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Research paper

Quantitation of Methanol & Acetaldehyde from Raw & Black Garlic by Headspace GC with PLOT Column

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Abstract

Background/Objectives: Black garlic is produced by heating a garlic at 60~70°C for a couple of weeks. Methanol and acetaldehyde are toxic chemicals that could be produced naturally from plant materials.
Methods/Statistical analysis: Methanol and acetaldehyde contents in the raw and black garlic were measured. Manual static headspace (HS) sampling method was established and GC analysis with PLOT column was applied. Black garlic was prepared in a rice cooker at 60~70°C for three weeks followed by drying for two weeks. The ratio of water and mashed garlic for the preparation of the garlic extract was optimized.
Findings: The optimum ratio between the garlic sample and water was 1:2 for the preparation of headspace liquid sample. The HS sample in the 20mL vial (containing 3 mL of garlic extract) was analyzed. Acetaldehyde detected as large amount comparing to methanol was almost gone in black garlic. It might be too volatile to survive in that harsh condition such as heating at 60~70°C for a couple of weeks. The analyzed methanol content in the black garlic was about 6 times higher than the raw garlic. That means the methanol derived from the pectin might be increased even at the relatively mild heating condition. If two weeks of the drying period was considered, actual amount of methanol occurred during the heating process might be higher than that. The naturally occurring methanol from the plant materials containing pectin is very popular in our dish. Moreover, the heating process is the essential part of cooking. Hazardous chemicals such as methanol are more likely to be present during common cooking processes.
Improvements/Applications: The manual headspace sampling is very simple and easy to apply even without the expensive instruments specifically dedicated for the automatic headspace sampling. Moreover, the PLOT column is also a very efficient tool even without the concentration trap to decrease the peak width. The method could be applied in any laboratory if they have a GC-FID.

Keywords: *Black garlic, Methanol, Acetaldehyde, Headspace sampling, GC-FID, PLOT column.*

1. Introduction

Garlic (*Allium sativum*) has been known as a seasoning food and folk medicine since prehistoric era. It is believed that the ancient Egyptians consume garlic as a daily diet since the well-preserved garlic was found in Tutankhamun's tomb. The Codex Ebers (the authoritative medical text of the era) prescribed garlic for the various illness, such as abnormal growth, circulatory ailments, etc.[1]. South Korea was the 5th country, producing garlic much in the world (in 2016). Garlic has been shown to have antibacterial, antihypertensive, antitumor and antimutagenic and antioxidant effects[1]. Garlic has been registered as a health functional food (HFF) material for improving blood cholesterol in Korea. Alliin (0.6~1.0g) is the indicative compound for assuring the quality of HFF garlic. Black garlic (BG) is turned as black during the process by maillard reaction rather than caramelization. The presence of maillard reaction products might be related to the increase of antioxidant activity of BG[2]. The taste of jelly like BG is sourly sweet[3]. The recipe (process) is the simple heating at low temperature for a couple of weeks in a cooking device such as rice cooker. BG has been studied for the biological activities such as antioxidants, anti-carcinogenic, anti-inflammatory, anti-diabetic, and etc.[4]. The pharmaceutically active compounds in garlic and other Allium species are categorized as one of three groups having pungent odor, such as vinyldithiines, allyl sulfides, and ajoenes[5]. The volatile compounds that contribute to the flavors of plant materials are formed by the intrinsic metabolic pathways including ripening, storage, etc. And some of them could be produced during the cooking or processing, especially by the heating process. Woo etc. analyzed the volatile compounds from the heated garlic, and detected the 25 compounds such as 2-butanal, 3-hexanal, dimethyl disulfide, 2-methyl-2-butenal etc.[6]. One of the major findings of them was the amount of sulfur related compounds such as methyl disulfide, allyl methyl disulfide, methyl trisulfide, allyl disulfide, and 3-(methyltrisulfanyl)prop-1-ene decreased as heating time extended. And Kim etc. analyzed the mass spectral ion counts of the garlic samples treated by a various heating methods including the process of BG[7]. Among the detected twenty six volatile compounds, only fourteen and nineteen compounds were survived in the aged BG clove and aged-crushed BG, respectively. Most of the analytical studies for the volatiles in garlic or processed garlic are dedicated to the analysis of the biologically potent active compounds. However, almost no researches have been made for the potentially hazardous chemicals could be made or diminished during cooking or manufacturing process.
Acetaldehyde and methanol must be the major naturally occurring hazardous chemicals in plant materials. Acetaldehyde is an aldehyde with the formula C_2H_4O (MW 44.0526, CAS Number

