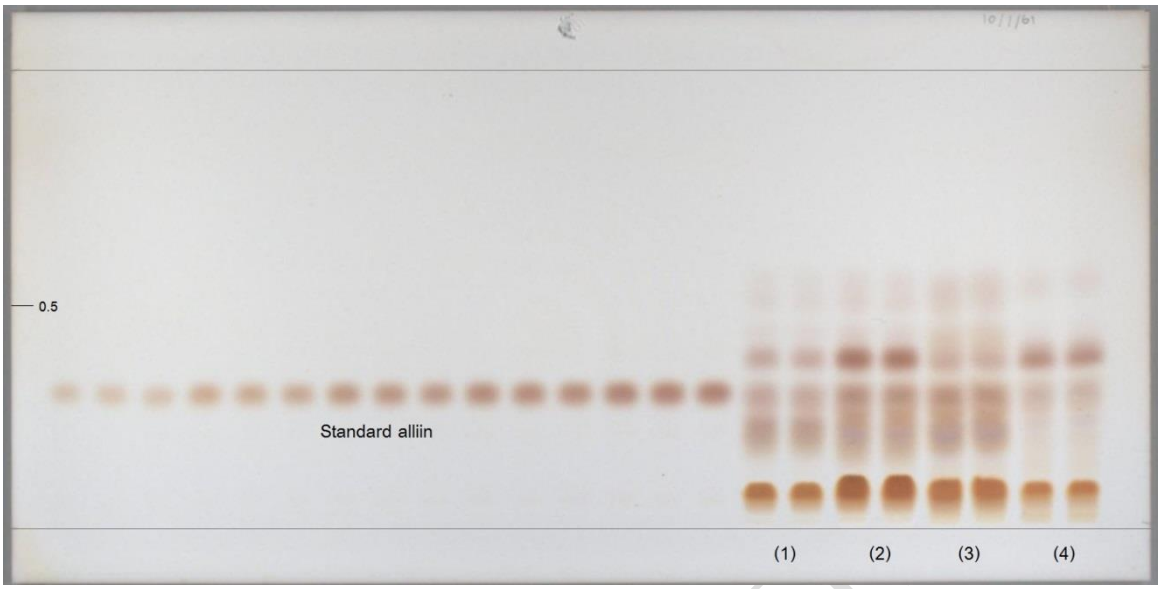


Figure 2 HPTLC chromatogram of standard alliin and garlic

Table 1 Alliin contents in garlic samples

Samples	Alliin (%w/w)	Alliin (mg/g)	Sig*
(1) Fresh garlic	0.26 ± 0.01	2.57 ± 0.12	-
(2) Lyophilized garlic	0.38 ± 0.00	3.81 ± 0.05	0.053
(3) Garlic capsule 1	0.49 ± 0.01	4.89 ± 0.15	0.012
(4) Garlic capsule 2	0.41 ± 0.01	4.10 ± 0.07	0.025

*Significant level at *p*-value < 0.05 compared with fresh garlic

HPLC chromatograms of fresh garlic and garlic products were overlay as showed in Figure 3. Since there were no functional groups in organosulfur compounds, they showed less UV absorbance. In this study there was no derivatizing step before HPLC analysis and HPLC condition still cannot completely resolve the separation. Therefore, MS and MSMS were used to identify some organosulfur compounds in garlic samples. The molecular weights, molecular ions and fragment ions of identified organosulfur compounds were displayed in Table 2. The identified organosulfur compounds were similar in fresh garlic and garlic products; therefore, some representative MS and MS/MS spectra are showed in Figure 4-8.

