The changing profiles of organosulfuric compounds during black garlic processing

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Abstract. Black garlic is popular as food supplement as well as herbal medicine due to its rich and beneficial chemical contents. However, the chemical changes during the process are rarely investigated. The purpose of this study was to determine the effect of heating time on sulfur-containing compound profiles and antioxidant activity during fermentation process at 60°C by solvent extraction followed by GC-MS analysis, as well as antioxidant activity, using DPPH method. The result indicated that black garlic underwent changes of sulfur-containing organic secondary metabolites, such as allicin and its derivative compounds. The process also increased antioxidant activity in black garlic.

1. Introduction
Garlic (Allium sativum) are used worldwide, commonly cooking and spice. It has unique taste and also many benefits due to its nutritional value, that makes garlic one of the best daily healthy foods [1, 2]. In addition to being used as a food ingredient, garlic can be used as herbal medicine, for example, in lowering high blood pressure, relieving headaches, lowering cholesterol levels in the blood, and ulcer medication. In fact, recent studies have found that garlic can be used as a cure for cancer and diabetes [3, 4].

Garlic has a biological effect, presumably because of the content of flavonoids in it. Some flavonoids containing sulfur can protect cells from damaging effects. The organosulfur content contained in garlic includes: S-alk (en) -il-L-Cysteine Sulfoxide (ACSOs), S-methyl-cysteine sulfoxide (methiin), S-(1-propenyl) -cysteine sulfoxide (isoalliin), S-propyl-cysteine sulfoxide (propine), (1S, 3R, 5S) -5-methyl-1,4-thianaze-3-carboxylic acid 1-oxide (cycloalliin), volatile sulfur compounds such as allicin, sulfur compounds fat-soluble substances such as diallyl sulfide (DAS) and diallyl disulfide (DADS), allil cysteine (SAC). Among these organosulfur compounds, there is a compound that causes garlic to have a bad taste, smell, and aroma. This causes people to be reluctant to consume it directly, even though they already know the many benefits contained in garlic.

Aged black garlic is a type of fermented product made from spontaneous fermentation of garlic under certain circumstances [5, 6]. Heating garlic into black garlic is carried out at controlled temperatures and humidity for approximately one month [7]. There have been reported that a consortium of bacteria is responsible for the fermentation. The bacteria that help the fermentation process includes the strains of Bacillus subtilis, Bacillus methylotrophicus, and Bacillus amyloliquefaciens. These bacteria are able to ferment glucose, lactose, sucrose, and polysaccharides in garlic to produce acid, and have the ability to withstand heat [6]. After the fermentation process with
heating, the unpleasant odor in onion has been reduced because the levels of allicin before and after being heated decreases. The thermal process induces many chemical reactions in garlic, such as enzymatic browning and Maillard reactions, causing the color to change from white and yellow to dark brown [8]. In addition, the heating process can also affect antioxidant activity in garlic [7]. The process of fermentation is actually very complex and the chemical content changes in content and composition.

The odor on the onion is caused by the presence of alicin compounds. Alicin compounds, the strong odor of garlic, are derived from changes in alliin compounds. Changes in alliin compounds to alicin are catalyzed by the allinase enzyme. The results of a study conducted by Chhabria and Desai [9] showed that, allinase enzymes denatured at temperatures above 50°C, so that to stop the catalytic process of alliin to become alicin, the onion must be heated at a temperature of around 60°C.

There were also many analytical chemistry methods were employed to reveal components in biological materials, depending on the characterization as well as applications, related to natural products as well as man-made materials [10, 11]. Most of used analytical chemistry methods were based on separation and characterization by gas chromatography as well as mass spectrometry. The separated components of garlic was assigned by those methods and instrumentation that has big capability for analysis similar samples [12 - 14]. In the future analytical chemistry plays important roles in analyzing natural substances during processes.

Previous research on levels of alicin compounds has been carried out by Zhang et al. [7] showed dynamics in garlic composition during treatment to black garlic. There has also been no study that explains the changes from alicin compounds to other organosulfur compounds during the fermentation process. Therefore, this study aimed to determine the effect of heating time on changes in organosulfur compounds, in order that the unpleasant odor in garlic decreases and even disappears and find out the percentage inhibition of antioxidant compound during fermentation of onions from garlic.

2. Materials and Method
2.1. Preparation black garlic
The material used in this study was around 800 g of garlic, without washing and peeling. This was purchased in a supermarket in Malang city, East Java, Indonesia. Methanol p.a (E.Merck) was used as the solvent in extraction process. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was the free radical in the analysis of the percentage inhibition of antioxidant compounds, according to Gadow et al. [15] with modification.

