Makara Journal of Science

Volume 27
Issue 2 *June*

Article 8

6-25-2023

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Recommended Citation

Wonorahardjo, Surjani; Sari, Dian Puspita; Salsabila, Arini; Estiyawati, Estiyawati; Yuliani, Dewi; Wijaya, Anugrah Ricky; Suharti, Suharti; Kusumaningrum, Irma Kartika; Maharani, Chariztya Anggita; and Noviyanti, Thjiong Angelina (2023) "Physicochemical Changes and Role of Analytical Chemistry in Black Garlic (Allium sativum L.) Processing," *Makara Journal of Science*: Vol. 27: Iss. 2, Article 8.
DOI: 10.7454/mss.v27i2.1333
Available at: https://scholarhub.ui.ac.id/science/vol27/iss2/8

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Makara Journal of Science, 27/2 (2023), 148–159
doi: 10.7454/mss.v27i2.1333

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Received March 17, 2022 | Accepted May 10, 2023

Abstract

Black garlic is a traditional spice having potential of herbal medicine; however, continuous heating darkens its color and changes its taste due to chemical processes and new equilibrium in the system. In this study, fresh garlic was processed into black garlic at 60 °C for 24 days. Changes in physical and chemical parameters, such as browning and protein content, reducing sugar content, and antioxidative components were recorded. Protein content was measured by Kjeldahl method, brown color development was analyzed using a color reader, reducing sugar was examined using a dinitro salicylic DNS reagent, and antioxidative activities were studied with a diphenyl picrylhydrazyl DPPH reagent in terms of their percent inhibition. Gas chromatography – mass sepctrometry (GC–MS) and Liquid Chromatography-High Relosultion Mass Spectrometry (LC–HRMS) were performed to determine the chemical profiles. Results showed a number of interesting features. The protein and sugar contents increased up to 3 and 4 times that of the initial values, respectively during processing. The antioxidative properties improved in the later stages. The chemical profiles of volatile and nonvolatile components were altered in their final stage. Sulfur and nitrogen-containing nonvolatile components were relatively stable during heating, but their compositions changed. The natural chemical changes reflect the biological potential dynamics in biological processes as herbal supplements. The reflection of the processes might go further into food chemistry and the food industry. This needs an interdisciplinary approach, expanded to biological concepts and safe, ethical industrial processes.

Keywords: antioxidative components, black garlic, browning intensity, chemical profiles, chromatography–mass spectrometry, protein content, sugar content

Introduction

Over the centuries, various species of genus *Allium*, including garlic (*Allium sativum* L.) have been used as vegetables, spices, and herbal medicines to cure various ailments. They are a part of nature and healing substances for humans and other living beings. They are often used as herbal medicine to prevent and cure various diseases [1, 2]. Garlic has antibacterial, antifungal, hypolipidemic, hypoglycemic, antithrombotic, antioxidant, and anticancer properties [3] It is often used as a supplement because it contains organosulfur compounds with distinctive aromas and tastes (mostly allicin compounds) [4]. Black garlic is made by heating the garlic at a constant temperature and maintaining its humidity for several weeks [2]. Many reports have focused on the chemical compounds of garlic prepared using various experimental setups [5, 6], but only a few studies have reviewed the chemical processes during heating.

Two kinds of reaction occur during black garlic processing: the first is enzymatic reactions aided by endophytic bacteria and fungi [1] and the second is nonenzymatic reactions [7, 8], primarily Maillard reactions [6]. A report on the composition of volatile components during black garlic processing showed that organosulfur compounds decompose into small and light compounds [2]. Endophytic fungi and bacteria were also observed during the process [9]. A high acid content was observed at the late stage of black garlic processing.

The chemical profile of black garlic contains many polysaccharides, reducing sugars, proteins, phenolic compounds, organic sulfur compounds, and melanoidin [10], [11]. The variables of the Maillard reaction include substrates (reducing sugars and proteins), pH, water content, and temperature [12]. Heating garlic results in its physical changes, such as the texture shifting from hard and crunchy to soft and the color shifting from light to

dark [8, 13]. To date, no attempts have been made to correlate browning intensity with the Maillard reaction, protein content, and individual components to determine alterations during black garlic production. Given that amino acids play an essential role in the Maillard reaction, a correlation must occur among the browning intensity, color, protein content, and individual components that are altered during black garlic processing.

Organosulfur compounds are bioactive substances that inhibit cancer, cardiovascular disease, and age-related diseases [14]. Inhibition/percent damping (free radical capture activity) calculation can be used to determine the antioxidant activity of the sample through the magnitude of the DPPH radical absorption barrier (2,2-diphenyl-1-picrylhydrazyl). Bacteria and fungi also pave the way for research into enzymatic processes [1, 9]. However, publications that track the daily changes of these compounds and discuss them thoroughly within the scope of interdisciplinary science are rare. The primary aim of the current study is to determine the chemical and physical changes in black garlic processing. Its underlying goal is to show that science can provide a comprehensive description of nature.

In this study, the processes were studied by the means of analytical chemistry. Apart from physical appearance and changes in biochemical functions, analytical methodologies using instrumentation and conventional methods reveal chemical compounds. When a process can be documented, it can be emulated for scientific purposes. In this way, the objective function of science can be revealed and continuously updated from time to time. This concept might also be of interest to other sciences' education or disciplines and science philosophies, that have emerged in the last decades [15]. Chemical changes in black garlic processing show its complexity from different angles and how it is a part of the natural process that must be investigated as a research material for eco-reflective science [16, 17].