Table 2 Molecular ions and fragment ions identified from LC-MSMS detected in garlic cloves

Compounds	Molecular weight	Molecular ions $[M+H^+]^+$, $[M-H^+]^+$	Fragment ions
Alliin	177	178.1, 176.1	88, 70.6
SAC	162	-	145, 73.6
γ-glutamyl-*S*-methylcysteine	264	265.0, 263.0	245.1, 133.3, 86.6
γ-glutamyl-*S*-1-propenylcysteine	291	291.0	274, 234, 170.2, 162.2, 145.2
γ-glutamyl-*S*-2-propenylcysteine	291	291.0	274, 228, 162.2, 145.2
γ-glutamyl-*S*-propenylcysteine-*S*-oxide	305	305.0	286.1, 215.1, 189.2
γ-glutamyl-*S*-propylcysteine-*S*-oxide	307	307.0	217.1, 178.1, 130.2

All garlic samples showed molecular ions corresponding to molecular weight of standard alliin which was eluted at RT 1.4 minute (Figure 4A, 5A). Its fragment ion was m/z 88 which further fragmentation to obtain m/z 70.6 as a base peak (Figure 4B). However, in garlic samples the molecular ion was clearly seen in positive mode with high relative abundance comparing with in negative mode. Fragment ion m/z 162 was corresponded to molecular weight of either SAC or allicin. Then this fragment ion further fragmented to give a fragment ion of m/z 145, and 73.6, thus it indicated a loss of CH_2=CH-CH_2-S moiety of SAC (Figure 5B). Molecular ion m/z 264 was clearly detected in both positive and negative modes of fresh garlic and garlic products (Figure 6A). Its fragment ions were 133 and 86 corresponding to loss of glutamyl ($C_5H_8NO_3$) and carboxylic groups (Figure 6B). The molecular ion m/z 291 was corresponding to either γ-glutamyl-*S*-1-propenylcysteine or γ-glutamyl-*S*-2-propenylcysteine. There were two peaks eluted at different retention times but showed the same molecular ion (m/z 291). Its fragmentation showed two different patterns (Figure 7A and 7B). These MS spectra was similar to that of MS spectra obtained from FT ion cyclotron MS (Nakabayashi et al., 2016) which had a higher resolution than ESI MS in our laboratory. The fragment ion m/z 162 was also corresponding to propenylcysteine moiety ($C_6H_{12}NO_2S$). The product ions m/z 170 and 234 were corresponding to $C_8H_{12}NO_3$ and $C_8H_{12}NO_5S$, respectively. These results indicated that one is γ-glutamyl-*S*-2-propenylcysteine (Figure 7A) and the other was γ-glutamyl-*S*-1-propenylcysteine (Figure 7B).

In addition, the molecular ions m/z 305 and 307 were observed. The product ion m/z 215 was corresponding to a loss of mass 90 (C_3H_7SO), so this MS spectra indicates γ-glutamyl-*S*-propenylcysteine-*S*-oxide (Figure 8A). However, it still could not differentiate 1- or 2- propenyl compounds. In the other MS spectra it indicates a small m/z 88, so it could be γ-glutamyl-*S*-propylcysteine-*S*-oxide (Figure 8B).

5. Discussion

The content of alliin was affected by garlic cultivars, planting conditions (i.e. soil, fertilizer), processing of garlic and sample preparation of analytical methods. Fresh garlic cloves contain 0.25-1.15% alliin while garlic products under different dried processes contain 0.7-1.7% alliin (Lawson, Wood, & Hughes, 1991; Iberl, Winkler, Müller, & Knobloch, 1990; Mochizuki et al., 1989). Different garlic cultivars (i.e. white garlic, elephant garlic) contained 0.003-0.281 mg alliin/g garlic cloves (Apawu, 2009). Two brands of garlic capsules showed 4.10-4.89 mg alliin/g garlic cloves compared with 0.049-0.361 mg alliin/g garlic cloves of two commercial garlic tablets (Apawu, 2009). The alliin contents of fresh garlic and garlic products in this study showed similar results, although the alliin contents were lower than the ones of Indian and Chinese garlics (8.19-10.08 mg/g) (Siddiqui et al., 2016). In addition, 0.01 M hydrochloric acid- 90% methanol mixtures showed a better extraction solvent than hot water for sample preparation (Apawu, 2009). However, most articles had reported using organic solvent extraction (i.e. methanol, dichloromethane) to prepare samples for the analysis.