The equipment employed was built-from rice cooker (commercial brand Miyako) type MCM-606. This was equipped with temperature controller Autonics TC4S-N4R, with 15 A output from the Solid State Relay connected with FT-K-M6 type thermocouple cable so that the temperature can be kept constant during process. Other tools are UV-Vis spectrophotometry (Shimadzu) used as an instrument for testing the inhibition percentage from the absorbance of antioxidant compounds, and GC-MS (QP 2010 Plus from Shimadzu) which was used as an instrument that detects organosulfur compounds in onions by matching with Wiley 8 mass spectrum database. Helium gas was the mobile phase used in all experiments. One semi-polar column from Restek RTX 5-MS was used for the elution in a programmed temperature ramping up from 50°C to 250°C by 10°C per minute rate. No standard solution was used since no absolute quantitative analysis were planned.

2.2. Onion fermentation
As much as 800 g of garlic was being processed in the experiments. Each bulb of garlic was wrapped in aluminium foil and kept in the modified rice cooker at 60°C in constant condition. Fermentation was carried out for 24 days, while extraction was done every 3 days. The process started from day 0, 3, 6, 9, 12, 15, 18, 21, to 24. After extraction, they were analysed for chemical profiles and organosulfur compounds as well as the percentage of inhibition of antioxidant compounds.
2.3. Change analysis of organosulfur compounds
Onions was peeled and weighed as much as 5 grams and mashed to form thick pasta. The pasta was macerated in 20 mL of methanol. Maceration was done in the refrigerator for 1 day prior to injection to GC-MS. Chromatography experiments were set up with this setting: column temperature 50-250 °C with flow rate 10 degree/ minute. The components were analysed and matching to Wiley 8 library. Components with minimum 80 of similarity index (SI) were considered in this report. The same parameters were used to carried out the experiment every 3 days up to day 24. This method was modified from several experiments in previous used in several previous works [12].

2.4. Analysis of percentage inhibition in antioxidant compounds
The same amount crushed onion was macerated the same way, and as much as 4 mL were added 2 mL of 20 ppm 1,1-diphenyl-2-picrylhydrazil (DPPH) in methanol and put in 30 minutes incubation. Then the absorbance was measured at wavelength $\lambda = 517$ nm. Testing the percentage of inhibition of antioxidant compounds was done every 3 days.

3. Results and Discussion
3.1. Onion fermentation
The fermentation process can be seen by eyes due to developing darker color during process as can be seen in Figure 1 (a and b). Including to the physical development, increased density would also be observed from the hardness of the tissue.

![Figure 1. Garlic before (a) and after (b) heating](image)

Heating causes a Maillard reaction which causes the white garlic to become yellow then light brown until dark brown in color [14]. The brown color comes from melanoidin which is formed during process. The color intensity is very dependent on temperature and heating time. The higher the heating temperature, the darker the color of the garlic in a certain length of time. In this experiment, the color change in the garlic was observed every 3 days and volatile organosulfur compound was analysed as well as the inhibition percentage of the antioxidant compounds contained in the sample.

3.2. The effect of heating time on changes in organosulfur compounds from the chromatograms
Apart of physical appearance, the organic extract content was eluted, and they gave ideas about different chemical composition present in different stages of process. As in Figure 2, the fresh garlic showed a very rich components which were unresolved from each other. After heating process at 60°C, most of the volatiles were gone as can be seen in Figure 3, in which the next four experiments after day 3, 6, 9, and 12 were presented.
Some more chemicals disappeared after 6 days in Fig.3, but some new compounds appeared at later retention times. This suggested bigger molecules or less volatile components came during process. The real biochemistry routes are difficult to monitor since the study monitored the volatiles was only by GC MS. After 9 and 12 days, some new components appeared in the short retention times. This indicates more volatiles formed again from the fermentation process up to 9 days and 12 days, can be from any mechanism.

Figure 3 gave the tendency of components in earlier retention time were dominant in the black garlic at older time of heat fermentation. These new volatiles can be from bigger molecules degradation due to fermentation or heat. The back color of the garlic was persistent so that one can be made sure the chemical composition of black garlic volatiles is not the same as the fresh onion. The density was higher too since the physical appearance suggests shrinkage process. This also due to the heat, made the water molecules gone. The end tissue was dry and hard, as can be seen in Figure 1. At older stage of the process, after 21 and 24 days in Figure 4, most volatiles disappeared as the signals were smaller compared to the previous chromatograms.

Based on the MS analysis results one can mapped the presence of organosulfur compounds and their numbers by calculating the area under the peaks of each compound. The names of the compounds and their percentages can be seen in Figure 5. The MS matching were done and the suggestion of at least 80% similarity were counted. The categorization of chemicals was done based on functional groups they have. Sulfur-containing compounds were put into account since those are the ones responsible for
may good properties of black garlic. Figure 5 shows the relative similar compounds present in the process. The calculation was based on areas under each peak.