Materials and Methods

Garlic was obtained from a local supermarket in Malang, Indonesia. The following chemicals of p.a. grade were bought from E. Merck or Sigma Aldrich: Na_2CO_3, NaOH, $CuSO_4.5H_2O$, sodium-potassium-tartrate, Folin–Ciocalteu reagent, phosphate buffer solution (PBS), buffered acetic acid, ammonium sulfate, NaOH–HgO, KOH, NaOH, $Na_2S_2O_3$, HCl, H_2SO_4, H_3BO_4, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, DNS reagent, and iodine. UV-vis experiments were performed on a Shimadzu UV-1800 spectrophotometer. Browning intensities were examined by a Konica Minolta CR-10 color reader. A rice cooker equipped with a TC4S temperature controller for black garlic making was used with a 15 A solid-state relay output connected using an FT-K-M6 thermocouple. GC–MS was conducted on a Shimadzu QP2010S type. LC–HRMS was performed using Thermo Scientific Dionex Ultimate 3000 RSLCnano with a micro flow meter. The garlic skins were not removed, and each clove was wrapped in an aluminum foil before the heat treatment in the modified rice cooker. The temperature was maintained at 60 °C for 24 days.

Analysis of browning intensity. Black garlic samples at days 0, 6, 12, 18, and 24 of fermentation times were randomly selected. The peeled samples were placed in a transparent container to be measured using a color reader. Duplo measurements were performed on each sample. The measurements produced a notation for L, a, and b. Standard values appeared as L as white, a as red–green, and b as yellow–blue colors. L represented the brightness parameter (achromatic color, 0 = black to 100 = white). The chromatic color of red–green mixture was expressed as a+ (positive), a value between 0–100, indicating a red color, and a− (negative), a value between 0–(−80), indicating a green color. The chromatic color of yellow–blue mixture was expressed as b+ (positive) a value between 0–70, indicating a yellow color, and b− (negative), a value between 0–(−70), indicating a blue color. The brightness (L) value obtained showed the overall browning intensity where L = 0 was dark and L = 100 was light or white. If L decreases, then the sample is brown or dark; If L increases, then the sample is light or white.

Analysis of total protein content. The protein content was determined using the Kjeldahl's method by dissolving ±2 g of the sample in 2 mL of PBS, followed by the addition of 2 mL of concentrated H_2SO_4 and ±2 g of Na_2SO_4–HgO catalyst (20:1). The mixture was boiled in a digestion device until the solution was clear (±4 h), and the treatment was continued for 30 min. When the digestion was completed, 35 mL of distilled water and 8.5 ml of NaOH–$Na_2S_2O_3$ were added to the mixture. In brief, 6.5 mL of 4% H_3BO_3 solution was placed in an Erlenmeyer flask, and a few drops of methylene red or methylene blue indicator were added into the prepared distilled distillate container. Distillation was stopped at ±25 mL, and the mixture was titrated using 0.1 N HCl. The protein content of the sample was calculated. The conversion factor for protein from this sample was 6.25.

Analysis of reducing sugar content. First, a 1,3 dinitro-salicylic acid (DNS) solution was prepared by dissolving 0.5 g (DNS) into 10 mL of NaOH 2M and homogeneous stirring the solution before adding 10 g of Na–K tartrate and phenol each and 20 mL of distilled water to a 50 mL solution. Standard solutions of 500 ppm concentration were prepared by dissolving 0.05 g glucose into 100 mL of water. Other concentrations were prepared by diluting the solutions to 150–450 ppm. Afterward, 1 mL of these solutions were added to 1 mL of DNS solution and 10 mL of water, boiled to 100 °C, cooled, and measured as standard solutions at 540 nm wavelength.

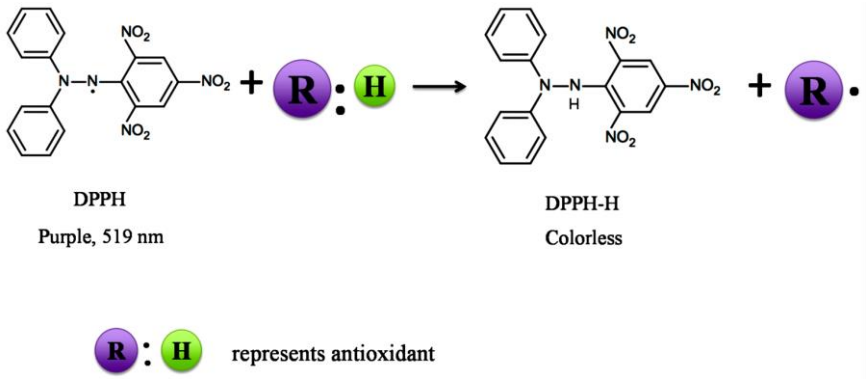


Figure 1. Reaction Mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with Antioxidant. R:H = Antioxidant Radical Scavenger; R = Antioxidant Radical

In brief, 1 mL each of the garlic extract solutions was transferred to a test tube and then mixed with 1 mL of DNS reagent and distilled water before boiling in a water bath at 100 °C. The mixtures were cooled in running water and measured at a wavelength of 540 nm. Reducing sugar content was tested every 6 days.

Analysis of antioxidant activity. In brief, 5 g of garlic without the skin was mashed using a mortar and pestle, followed by maceration in 20 mL of methanol for 1 day in the refrigerator and subsequent filtering. Afterward, 1 mL of the filtrate obtained from the filtration was added with 30 mL of reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) 20 ppm in methanol. The reaction between DPPH and antioxidants is successful when the purple color of DPPH changes to yellow. The yellow color comes from the picryl group. The reaction between DPPH and antioxidants is illustrated in Figure 1. The method was adopted from Gadow [18]. The greater the percentage of DPPH that undergoes reduction, the stronger the antioxidant activity. The percentage of inhibition represents the inhibition effect of an antioxidant against free radicals and was obtained from the calculation of absorbance measured using a UV-Vis spectrophotometer and converted to percent inhibition values [18].