Although in some articles mentioned that ACSO and SAC may not present in garlic capsules (Ramirez, Locatelli, Gonzalez, Cavagnaro, & Camargo, 2017), in this study MS spectra identified alliin and SAC in garlic products as well as in fresh garlic. Identified organosulfur compounds (Table 2) were found in lyophilized garlic, garlic capsule1 and garlic capsule 2 but they were slightly different in relative abundance. In addition, methiin (m/z=150) was found in garlic capsule 2. Isoalliin is a trans isomer of alliin and they are the same molecular weight and showed similar product ion m/z=88. HPLC condition in this study cannot well separate these compounds and LC-ESI/MS was not sensitive enough to differentiate them and isotope also. The drying process either in the oven or in the lyophilizer can maintain some organosulfur compounds as in fresh garlic. Moreover, HPTLC is an efficient analytical method to determine sulfur containing compounds in garlic while LC-MSMS can be used to characterize individual organosulfur compounds.

6. Conclusion

Fresh garlic and garlic products are used for medicinal applications. TLC and HPTLC are rapid and reliable methods to simultaneously identify active sulfur containing compounds in garlic and its products. The alliin contents in garlic products may be useful for prescription according to its indication. The MS and MS/MS spectra indicated that fragmentation patterns of the organosulfur compounds in garlic products showed similar profile as in fresh garlic**.** Only garlic capsule 2 showed a mass of methiin.

Therefore, some bioactive organosulfur compounds still maintain in these products after a drying process either by lyophilized or mild heating processes.