![Graph showing changes in garlic organosulfur compounds from GC-MS results](image)

Figure 5. Changes in garlic organosulfur compounds from GC-MS results
On day 0 or the fresh garlic, there were 5 organosulfur compounds detected, including 2-Vinyl-[4H]-1,3-dithiin with the highest percentage of 99.5011%, 1-methyl-5- (metilsulfonyl) -1H-Tetraa zole, trisulfide-methyl 2-propenyl, 1,2-Dithiin, 3-ethenyl,3,4-dihydro, and trisulfide, di-2-propenyl. After some days heating, the patterns were different. All three trisulfide compounds, di-2-propenyl, still appeared, but with a higher percentage of 84.2439%. In addition, a 1-butene-1-sulfonic acid compound is formed, ethyl ester, (z) - of 15.7561%. On the 6th day, only 1 organosulfur compound was detected, namely Silane, trimethyl [4- (4-methylphenyl) -3- (phenylsulfanyl) butyl for 100% organosulfur compounds and 65.66% of all volatile compounds. While on the 9th day of the trisulfide compound, di-2-propenyl reappeared with a percentage of 2.473% of all volatile compounds. On the 12th day there was a 3.098% 2-propenythioacetoniitrile compound of all volatile compounds detected. On day 15 tributylstannylmethyl thiobenzoate appeared as 7.3957% and bicyclo [4.1.0] hept-3-ene-2-thiol, 3,7,7-trimethyl-, [1s- (1.alpha, 2. alpha, 6. alpha.)] - as many as 92.6053%. On day 18 bicyclo compounds [4.1.0] hept-3-ene-2-thiol, 3,7,7-trimethyl-, [1s- (1.alpha, 2.alpha, 6.alpha.)] - still there are almost the same number of 92.2134% of all existing organosulfur compounds. In addition to these compounds there are also s-phenyl (2s, 1’s)-2- (2-nitro-1-phenylethyl) -1 -1 (1-phenylethyl) aziridine-2-carbothioate compounds of 7.78966%. After that, on days 21 and 24 there were no organosulfur compounds that could be identified by GC-MS.

During the fermentation process, allin compound will be degraded due to heating. Allin can be degraded to other organosulfur compounds, including (E) -ajoene, (Z) -ajoene, 3-vinyl-4H-1,2-dithiin, 2-vinyl-4H-1,3-dithiin, and diallyl disulfide. However, based on GC-MS data, the allicine derivatives identified were only 1,2-Dithiin, 3-ethenyl-3,4-dihydro, 1,2-Dithiin, 3-ethenyl-3,4-dihydro compounds identified in the 0-day after continuous heating can be decomposed into simpler organo compounds. Some reports also discussed about several similar organo-sulfur components in garlic processing [15] depending on various conditions.

3.3. The effect of heating time on percentage inhibition of antioxidant compounds

Previous studies yielded that the content of antioxidant compounds in black garlic increased compared to raw garlic as also obtained previously [6, 7]. Antioxidant compounds that increase by heating are total phenolic, 5-hydroxymethylurifural, allyl sulfide, allyl disulfide, etc. Black onions contain polyphenol compounds with levels 2-4 times higher and total flavonoids content 2-5 times higher than raw garlic [16]. This is in good agreement with the experiment, the percentage of inhibition decreased upon heating to black garlic as can be seen in Fig.6. The percentage value of inhibition of antioxidant compounds starting from day 0 to day 24 continued to decline. The lower the inhibitory value is, the free radicals (DPPH) were highly reduced. This shows that the longer the process of heating the garlic, the more antioxidant compounds are formed. The percentage inhibition value also describes antioxidant activity during the fermentation of black garlic.

![Figure 6. Percentage inhibition of black garlic](image-url)
The figure indicated the potential of black garlic in giving good performance as herbal medicine or food supplement. Under optimum fermentation condition better good and beneficial chemicals formation can be expected. Some components can be isolated as well for good purposes, given the changing profile of chemicals during process by chromatographic separation.

4. Conclusions
During black garlic making, the process under continuous heat and certain humidity, chemicals profile of garlic changes significantly. The process also indicated organosulfur compounds decomposed into other smaller and lighter organosulfur compounds. In term of organosulfur volatile compounds, earlier heating process around 60°C was able to reduce almost all organosulfur components of the garlic. In this early process, most of the source of unpleasant odor were eliminated. The longer heating causes the percentage inhibition value of antioxidant compounds to decrease in order that more free radicals are reduced by antioxidant compounds. Gas chromatography was adequate to observe volatile components in garlic. Other bigger molecules can be followed by other chromatography methods.

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