The investigation of the effect of freezing pretreatment on properties of black garlic produced from Kastamonu garlic

Fatma Kandemirli1 ©, Nesrin İçli2 ©, Temel Kan Bakır3 ©, Bahar Nazlı1 ©, Serap Aydın4 ©

Cite this article as:

Abstract
Black garlic is obtained by fermentation of fresh garlic in condition under controlled high humidity and temperature. The aim of this study is to investigate the effect of freezing as pre-treatment process on black garlic produced from fresh Kastamonu garlics in terms of various antioxidant activity parameters, phenolic and flavanoid contents, fermentation time and HMF formation. The antioxidant properties analysis results of frozen and non-frozen black garlic samples were shown that higher than those of white garlic and it was observed that total antioxidant capacity, the total phenolic compound and total flavanoid content values of black garlics increase with the storage period under fermentation conditions. In addition, frozen garlics were found to have higher total antioxidant activities on the 25th and 30th days (9.56 mg AE/g and 10.40 mg AE/g, respectively) of the fermentation than the activities of non-frozen garlics the on the 35th day (8.49 mg AE/g) of fermentation. The toxic compound HMF formation was found of the frozen sample at as high as 110 mg/kg in end of the 35th day. In conclusion, the use of pre-frozen garlics in black garlic production will be more efficient in respect of antioxidant properties and time and cost saving.

Keywords: Black garlic, Kastamonu garlic, Antioxidant activity, HMF
Introduction

Nowadays, people have interest in foods with strong natural antioxidants and polyphenolic contents in order to maintain a healthy and balanced diet. Especially the use of natural products in the treatment of diseases increased the clinical trials of these products. (Ercişli, S. 2008). It has been reported that garlic reduces the risk of breast cancer, the risk of brain haemorrhage and the risk of heart attack and slows down the progression of these diseases (De La Cruz and Garzia, 2007). Garlic (Allium sativum) is widely used in the world and is known as a spice with important polyphenolic contents. In addition, the anti-bacterial property of garlic has been known for many years (Marchese et al., 2016). It is believed that the medical and beneficial properties can be attributed to certain components present in garlic and extracts, and many studies are associated with organosulfur compounds. (Tumors et al., 2006).

There are studies showing the changes in phenol content and antioxidant capacity according to the processing and storage life changes of garlic containing products (Queiroz et al. 2009). Black garlic, a garlic product, has been widely used in health and cosmetics in recent years. Black garlic is obtained by keeping fresh garlic under controlled temperature and humidity for a while. The processing time varies between 5-45 days depending on the temperature. It is kept in the temperature range 60 °C to 90 °C during the process and under controlled humidity with no additional treatment and additives (Toledano et al. 2016). When the garlic is cut or crushed, the allicin which produces a sharp smell of garlic occurs (Marchese et al., 2016). This sharp flavor and the smell of fresh garlic limits the use of garlic. Black garlic does not have this pungent odor and some bioactive properties such as increased antioxidant properties vary. (Li et al., 2015). 5-Hydroxymethylfurfural (HMF) content, allicin content, amino acid, nitrogen compounds, total acid content and total phenol content were determined by Zhang et al. The results of HMF analysis and total phenolics showed that a significant difference was observed in the samples that had darkened at different temperature values (Zhang, et al., 2016). Kimura et al. investigated the production, bioactivity and applications of black garlic (2017). When compared to the biological activity of black garlic and fresh garlic, black garlic is said to have advantages such as antiallergic, antioxidant and anti-cancer properties (Kimura et al., 2017). It was stated that the use of black garlic had no adverse effect on the medical field or the food industry and that the biological activity of black garlic could be increased by fermentation using yeast (Jung et al., 2011). Exposing the garlic to high temperatures in the blackening process will destroy the cell wall. The process of freezing the cell wall by pretreating the garlic before exposure to high temperatures influences the darkening process. The samples exposed to freezing were observed to darken approximately twice as fast. It was shown that the study reduced the blackening time and TPC, HMF-5 affect the sugar content change and amino-N content (Li et al., 2015).