75-07-0) that is a very early eluted by gas chromatography (GC) with retention index (RI) of 343~427 on the non-polar columns such as Squalane, Apiezon L, OV-101, SE-30, HP-5, etc. and RI 655~750 on the polar columns such as Carbowax 20M, DB-Wax, PEG 20M, FFAP, etc.[8]. Methanol is the most light alcohol with the formula CH_4O (MW 32.0419, CAS Number 67-56-1) that is also a relatively early eluted GC peak with RI of 330~408 on the non-polar columns and RI 860~920 on the polar columns[9]. Even though acetaldehyde and methanol are the typical volatile compounds, they are not eligible to be detected by GC without the careful manipulation of the analysis conditions such as column, oven condition and sample pre-treatment methods. It must be the reason why the name of these two compounds are rarely in the list of the volatile compounds detected in garlic and its related materials. Acetaldehyde is reasonably anticipated to be a human carcinogen, in other words, is regarded as a probable human carcinogen (Group B2), based on the result of the studies in rats (nasal tumors) and in hamsters (laryngeal tumors) though inadequate human cancer studies[10,11]. However, acetaldehyde is one of the naturally occurred compounds during fermentation and is found in low levels in certain foodstuffs such as dairy and soybean products, pickled vegetables, non-alcoholic beverages, etc. And industrially, acetaldehyde is used in the production of dyes, resins and perfumes, etc. It is also used as a preservative to keep fruit and fish fresh. And it is a flavoring agent for food and as a denaturant for alcohol (in fuel), for gelatin (for hardening), and as a solvent in the rubber, tanning, and paper factories[10]. Acetaldehyde is also a fermentation volatile undesirable in excess amount and contribute to off-flavors and aroma. However, the presence of lower levels of it may enhance apple aroma and flavor[12]. It should be noted that a population exposed to environmental sources of acetaldehyde may be adding to a body burden of this compound produced by normal metabolism and by such life-style habits as cigarette smoking and ethanol consumption[13]. Methanol is a well-known chemical inducing vision impairment and coma followed by death. The healthy human may intake the exogenous methanol via fruits, vegetables, and alcoholic beverages, mainly. Methanol is also made when pectin is broken down by microscopic organisms in the digestive tract, after fruit or vegetables are eaten[14]. It has been reported that apples contain approximately 1% pectin, and that consumption of 1 kg of apples typically results in release of 0.5 g of methanol in the human body[15]. The California Office of Environmental Health Hazard Assessment (OEHHA) added methanol to Proposition 65 (in March 2012). OEHHA proposed the maximum allowable dose level (MADL) of methanol as 23 mg per day for ingestion, that is same as from 1 can diet soda containing aspartame, 10 cigarettes, etc.[16]. Aspartame (E 951) is the α-aspartame consisted with an L-phenylalanine methyl ester and L-aspartic acid having an amino group (at the α-position of the peptide bond). The hydrolyzed (or degraded) metabolites of α-aspartame are L-phenylalanine, aspartic acid, methanol, etc. Hydrolysis of aspartame releases a corresponding 10 % of methanol (by weight). The exposure to methanol was estimated by EFSA panel with the raw individual food consumption data in the comprehensive DB and using the occurrence data. In that estimation, the exposure of methanol from endogenous pathways was over 80%, and aspartame and the natural food sources took under 10% each[17]. In plants, pectin (mostly in cell wall) methylesterase enzyme produces methanol by demethylation[18]. However, the enzymatic reaction is not the only source of methanol formation. Diaz etc. reported that heating pectin solutions led to an accumulation of methanol, indicating pectin de-esterification[19]. Massiot etc. studied about the degradation of highly methyl esterified apple pectin with an endocytic polygalacturonase and pectin methylesterases from Orange or *Aspergillus. niger*[20].