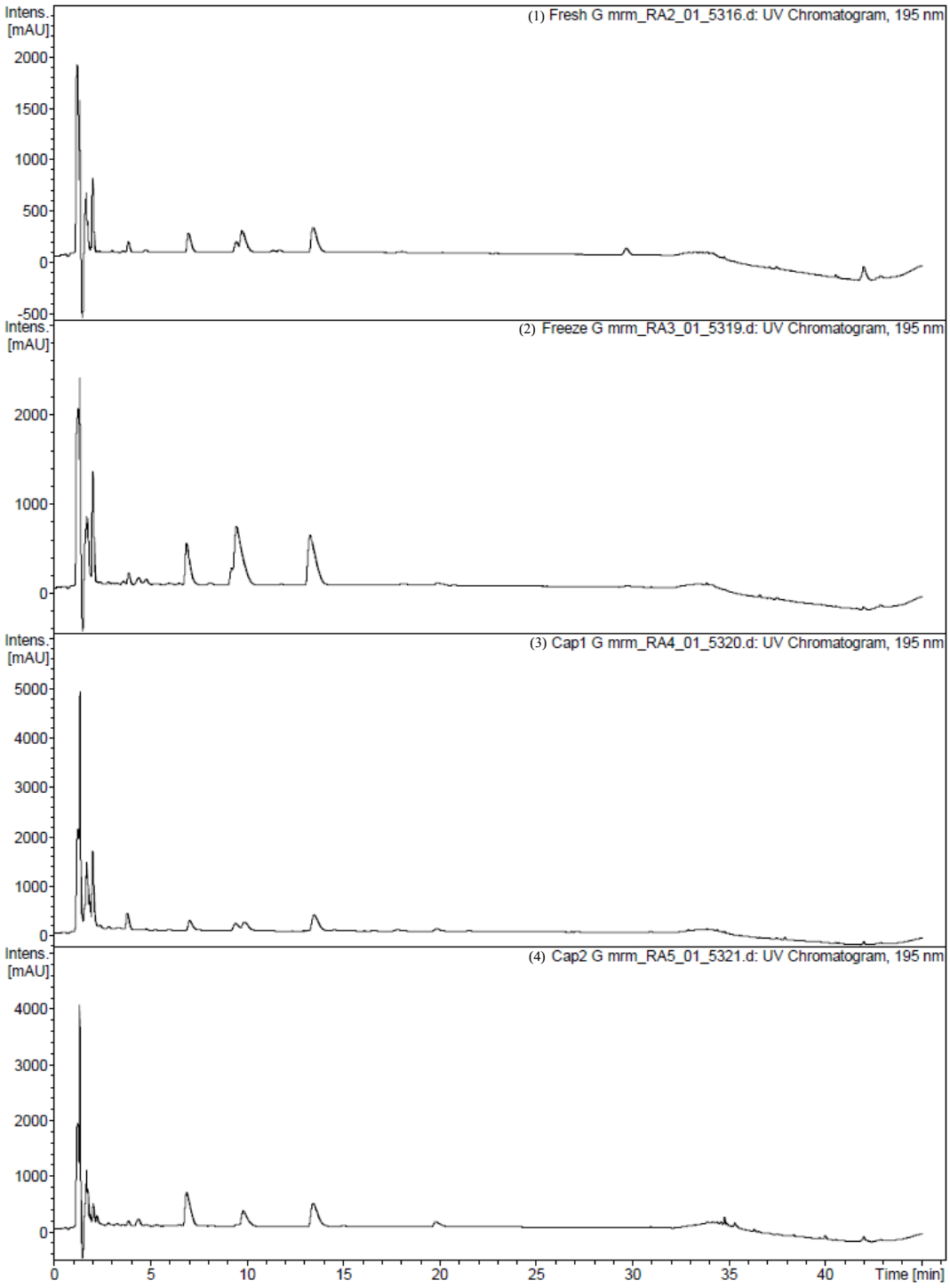


Figure 3 Overlay HPLC chromatograms of fresh garlic and garlic products

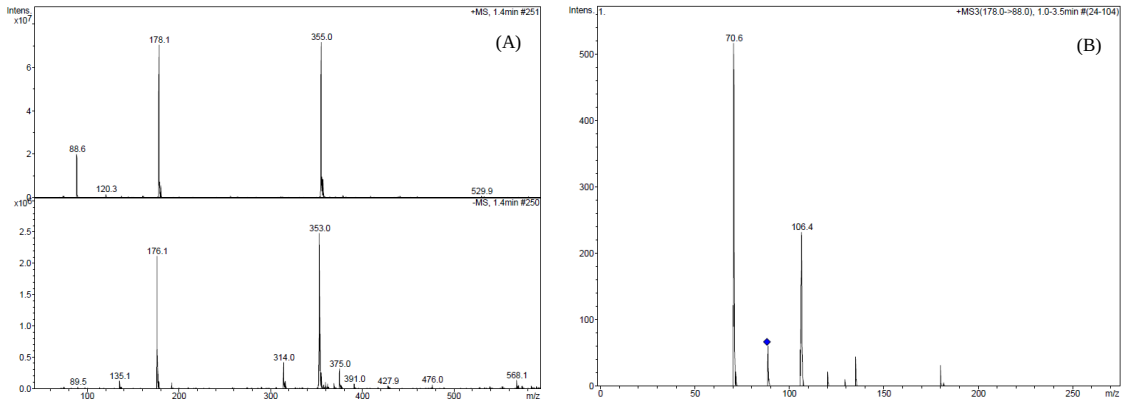


Figure 4 (A) MS spectra of standard alliin (MW=177.2) in positive and negative mode (B) Fragment ion of m/z 88