Absorbance was measured at a wavelength of 514 nm every second from 0 to 4–5 minutes after the sample was mixed with the DPPH reagent. Furthermore, this antioxidant compound's inhibition percentage was tested in every sample. The absorbance data were used to show the free radical scavenging activity. However, from a qualitative view, the dropping values of absorbances already show the antioxidants' power.

Analysis of volatile and nonvolatile compounds by GC–MS and LC/HRMS. Garlic and black garlic tissues were crushed and filtered using methanol. The chromatograms of the compounds were matched with the WILEY 8 and NIST 12 libraries of GC–MS and mzCloud MS/MS library for LC–HRMS. These compounds were then selected according to the similarity index (SI). The amount of each component was calculated from the percent area.

Results and Discussion

Black garlic preparation. Garlic was heat-treated without removing the skin, to protect the enzymes, such as its antioxidant content.

Garlic experiences changes in texture and color during processing. A long heating time results in reduced water content, rigid texture, tendency to shrink or wrinkle, and a smooth texture of the bulbs. The dark color has appeared gradually. In addition, the black garlic has a milder pungent taste and aroma compared with the fresh garlic. Black garlic also tends to have a smooth sweet and sour taste, different from raw garlic that is spicy and hot (private communication).

Intensity of browning during black garlic processing. Color measurements were carried out, and Table 2 shows the brightness level of garlic as L values and a and b parameters.

The decrease in L values shows the brightness of the black garlic and is associated directly with the chemical changes inside. Meanwhile, a (+) and (−) indicate red and green colours, respectively, and b (+) and (−) are for yellow and blue colours, respectively. The colour would stay within these a and b value ranges. Some large molecule components, especially carbohydrates and oligosaccharides, undergo decomposition, and new short-chained chemical compounds appear [19]. Other components, such as organosulfur, would appear as small components with different properties [2], similar to any food product. On day 18 of black garlic processing, high amounts of antioxidants appear because of the process, and this phenomenon is correlated with garlic darkening colours. This chemical change in odor is observed daily. The decreased profiles in the brightness level of the black garlic are shown in Figure 2.

Table 1. Color Profile of Peeled Garlic During Processing

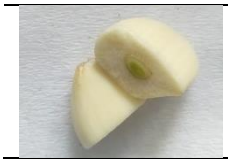
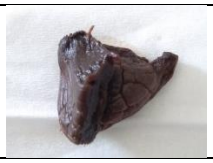
Garlic (day)				
0	**6**	**12**	**18**	**24**
				

Table 2. Value of Garlic (G) Color at Days 0, 6, 12, 18, and 24 of Processing

	L		a		b	
Sample	**Average**	**STDEV**	**Average**	**STDEV**	**Average**	**STDEV**
G-D0	80.84	0.0306	2.13	0.0100	31.78	0.0115
G-D6	24.16	0.0200	9.06	0.0115	15.34	0.0153
G-D12	4.29	0.0058	1.87	0.0153	1.34	0.0058
G-D18	3.86	0.0153	0.34	0.0153	1.08	0.0058
G-D24	1.97	0.0404	−0.74	0.0153	0.68	0.0058

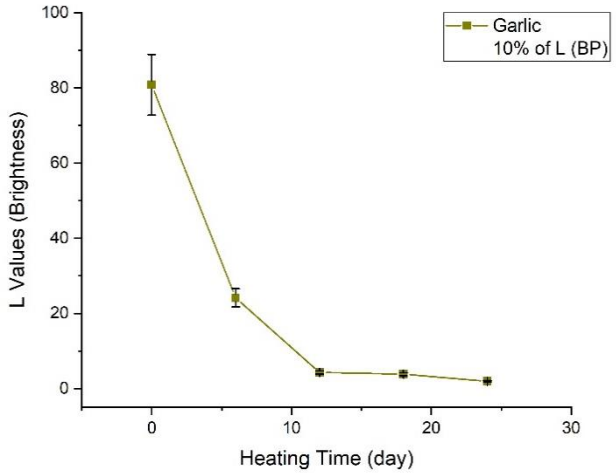


Figure 2. Changes in the Brightness Levels of Black Garlic During Processing

Garlic has exhibited a rapid darkening process (Table 1). However, after day 7, the decrease in L values has flattened. In this case, the chemical changes might have slowed down. Further detailed experiments should be performed to reveal the natural mechanism of the process and how to stop at the optimum condition according to the aims. The next project would be stopping the process at the right time and maintaining the best condition for a long period.

Although the darkening process is assumed to result from melanoidin presence, the carbohydrate and nitrogen compounds (proteins) were still analyzed [20]. The longer the heating process of garlic, the more melanoidin compounds are formed as observed from the brown-colored texture of the garlic. This phenomenon was indicated by the decline in L values (brightness).

Given that a(+) indicates red, a(−) indicates green colors, b(+) indicates yellow color, and b(−) indicates blue color, the color composition of black garlic is dominated by red and yellow as indicated by the positive a and b values (+). The longer the process, the darker brown the garlic color until it becomes close to black. Red and yellow cannot directly show the compounds, but their mixture cannot be directly associated with the chemical changes. A tricky separation and analysis method is needed to show the chemical shift profiles.