For the matter of volatile (such as methanol and acetaldehyde) analysis, a various methods are available depending on the sample preparation and instrumental analysis. Methanol is one of the common high-performance liquid chromatography (HPLC) solvent. Moreover because the lack of chromophore, a common instrument, HPLC-UV can't be used for the analysis of methanol without derivatization. That is why HPLC with the reflective index (RI) detector was adapted for the analysis of alcohols such as methanol[21,22]. However, the lowest concentration of detectable for methanol was high as reported as over 50 mg/L by HPLC-RI detector. Acetaldehyde also could not be analyzed by HPLC without derivatization. A typical derivatization reagent for carbonyl compound, hydrazine (such as 2,4-dinitrophenylhydrazine) easily derivatize carbonyls (in acidic media) to the respective hydrazones, those are well separated in reversed phase HPLC and well detected by common detectors such as UV-Vis detector[23,24]. However, the simplest method for the detection of methanol and acetaldehyde is the GC-Flame Ionization Detector (FID) analysis. Oh etc. analyzed methanol content in liquors by the static headspace (HS) followed by GC-FID analysis with porous-layer open tubular (PLOT) column (that is a GC capillary column with high theoretical plate number)[25]. Recently a highly polar PLOT column coated with particles of divinylbenzene ethylene glycol/dimethylacrylate (Rt®-U-BOND) was adapted for the analysis of acetaldehyde and methanol even from their deuterated isotopologues[26]. The high efficiency of PLOT column could make possible of GC analysis for the very volatile compounds (like methanol and acetaldehyde), even without the cold-trap minimizing the peak width of the gaseous compounds. The biggest difference between HPLC and GC must be the physical form of the mobile phase. GC is using gas as the mobile phase, not like HPLC in which only liquid could be the mobile phase. Therefore the gaseous sample could be delivered to GC column even without dissolving it in the liquid solvent. The gas solid chromatography (GSC) equipped with a column packed with the polar supports was the typical and classical analysis tool for the gas-phase separation. However, after starting off the new era of the capillary column that took the baton from the packed column, the solid adsorbents (such as molecular sieve, porous polymer, carbon, silica gel, alumina etc.) for the gas-phase separation were coated in the wall of the open-tubular capillary column[27]. That is the birth of PLOT column. Because the similarity of PLOT column's separation mechanism "adsorption" with GSC, PLOT column is adequate for the gas-phase separation. Before the GC analysis, sample preparation method must be carefully selected. The most common injection method for GC is the liquid injection. However, the liquid solvent extract used to contain various hindrances such as sugars, fats and etc. Therefore the complex sample preparation steps removing those inhibiting compounds such as liquid-liquid extraction, solid-phase extraction, distillation, saponification and etc. are needed. On the other hand, even without those cumbersome steps, the direct analysis of the equilibrium HS above the sample in a vial, is a simple ideal method for the volatile analysis[28]. HS sampling is categorized as dynamic and static methods. The static HS does not have the concentration step, not like the dynamic HS does. However, even though the dynamic headspace sampling has high sensitivity due to concentration effect of volatiles by solid adsorbent having high affinity to the target chemicals, the carry-over of the target components derived from the dynamic HS device (such as the purge and trap tubing) provoke some analytical difficulties often. Moreover the insufficient adsorption of acetaldehyde on a adsorbent like Tenax was reported in a recent research[29]. Therefore static HS might be the right choice for the analysis of methanol and acetaldehyde in the garlic samples. Static HS has the advantage of injecting samples manually even without the high-priced state-of-the-art instrument. Therefore the static HS could be adapted in any laboratory having GC-FID without further investment. In this study, methanol and acetaldehyde contents in raw garlic (RG) and BG were analyzed by the manual static HS and GC-FID (with PLOT column), to figure out the variation of the amount of those volatile hazardous components by the cooking process of low-temperature and long period.