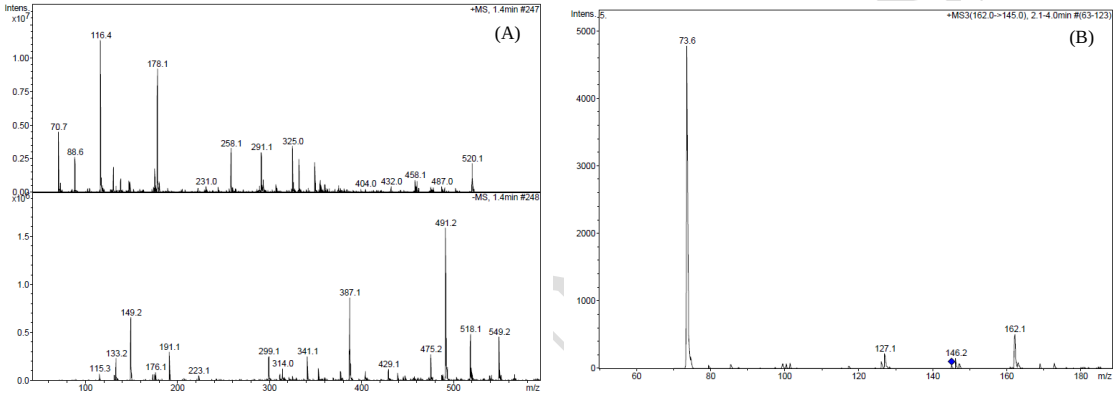


Figure 5 MS spectra of fresh garlic showed (A) molecular ion of alliin ($[M+H]^+$ =178) and (B) fragment ion of m/z 162 (SAC)

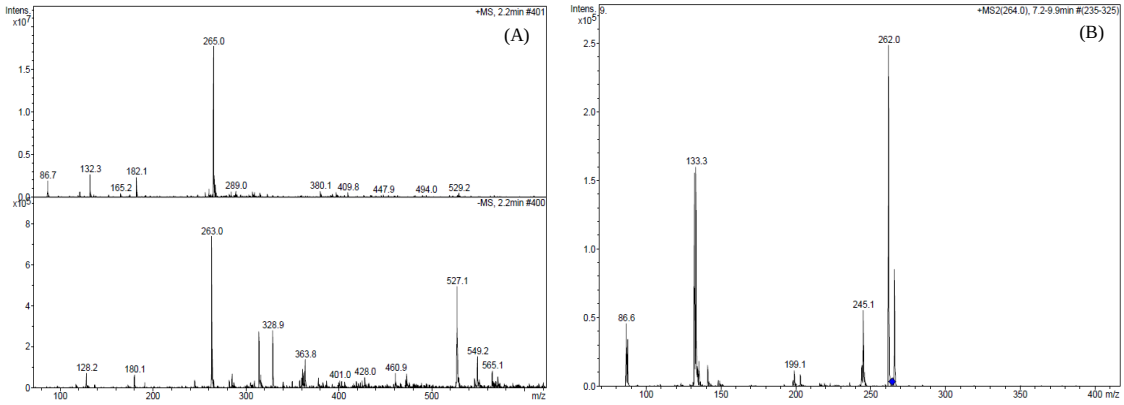


Figure 6 MS spectra of lyophilized garlic showed (A) molecular ion of γ-glutamyl-*S*-methylcysteine ($[M+H]^+$ =264) and (B) its fragment ions (m/z = 245, 133, 86)