Volatile components of black garlic during processing. Another set of experiments was performed to determine the rough chemical profiles of components during the heating process. In GC analysis, the profiles of volatile components can be described in detail for each compound. Volatile components contribute to the unique odor of the black garlic during processing. The exact amount cannot be discussed because the compounds were not quantitatively examined. However, the behavior in a group of chemical compounds can be analyzed from the profiles. Some ways can be adopted to categorize available components as shown in Figure 3. Table 3 presents the three main components for each group for days 0, 6, 12, and 18.

Natural garlic (day 0) has various components but is dominated by sulfur-containing compounds. The dominant components are altered daily during black garlic processing. Regular garlic before the process has higher percentages than acids, alcohols, ethers, esters, phenolic components, and sugar content. Sulfur components give a strong and unique garlic odor and are gradually lessened daily during black garlic processing. However, evidence has shown that the sulfur components are still present up to the end of the processing as volatile components [2]. As a natural process, this complex situation occurs in the tissues. Although the total acidity increases [9], the volatile acids seem to disappear (Figure 3) and ether, hydrocarbons, and ketones have emerged as the main components. Hydrocarbon compounds also appear in large quantities after day 6; before, they existed in small percentages. The total acidity must be attributed to other components, such as the nonvolatile ones that will be discussed further. All these results illustrated the complex possibilities of recording the process from a chemical perspective. Although the color of garlic has darkened, small compounds with oxygens such as ketones and aldehydes bring the antioxidative properties, which will be discussed further in this paper.

The specific components of each group are tabulated in Table 3. They are based on WILEY 8 and NIST library and have the highest SI when matched with the databases.

Profile of nonvolatile components of black garlic during processing. The gaseous mobile phase in a chromatography column cannot elute nonvolatile components. Despite being present in the samples in considerable amounts, these large components will not appear in the gas chromatogram. Thus, their analysis is critical. These large components were separated using liquid chromatography. After some categorization, their composition is presented in Figure 4.

LC–HRMS results showed different tendencies for the components. Liquid chromatography separates large molecular weight components depending on their solubility. Amines, amides, and esters appear in considerable amounts up to day 24 of the processing, except amines (Figure 4). Protein degradation occurs for small-molecular-weight compounds during the processing while garlic is still giving pleasant and milder odors. Sugar components provide the sweet smell and smooth texture for the darkened tissues. Other oxy components, such as aldehydes and ketones, have persisted until the end of the stages. Finally, the suitable components of garlic providing herbal effects were detected. The specific components of each group are tabulated in Table 4. They are based on the mzCloud library and have the highest SI when matched with the database.

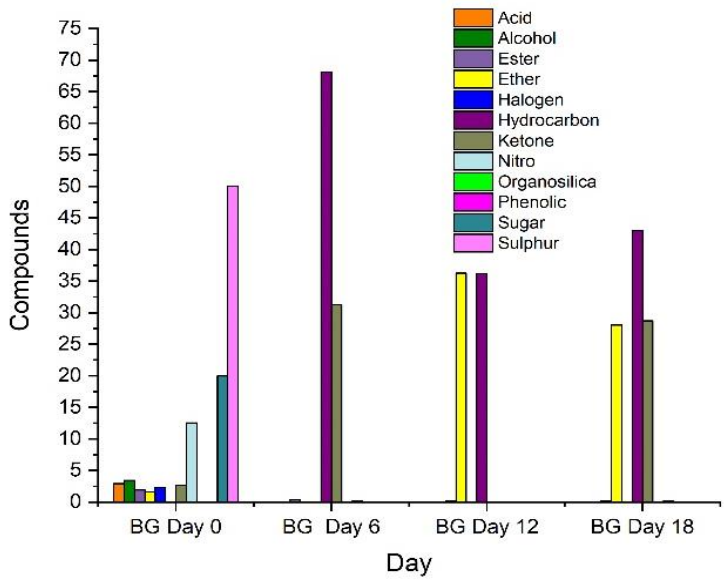


Figure 3. Profile Changes of Volatile Components During Black Garlic Processing

Table 3. Three Main Components of Each Group Appeared in the Chromatograms of Black Garlic During Processing