2. Materials and Methods

2.1. Materials and Sample Preparation

The analytical grade acetaldehyde and methanol standard materials purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).The HPLC grade water was purchased from Burdick & Jackson (Muskegon, MI, USA). RG (cultivated in Haenam, Jeonam, S.Korea) was purchased in G-market. The whole bulb of RG was put in a rice cooker (CRP-FA0621MR, Cuckoo, Yangsan, Geongnam, S.Korea) equipped with a wicker tray, on where RG was cooked for three weeks (at 60~70 °C) followed by drying for two weeks in the shade. After dehulling of the dried BG, it was crushed and homogenized into shatters by a multi-purpose mini mixer (MCH-300, Dongyang Magic, Seoul, South Korea). The crushed BG was measured and it is mixed with HPLC grade water with 1:2 ratio. The BG and water mixture was sonicated for 20 min and filtered by the Büchner funnel with the Whatman Grade 2 filter paper (Whatman plc, Maidstone, UK) by gravity (Fig 1). RG was treated same to BG from the crushing step. The final each 3 mL of filtrate was moved to the 20mL size headspace vial for GC-FID analysis.

2.2. Instrumental Analysis

The headspace sampling and GC analysis was followed to Oh's method[25]. The 20mL size HS vial (Agilent Technologies, Böblingen, Germany) was positioned inside of a 100 mL size beaker filled with the preheated sea sand (Daejung, Daegu, Korea) at 70°C, and the beaker was put into the 70°C oven for 10 min. After taking out the beaker, the pre-heated 2.5mL size headspace syringe (2.5MDF-GT, SGE, Ringwood, VIC, Australia) needle was inserted to the headspace pushing 1 mL air to the headspace and waited for 1 min. The headspace gas was taken for 30 seconds and injected to GC-FID (7890 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with CP-PoraPLOT U (25m × 0.25 mmI.D., 8μm d_f + 2.5m Partial Trap) column (Varian, Palo Alto, CA, USA). The mobile phase of GC was nitrogen (1.0 mL/min). The injection was performed by the splitless mode (with purge delay time, 1 min) at 140°C. The temperature of FID was 190°C. The oven temperature condition No. 1 was started from 40°C (2min) and ramped to 120°C with 20°C /min. And the temperature was increased to 190°C by 10°C /min and held for 5 min. The oven temperature condition No. 2 was started from 40°C (2min) and ramped to 190°C by 10°C /min and held for 5 min. The calibration curves for methanol and acetaldehyde were prepared by 4 points (1, 5, 10 and 25μg/mL) and 5 points (5, 50, 100, 130 and 150μg/mL), respectively.

Fig. 1: Sample preparation process of black garlic (mixing water, sonication, and filtration). The final filtrate was moved to headspace vial for the GC-FID static headspace injection

2.3. Statistical Analysis

The statistical analyses such as relative standard deviation, etc. were carried out using the Sigma Plot version 13(Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

The manual HS analysis by CP-poraPLOT U column for acetaldehyde and methanol was quite successful as the two peaks were separated with resolution over 1.5 in two oven temperature conditions. For the oven temperature condition No. 1, acetaldehyde and methanol were eluted at 8.94min and 9.01min, respectively, where the resolution was 1.6. On the other hand, at the oven temperature condition No. 2, the retention time (RT) of two peaks were 11.99min (methanol) and 12.14min (acetaldehyde). Those two peaks were separated with resolution 2.6 that is enough for the quantitative analysis. Therefore, to guarantee enough selectivity, the oven temperature condition No. 2 was adapted for the analysis of methanol and acetaldehyde in this study. The calibration curve linearities for methanol and acetaldehyde were 0.9994 and 0.9998, respectively. These values were quite enough to satisfy the good accuracy of the quantitative analysis. The relative standard deviation (RSD) % derived from the triplicate analysis for methanol (at 10μg/mL) was 3.6% while RSD% for acetaldehyde was 4.7% at 100μg/mL. The biggest bias source for the manual HS analysis was the tightness of HS vial cap where the most leak could be made during the heating process. And the second source of bias was the contacting of the metal cap part with air. Therefore the careful manipulation of cap positioning inside of the sea sand beaker was made to keep the metal cap part be inside of sea sand. The LOD and LOQ for