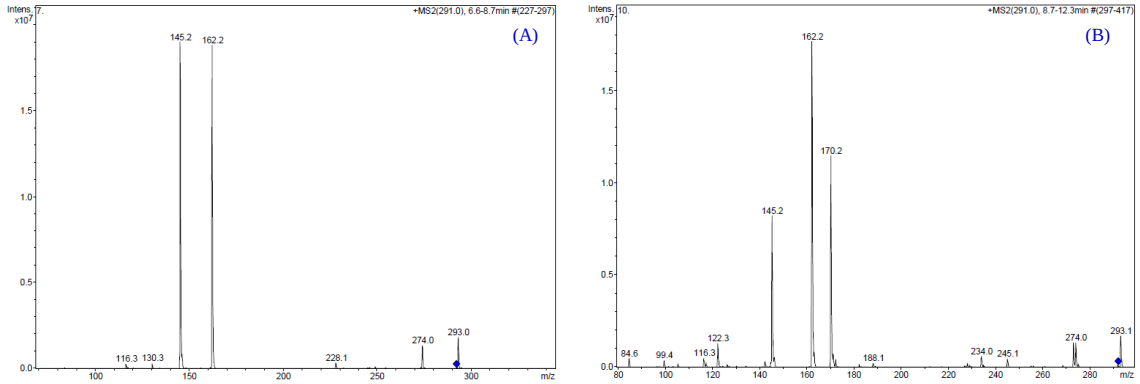


Figure 7 MS spectra of garlic capsule 1 showed (A) fragment ions of m/z 291 at RT 7.5 min and (B) at RT 10 min

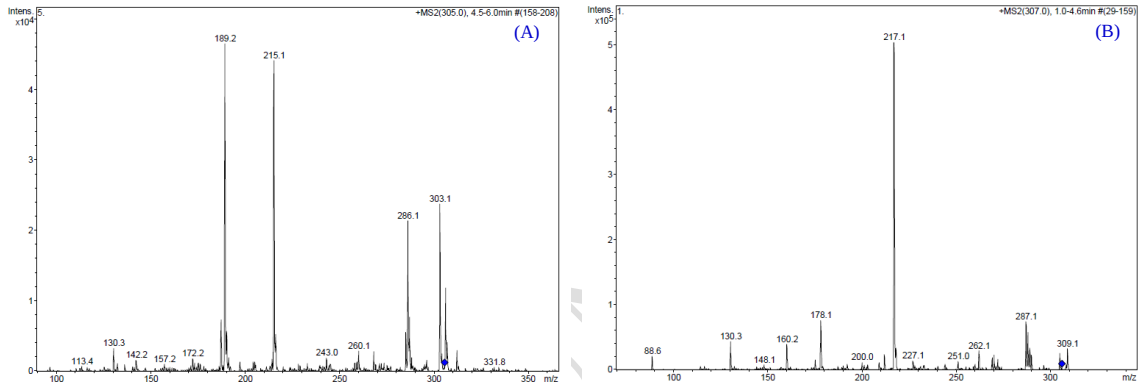


Figure 8 MS spectra of garlic capsule 1 showed (A) fragment ions of m/z 305 and (B) fragment ions of m/z 307

7. Acknowledgements

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8. References

Apawu AK. (2009). *Reversed-phase HPLC determination of alliin in diverse varieties of fresh garlic and commercial garlic products* (Master's thesis, East Tennessee State University, USA)

Arnault, I., Christidès, J. P., Mandon, N., Haffner, T., Kahane, R., & Auger, J. (2003). High-performance ion-pair chromatography method for simultaneous analysis of alliin, deoxyalliin, allicin and dipeptide precursors in garlic products using multiple mass spectrometry and UV detection. *Journal of Chromatography A*, *991*(1), 69-75.

Benavides, G. A., Squadrito, G. L., Mills, R. W., Patel, H. D., Isbell, T. S., Patel, R. P., ... & Kraus, D. W. (2007). Hydrogen sulfide mediates the vasoactivity of garlic. *Proceedings of the National Academy of Sciences*, *104*(46), 17977-17982.

Dewi, A. D. R., Kusnadi, J., & Shih, W-L. (2016). Comparison of the main bioactive compounds and antioxidant activity from garlic water-soluble and garlic oil. *NRLS Conference Proceedings International Conference on Natural Resources and Life Sciences*. 20-34.

Fukao, H., Yoshida, H., Tazawa, Y. I., & Hada, T. (2007). Antithrombotic effects of odorless garlic powder both *in vitro* and *in vivo*. *Bioscience, Biotechnology, and Biochemistry*, *7*, 21.