Groups	day 0	day 6	day 12	day 18
Acids	Acetic acid	-	-	-
Alcohols	Phenol	1,3-Dioxolane-4-methanol, 2,2-dimethyl-, (S)-	(R)-(-)-2,2-Dimethyl-1,3-dioxolane-4-methanol	(R)-(-)-2,2-Dimethyl-1,3-dioxolane-4-methanol
	3,3-Diethoxy-1-propanol		Phenol, 2-methoxy-4-(1-propenyl)-	
	2-Furanmethanol			
Esters	But-3-enyl (E)-2-methylbut-2-enoate	3-Hexen-1-ol, 2,5-dimethyl-, formate,(Z)-	4,7-Dioxooctanoic acid, methyl ester	3-Hexen-1-ol, 2,5-dimethyl-, formate,(Z)-
	1,2-Cyclohexanediol, monoacetate	4,7-Dioxooctanoic acid, methyl ester	3-Hexen-1-ol, 2,5-dimethyl-, formate,(Z)-	4,7-Dioxooctanoic acid, methyl ester
	Glycine, N-(ethoxycarbonyl)-, ethyl ester	Diethyl Phthalate	Phorone	Phthalic acid, 4-bromophenyl ethyl ester
Ethers	Benzofuran, 2,3-dihydro-	-	Propane, 2,2-dimethoxy-	Propane, 2,2-dimethoxy-
			3,3-Diethoxy-1-propyne	Oxirane, [(2-propenyloxy)methyl]-
			5-Hydroxymethylfurfural	
Halogen compounds	4-[(2E)-3-Cyclopropyl-2-fluoroprop-2-en-1-yl]morpholine	1-Fluorooctane	1-Chloroundecane	1-Chloroundecane
	Butane, 1-(2,2-dichloro-3,3-dimethylcyclopropyl)-			
Hydrocarbons	-	Propane, 2,2-dimethoxy-	Toluene	Toluene
		Toluene	Cyclohexane, methyl-	Cyclohexane, methyl-
		Cyclohexane, methyl-	o-Xylene	o-Xylene
Ketones	Benzeneacetaldehyde	3-Penten-2-one, 4-methyl-	3-Penten-2-one, 4-methyl-	3-Penten-2-one, 4-methyl-
	5-Methoxypyrrolidin-2-one	2-Pentanone, 4-hydroxy-4-methyl-	2-Pentanone, 4-methoxy-4-methyl-	2-Pentanone, 4-hydroxy-4-methyl-
	2,5-Dimethylfuran-3,4(2H,5H)-dione	2-Pentanone, 4-methoxy-4-methyl-	Methyl Isobutyl Ketone	2-Pentanone, 4-methoxy-4-methyl-
Nitro compounds	Oxane-4-carboxamide, 2-propyl-	(S)-(+)-2-Amino-3-methyl-1-butanol	3-Isothiazolecarboxamide	-
	2-Propanamine, N-ethyl-N-nitroso-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	
	Cyclohexene, 1-nitro-			
Organosilica	-	Cyclotetrasiloxane, octamethyl-	2,6-Dihydroxyacetophenone, 2TMS derivative	Cyclopentasiloxane, decamethyl-
		Cyclopentasiloxane, decamethyl-	Cyclopentasiloxane, decamethyl-	Cyclohexasiloxane, dodecamethyl-
		Cyclohexasiloxane, dodecamethyl-	Cyclohexasiloxane, dodecamethyl-	2-(2',4',4',6',6',8',8'-Heptamethyltetrasiloxan-2'-yloxy)-2,4,4,6,6,8,8,10,10-nonamethylcyclopentasiloxane
Phenolic compounds	-			
Sugars	Sucrose	-	-	-
Organosulfur compounds	3-Vinyl-1,2-dithiacyclohex-4-ene	-	-	-
	(Z)-1-Methyl-2-(prop-1-en-1-yl)disulfane			
	(E)-1-Allyl-2-(prop-1-en-1-yl)disulfane			

The matched components for GC–MS and LC–HRMS (Table 3, Table 4) were taken as the possible component obtained by mass spectrometry after separation in the chromatography column. Many possibilities can be inferred from the mass spectrum analysis, which shows the stability of each component and the transition components that exist during the processing. Some intermediate components were analyzed in many ways [11], providing MS and nuclear magnetic resonance NMR [21] methods and enormous data information for the future.

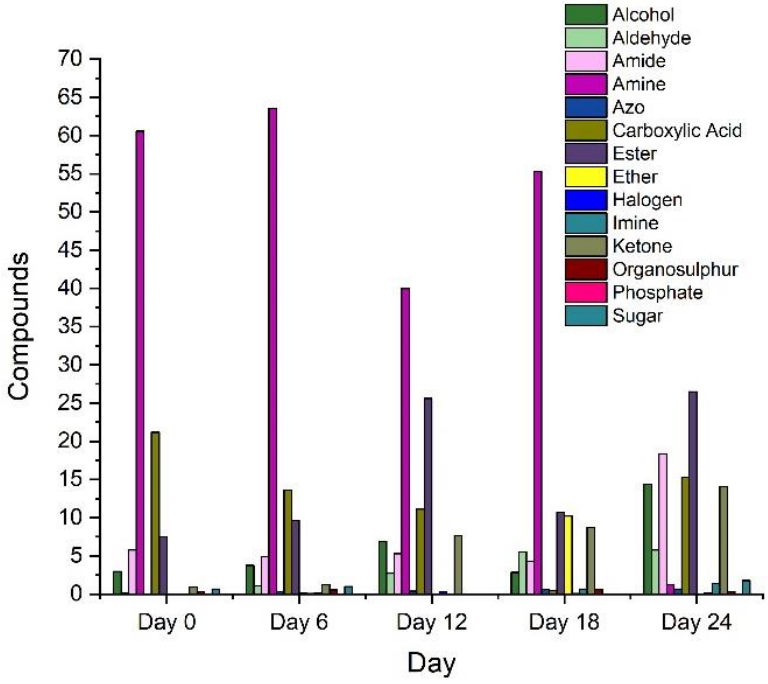


Figure 4. Profiles of Nonvolatile Components During Black Garlic Processing

Table 4. Chromatograms Revealing the Three Main Components of Black Garlic During Processing

Groups	day 0	day 6	day 12	day 18	day 24
Alcohols	5-({[3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl}thio)-4-pentyl-4H-1,2,4-triazol-3-ol	5-({[3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl}thio)-4-pentyl-4H-1,2,4-triazol-3-ol	4-Aminophenol	4-Aminophenol	4-Aminophenol
	2,2,6,6-Tetramethyl-1-piperidinol (TEMPO)	Bis(4-ethylbenzylidene)sorbito	5-({[3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl}thio)-4-pentyl-4H-1,2,4-triazol-3-ol	5-({[3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl}thio)-4-pentyl-4H-1,2,4-triazol-3-ol	5-({[3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl}thio)-4-pentyl-4H-1,2,4-triazol-3-ol
	Bis(4-ethylbenzylidene)sorbitol	NP-016596	Bis(4-ethylbenzylidene)sorbito	1-Linoleoyl glycerol	1-Linoleoyl glycerol
Aldehydes	4-Hydroxybenzaldehyde	5-Hydroxymethyl-2-furaldehyde	5-Hydroxymethyl-2-furaldehyde	5-Hydroxymethyl-2-furaldehyde	5-Hydroxymethyl-2-furaldehyde
	3,5-di-tert-Butyl-4-	4-Hydroxybenzaldehyde		4-Hydroxybenzaldehyde	Pyridoxal
Amides	Stearamide	Stearamide	Stearamide	Stearamide	Stearamide
	Oleoyl ethanolamide	Oleoyl ethanolamide	Phenacetin	Oleoyl ethanolamide	Phenacetin
	N-Benzylformamide	Methanandamide	(3S)-3-(5-Methoxy-1H-benzimidazol-2-yl)-N-phenyl-1-pyrrolidinecarboxamide	Erucamide	Oleoyl ethanolamide