methanol were 0.4μg/mL and 1.0μg/mL, respectively. The LOD and LOQ for acetaldehyde were 0.1μg/mL and 1.0 μg/mL, respectively. Those values were estimated from the signal to noise value of 3 and 10 for LOD and LOQ, respectively.

After continuous heating of garlic clove in a rice cooker, the color of garlic changed as total black. The disgusting odor arisen during the cooking process must be due to lots of sulfur compounds in garlic. However, the smell disappeared during the two weeks of the drying process. The texture of BG was more sticky than RG, therefore it was not easy to make the homogeneous matter. Therefore, mixing with water and sonication treatment were tried. To optimize the ratio of water volume to BG, "BG:water" ratio was optimized (1:2, 1:3 and 1:4 compared) for the detected amount and the precision (RSD%). The result is shown in Fig. 2. The highest average amount of methanol was 17.5 mg/L which was acquired in the sample of "BG:water = 1:2". And the average methanol amount detected in 1:3 and 1:4 sample was 12.3 and 9.0 mg/L, respectively. Those detected amounts were 93% and 85% of the theoretical amounts (13.3 mg/L and 10.6 mg/L, respectively) acquired by diluting the BG by water from 1:2 to 1:3 and 1:4. As a result of one-way ANOVA test for the detected average methanol amounts, the *P* value between 1:2 and 1:3 results was <0.001 (*P*>0.05). This statistical evaluation result show those mix ratios induce the different result of methanol quantitation while 1:3 and 1:4 results were not significantly different by *P*=0.088 (*P*>0.05). Moreover the RSD% of the detected methanol amounts in 1:3 and 1:4 samples (20.7% and 28.6%, respectively) were larger than 14.6% of the sample "BG:water = 1:2". On considering the detection level and analytical precision, "BG:water = 1:2" was adapted for the sample preparation.

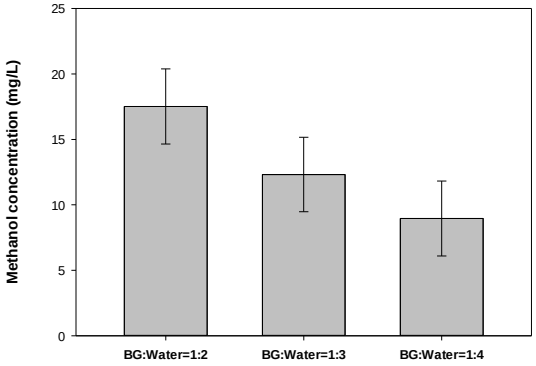

Fig. 2: The methanol concentration in a black garlic according to the mixed water volume ratio

The Fig. 3 is the chromatograms of RG and BG HS samples separated on CP-poraPLOT U column. As shown in (A) chromatogram of RG, large acetaldehyde peak was observed (the mean concentration was 26.4±8.2 mg/L) while methanol peak was comparatively small. The concentration of acetaldehyde detected in RG was 4.7 times higher than the value detected by Uebelacker and Lachenmeier[29]. On the other hand, methanol eluted out as the comparatively large peak than the trace peak of acetaldehyde in chromatogram (B) of BG. These results present that the most of acetaldehyde might be gone during the long processing of heating at 60~70°C and drying. On the contrary to acetaldehyde, methanol must be formed during the heating process. Diaz etc. mentioned that the major non-enzymatic process of methanol formation is β-elimiation, acid hydrolysis and demethylation of pectin and pectate. Additionally, those reactions could be affected by pH, temperature and the degree of methylation of pectin[19].