Galeone, C., Pelucchi, C., Levi, F., Negri, E., Franceschi, S., Talamini, R., ... & La Vecchia, C. (2006). Onion and garlic use and human cancer. *The American Journal of Clinical Nutrition*, *84*(5), 1027-1032.

Gebhardt, R., Beck, H., & Wagner, K. G. (1994). Inhibition of cholesterol biosynthesis by allicin and ajoene in rat hepatocytes and HepG2 cells. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1213*(1), 57-62.

Gebhardt, R. (1995). Amplification of palmitate-induced inhibition of cholesterol biosynthesis in cultured rat hepatocytes by garlic-derived organosulfur compounds. *Phytomedicine*, *2*(1), 29-34.

Gebreyohannes, G., & Gebreyohannes, M. (2013). Medicinal values of garlic: A review. *International Journal of Medicine and Medical Sciences*, *5*(9), 401-408.

Iberl, B., Winkler, G., Müller, B., & Knobloch, K. (1990). Quantitative determination of allicin and alliin from garlic by HPLC. *Planta Medica*, *56*(03), 320-326.

Jaber, M. A., & Al-Mossawi, A. (2007). Susceptibitity of some multiple resistant bacteria to garlic extracts. *African Journal of Biotechnology*, *6*(6), 771-776.

Koch, H. P., & Lawson, L. D. (1996). *Garlic: the science and therapeutic application of Allium sativum L. and related species* (Vol. 683181475). baltimore, Maryland: Williams & Wilkins xv, 329p. ISBN.

Kubec, R., & Dadáková, E. (2009). Chromatographic methods for determination of S-substituted cysteine derivatives—A comparative study. *Journal of Chromatography A*, *1216*(41), 6957-6963.

Lanzotti, V. (2006). The analysis of onion and garlic. *Journal of Chromatography A*. 1112, 3-22.

Lawson, L. D., Wood, S. G., & Hughes, B. G. (1991). HPLC analysis of allicin and other thiosulfinates in garlic clove homogenates. *Planta Medica*, *57*(03), 263-270.

Ministry of Public Health. (2016). Thai Herbal Pharmacopoeia. The Agricultural Co-operative Federation of Thailand. Ltd., Bangkok, Thailand.

Mochizuki, E., Nakayama, A., Kitada, Y., Saito, K., Nakazawa, H., Suzuki, S., & Fujita, M. (1988). Liquid chromatographic determination of alliin in garlic and garlic products. *Journal of Chromatography A*, *455*, 271-277.

Nakabayashi, R., Sawada, Y., Aoyagi, M., Yamada, Y., Hirai, M. Y., Sakurai, T., Komoi, T., Rowan, D.,D., & Saito, K. (2016). Chemical assignment of structural isomers of sulfur-containing metabolites in garlic by liquid chromatography-Fourier transform ion cyclotron resonance-Mass spectrometry. *The Journal of Nutrition*, *146*(Suppl), 397S-402S

Ohaeri, O. C. (2001). Effect of garlic oil on the levels of various enzymes in the serum and tissue of streptozotocin diabetic rats. *Bioscience Reports*, *21*(1), 19-24.

Ramirez, D. A., Locatelli, D. A., Gonzalez, R. E., Cavagnaro, P. F., & Camargo, A. B. (2017). Analytical methods for bioactive sulfur compounds in *Allium*: An integrated review and future directions. *ournal of Food Composition and Analysis*, *61*, 4-19.

Siddiqui, N. A., Mothana, R. A., & Alam, P. (2016). Quantitative determination of alliin in dried garlic cloves and products by high-performance thin-layer chromatography. *Tropical Journal of Pharmaceutical Research*, *15*(8), 1759-1765.

World Health Organization. (1998). *WHO Quality control methods for medicinal plant materials*. Geneva. Switzerland.