Table 4. ***Continue***

Groups	day 0	day 6	day 12	day 18	day 24
Amines	L-(+)-Arginine	L-(+)-Arginine	Choline	Choline	Dibenzylamine
	Choline	D-(+)-Proline	DL-Arginine	DL-Arginine	Norfenefrine
	Adenosine	Choline	D-(+)-Proline	D-(+)-Proline	Hexamethylenetetramine
Azide	-	-	-	2-[(2,3,4,5,6-Pentamethylbenzyl)thio]ethanohydrazide	-
Azo-compound	-	Azobenzene	Azobenzene	Azobenzene	Azobenzene
Carboxylic acid	L-Pyroglutamic acid	2-[3-methyl-2-(methylimino)-4-oxo-1,3-thiazolan-5-yl]acetic acid	L-Pyroglutamic acid	L-Pyroglutamic acid	L-Pyroglutamic acid
	trans-3-Indoleacrylic acid	3-(3,4-dihydroxyphenyl)propanoic acid	Muramic acid	2-[3-methyl-2-(methylimino)-4-oxo-1,3-thiazolan-5-yl]acetic acid	Trigonelline
	Valine	L-Pyroglutamic acid	1,4a-dimethyl-9-oxo-7-(propan-2-yl)-1,2,3,4,4a,9,10,10a-octahydrophenanthrene-1-carboxylic acid	L-Pyroglutamic acid	Betaine
Esters	Aspartame	Aspartame	NP-005196	NP-005196	NP-019811
	Diisobutylphthalate	NP-019811	NP-019811	NP-019811	Bis(2-ethylhexyl) phthalate
	Bis(3,5,5-trimethylhexyl) phthalate	Bis(2-ethylhexyl) phthalate	(1S,8S,9S,10S,13R)-6,9,10-trimethyl-2-oxo-4,14-dioxatetracyclo[7.5.0.0Âa,ÂaÂl.0Âl, â•·]tetradeca-3(7),5-dien-8-ylacetate	Aspartame	ethyl 2-{2-[(phenylsulfonyl)amino]-1,3-thiazol-4-yl}acetate
Ethers	-	Bisphenol A diglycidyl ether	-	-	-
		PEG n10			
Halogen compounds	-	Alprazolam	Alprazolam	6-Chloro-5-fluoro-1H-1,2,3-benzotriazole	5-Fluoro-3,5-AB-PFUPPYCA
				Alprazolam	
Imines	-	6-Methylquinoline	-	3-Hydroxypyridine	3-Hydroxy-2-methylpyridine
				3-Hydroxy-2-methylpyridine	3-Hydroxypyridine
				6-Methylquinoline	
Ketones	NP-020014	4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	1,9b-Dihydroxy-6,6,9a-trimethyl-5,5a,6,7,8,9,9a,9b-octahydronaphtho[1,2-c]furan-3(1H)-one	4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione
	Zearalenone	4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	Zearalenone	4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione
	Acetophenone	Acetophenone	4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	5-hydroxy-4-methoxy-5,6-dihydro-2H-pyran-2-one

Table 4. ***Continue***

Groups	day 0	day 6	day 12	day 18	day 24
Organosulfu compounds	2-[(2-chlorobenzyl)sulfanyl]-4,6-dimethylnicotinonitrile	[(2R,3S,4S,5R,6R)-3,4,5-trihydroxy-6-{[(2S,3S,4R,5R)-4-hydroxy-2,5-bis(hydroxymethyl)-3-{[(2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoyl]oxy}oxolan-2-yl]oxy}oxan-2-yl]methyl 4-hydroxybenzoate		Biotin	Biotin
		2-[(2-chlorobenzyl)sulfanyl]-4,6-dimethylnicotinonitrile			
Phosphates	Tributyl phosphate	-	-	-	-
Sugars	D-(+)-Maltose	D-(+)-Maltose	D-(+)-Maltose	-	D-(+)-Maltose
	α-Lactose	α-Lactose			

Total dissolved protein levels of black garlic. Gas and liquid chromatographies reveal the profiles of components and their changes during processing; however, the calculation from individual experiments and particular standard solutions is still needed. The total protein content was measured using Kjeldahl's method, which is based on any nitrogen component in the sample strongly correlated to the total protein content from the amino acids. When water was used as a solvent, black garlic has shown an increasing total protein content from day 0 to day 24 (Figure 5). The extraction before destruction using buffer solution (PBS) has led to better results than the same process using water as the solvent. However, Kjeldahl's method cannot explain specific proteins in the sample. Small nitrogen-containing molecules might also be included as they have appeared in GC–MS and liquid chromatography.