The methanol concentrations measured in RG and BG are presented in Fig. 4. After the ten trials of analyses for RG and BG sample, 12.9 mg/kg of the average methanol for BG was measured. It was about 6 times higher value than 2.2 mg/kg of methanol found in RG. The highest methanol amount found in RG was 4.3 mg/kg while the value in BG was 19.5 mg/kg. The RSD % of the 10 times analyses for each sample groups were 51.3% and 32.7% for RG and BG, respectively. These average methanol concentrations of RG and BG were significantly different with *P*<0.001 by the *t*-test. But the methanol amount detected in BG was not significantly higher than the amount which naturally exists in other plant materials such as fruits. The methanol concentrations of freshly squeezed fruit and vegetable juices were reported as the range from 12 mg/L to 200 mg/L[30]. But if the total amount of methanol is regarded as the sum of free and bonded (potential) form, the amount could be dramatically increased. The bonded form of methanol means CH_3O- form bound to higher molecules, those could be released as methanol in the human stomach and colon[30]. Therefore, the human intake of methanol occurred via both endogenous and exogenous sources may need to be continuously watched even in the future.

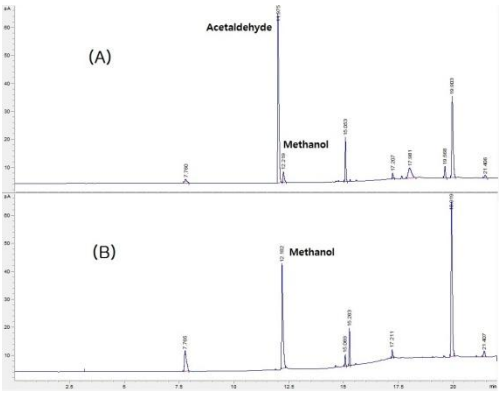

Fig 3: The GC-FID chromatogram separated on CP-poraPLOT U column for (A) raw garlic and (B) black garlic sample

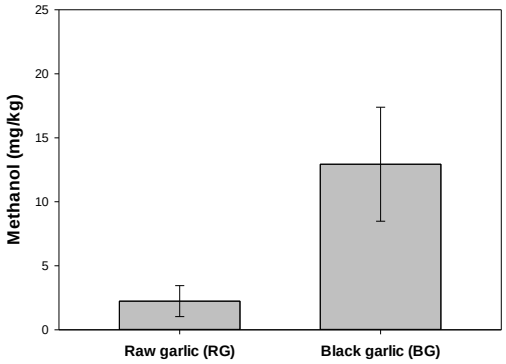

Fig. 4: The average methanol concentrations in the raw garlic and black garlic samples

4. Conclusion

The well-known hazardous materials, methanol and acetaldehyde were analyzed from garlic as raw form (RG) without cooking and black form (BG) after cooking. Cooking of garlic clove in a rice cooker (at 60~70°C) for three weeks and drying for two weeks were devoted to the preparation of BG. As a result of static HS analysis for the RG and BG samples, the different residual existence of methanol and acetaldehyde were observed. In RG, a relatively high amount of acetaldehyde (26.4±8.2mg/L) was detected, while it disappeared after cooking as BG. On the other hand, average methanol amount detected in RG and BG were 2.2mg/kg and 12.9mg/kg, respectively. Almost 6 times higher amount of methanol formed during the cooking process for BG. Even though the amount of methanol found in BG was the naturally occurring level in other plant materials, the bonded form of methanol could be released in our body during the digestion process, therefore the continuous interest on this toxic chemical might be needed. The manual headspace injection to GC-FID (with PLOT column) for the analysis of methanol and

acetaldehyde was easy and cost-efficient enough to satisfy to be used at the QA/QC.

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