Analysis of the total protein content of black garlic with PBS showed an increase in total protein level for the garlic heated from day 0 to day 24. This increase can also be caused by new protein synthesis and enzymatic hydrolysis reactions—the damage to the antioxidant activity of amino acids in nonenzymatic Maillard reaction.

In addition to the nonenzymatic reaction, namely, the reaction between amino acids (proteins) and reducing sugars which plays a role in producing melanoidin, breakdown or denaturation of proteins occurs by the act of some enzymes (denaturation of alliinase, causing a spicy taste in raw garlic and other sulfur-containing compounds). Alliinase is an enzyme found in garlic [2] and has a function in the carbon-sulfur group. Alliinase catalyzes the chemical reaction of S-alkyl-L-cysteine S-oxide to produce volatile compounds providing the pungent odor and taste in garlic. Sulfur-containing components were also detected from gas and liquid chromatography (Figures 3 and 4).

Relationship between browning intensity and protein levels. Proteins play a role in enzymatic and nonenzymatic reactions that occur in the Maillard reaction during the heat treatment for fresh garlic. In enzymatic reactions, proteins denature nonvolatile allinase enzymes into volatile allicin. Thus, the changes in organosulfur compounds (allicin derivatives) were analyzed [2]. Allicin compounds could not be identified in this study because allicin was degraded by heat in the GC system. Therefore, the changes in organosulfur compounds were observed, which are allicin derivatives, during garlic heating. The results showed that heating degrades allicin compounds. Allicin can be degraded into other organosulfur compounds, including (E)-ajoene, (Z)-ajoene, 3-vinyl-4H-1,2-dithiin, 2-vinyl-4H-1,3-dithiin, and diallyl disulphide. GC–MS showed that the allicin derivative compounds identified in black garlic are only 1,2-dithiin and 3, ethenyl-3,4-dihydro. When 1,2-dithiin and 3-ethenyl-3,4-dihydro compounds are heated into precursor compounds for a long time, they form other simple organosulfur compounds.

In nonenzymatic reactions, amino acids (proteins) and reducing sugars produce a dark color (Maillard reaction). The higher the protein content, the darker the browning intensity of the resulting black garlic. Protein acts as a precursor in the Maillard reaction during black garlic processing. The Maillard reaction produces melanoidin compound that generates the brownish color in black garlic. Therefore, the longer the processing for raw garlic, the higher the protein content, Maillard reaction, and resulting melanoidin content. As a result, the browning intensity of black garlic increases.

Reducing sugar content. Reducing sugar content can also reflect the nature of organic and natural product materials. In Figure 6, the sugar content is increased up to day 18 and then leveled off. The carbohydrate content in garlic undergoes complex reactions, including hydrolysis, and yields simple sugar during aging. According to some people, the taste of black garlic is sweet and its pungent odor gradually becomes mild day by day.

During black garlic processing, carbohydrates degrade into monosaccharides with the help of enzymes accompanied by heating. Fructan is unstable at high temperatures and under acidic conditions and is the most remarkable contributor to total reducing sugar content during black garlic processing. It is a non-structural carbohydrate found in garlic [11]. The enzyme fructan exohydrolase assists fructan degradation and is produced by bacteria-degrading polysaccharides, such as the members of genus *Bacillus.* A previous experiment found that bacillus bacteria appear during black garlic processing [9]. This sugar and other bioactive compounds were also found at high amounts in the later stages of black garlic processing [6].

Sugar content is associated with the Maillard reaction and induced by heating during black garlic processing [22]. Other possible explanations can explain the sugar content depending on the biochemistry of the process. Therefore, garlic shows dynamics in terms of carbohydrate chemistry.

Antioxidant activity. The antioxidant activity comes from compounds that mix while turning garlic into black garlic. Compounds whose composition constantly changes daily (Figure 2–7) are also observed from the drop in DPPH absorbance for each sample with different production times. The extracts contain all the components investigated by GC–MS and LC–HRMS discussed before for volatile and nonvolatile components without separation. The drastic changes of absorbances can be detected within seconds interval only at λ 514 nm. The best abrupt decrease in absorbance is observed for day 18 extract (green color) from the initial condition in the first 3 seconds of measurement. Fresh garlic (day 0) has shown around half of this value from day 18. The aged garlic (day 24) has less antioxidant activity than the one from day 18. These findings indicated that the general antioxidative power of the group of compounds present at that time. The excellent activity of black garlic can give information about the profile of chemical components during the processing.

The current findings are consistent with the previous result obtained using GC–MS and LC–HRMS to reveal different profiles during days 0 to 24 (Figures 3 and 4). Thus, sulfur-containing components were also investigated. A similar result is obtained for the day 18 extract with the highest content of organosulfur derivatives. The antioxidative properties are the highest for day 18 black garlic according to the inhibition data. However, the synthetic vitamins from the drugstore exhibit a more potent effect than expected because they are not herbal medicine (Figure 7). These synthetic vitamins show effective antioxidative properties compared with natural black garlic.

Many possible profiles can be obtained during black garlic processing. Amelia [2] looked into the details of organosulfur components in garlic. Some of the volatile compounds were lost due to heating at 60 °C. New compounds appeared in the profile after 9 and 12 days. The organosulfur compounds that appeared on the first day were 2-vynil-[4H]-1,3-dithiin, 1-methyl-5-(methylsulfonyl)-1H-tetra, trisulphide-methyl 2-propenyl, 1,2-dithiin, 3-ethenyl-3,4-dihydro and trisulphide, in-2-propenyl. At the 6th day of heating, only one organosulfur compound, trimethyl [4-(4-methyl phenyl)-3-(phenylsulfinil) butyl, has been detected in black

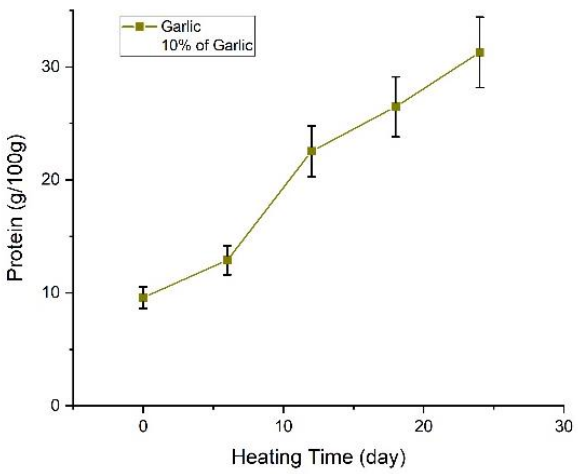


Figure 5. Total Protein Content of Black Garlic with PBS Solvent

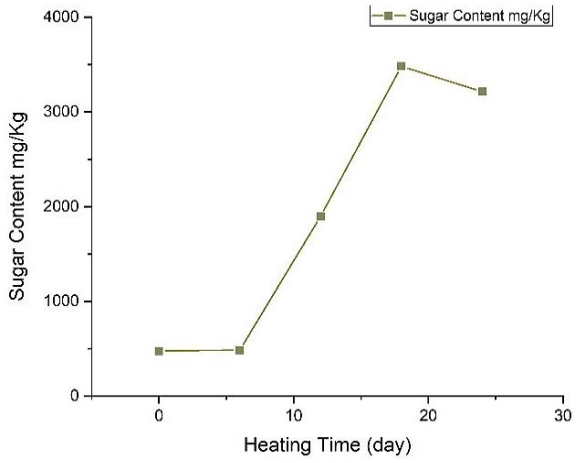


Figure 6. Reducing Sugar Content During Black Garlic Processing

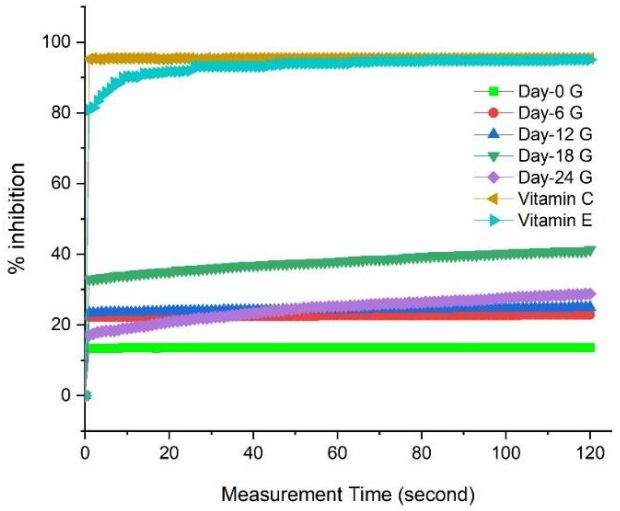


Figure 7. Percent Inhibition by Aged Garlic Compared with Vitamin C and E Supplements

garlic. On day 12 of heating, only 2-propenylthio acetonitrile can be detected. After days 21 to 24, no volatile sulfur compounds are detected by our instrumentation.

Several methods for analyzing black garlic processing were performed. They showed some advantages and disadvantages. Changes were detected in the microsystem during the processing. The presence of some critical chemicals benefits black garlic by acting as herbal medicine. Some people, including chemists and herbalists, follow that conclusion because they have witnessed this convincing phenomenon (as a result of the suitable methodology). Hence, the experts' voice can be misleading because the entire system and equilibrium were not considered. Much complexity exists outside the black garlic bulbs and takes action into these biological processes.

In equilibrium, changing each component would shift the state. An increase is observed in protein and sugar contents. Black garlic tends to be sweet with darkened color, and the physical and chemical changes explain these connections. An intensive follow up study can be performed using different methods in analytical chemistry. Additional studies in natural products employing a large amount of data and using spectroscopy methods and metabolomics are necessary [23]. Analytical chemistry provides information on these topics.

At this stage, chemistry alone is never adequate to explain natural changes. It is only a narrow, objective, and exclusive path in science. One can follow other narrow paths in biochemistry, organic chemistry, or even biological activities, but something more is often needed to explain natural products. An interdisciplinary way to see nature's biodiversity in modern life, including applying natural products in human activities, is considered. Chemoinformatics and bioinformatics, which generate big data for analysis, have become relevant in today's science [24], with more and more in silico studies being conducted nowadays. From an ethical point of view, additional attempts must be introduced because all systems in natural products are in equilibrium with their surroundings [25] and must be discussed in the frame of familiar natural laws.

Conclusion

The protein content of black garlic increased until the last day of the experiment (day 24). The higher the protein content, the faster the Maillard reaction and the more substantial the browning intensity. Reducing sugar content showed the same tendency, whereas the antioxidative properties were optimal on day 18 of the processing. Typical components from the chromatographic separation and mass spectrometry showed that the altered profiles during processing lead to the synthesis of the good constituents of herbal supplements.

Analytical chemistry can reveal and reflect dynamic changes occurring during black garlic processing. Optimization always occurs naturally within natural products. In this case, method development in analytical chemistry is necessary for future dynamic analysis. Multidisciplinary approaches are in demand at the moment. Many metabolites are changing, and the availability of the probes from many angles of analysis is crucial to reveal natural phenomena.

Acknowledgment

The Directorate General for Research and Civil Service, Ministry of Education and Culture under Fundamental Research Schemes funded this research.

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