The aim of this study is to investigate the effect of freezing pre-treatment before black garlic production process on antioxidant properties of black garlics such as DPPH radical scavenging activities, total phenolic content, total antioxidant capacity, total flavanoid content and ferric reducing antioxidant power (FRAP) and is observe to toxic HMF formation levels of frozen and non-frozen garlics on fermentation process time. Hence, the fermentation process was applied to frozen and non-frozen Kastamonu Garlic, an endemic plant, under optimum conditions compiled from the literature given above, for the production of black garlic. It was also investigated whether the pre-freezing treatment reduce or not the fermentation process time. This study is the first study about Kastamonu garlic on this subject and there is no study to investigate how the freezing process changes on various antioxidant activities during production of black garlic, in the literature review.

Materials and Methods

All analytical grade chemicals were purchased from Sigma-Aldrich Co. LLC. Deionized water was used to prepare all aqueous solutions. Absorbances were measured using a pair of identical quartet baths of 1 cm thickness using a SHIMADZU UV-1240 UV-Visible spectrophotometer (manufactured by Shimazu Corp., Kyoto, Japan). The SHIMADZU LC20-A Prominence high performance liquid chromatography (HPLC) device (manufactured by Shimazu Corp., Kyoto, Japan) was used for HMF analysis.

Preparation Procedure of Black Garlic

In order to look at the effect of the freezing process on the blackening process, 16 garlic bulb were frozen at -16 °C for 30 hours. After freezing, the frozen and non-frozen garlic were placed in a 60 °C special oven and under optimum conditions compiled from the literature for the blackening. One sample was taken on the 20th, 25th, 30th and 35th days of frozen and non-frozen garlic. All samples were stored at -16 °C for use in experiments.

Preparation of Black Garlic Extracts

Garlic extracts were prepared according to a standard protocol with minor modifications. 2.0 grams of garlic was taken from the garlic and crushed in porcelain mortar. It was then solved in 20 mL of a distilled water solution. After standing
at 4 °C for 24 hours, the mixture was filtered through filter paper. The resulting homogenate was centrifuged at 5000 rpm for 10 minutes (18°C). The last supernatant was removed (100 mg / mL). It was used for the measurement of all analysis parameters investigated in this study (Pedraza-Chaverri et al., 2004).

**DPPH Radical Scavenging Assay**

In this study, antioxidant activity of frozen and non-frozen garlic samples were investigated by DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging method at different storage times. The effect of ethanolic extracts of the garlic samples on the DPPH radical was monitored with a spectrophotometer at 517 nm. DPPH solution at concentration of 1.35x10⁻⁴ M was used as control solution. The absorbance change in the solution was measured at four different concentrations for each added garlic extract at 0.166-0.666 mg/mL. In addition, DPPH calibration solutions were prepared at 4.10⁻⁶ M-2.10⁻⁴ M concentrations by diluting the stock (4x10⁻⁴ M) DPPH solution. Calibration equation was found as y = (8.7±0.22)x10⁻³ x + 0.085. Thus, the percent radical scavenging activity was calculated by the following formula:

% inhibition = [(C₀ - C₁) / C₀] x100 (Bakir et al., 2018).

where C₀ is the absorbance in the presence of control absorbance and C₁ samples. Consequently, IC₅₀ values of inhibition change depending on concentration of garlic samples were obtained as suggested by Mukherjee et al., (2011).

**Determination of Total Phenolics**

The total phenolic component of the ethanol extracts of garlic samples was determined using the Folin-Ciocalteu reagent in the literature (Slinkard and Singleton, 1977) and the gallic acid-containing method as standard. For the application of this method, 4.5 mL of deionized water and 0.1 mL of Folin-Ciocalteu reagent were added. After 3 minutes, 0.3 mL Na₂CO₃ (2%) solution and 0.1 extract solution were added and vigorously shaken. After a 2 hour waiting period, the absorption was measured at 760 nm. The concentrations of the phenolic compounds were calculated according to the following equation, obtained from the standard gallic acid (GA) graph:

Absorption = 0.537 GA (μM) + 0.03, \( R^2 = 0.999 \)

**Total Antioxidant Capacity Analysis**

Reduction of Mo (VI) to Mo (V) and formation of green colored phosphate / Mo (V) complex in acidic environment are the basis of the method. 500 mg/L ascorbic acid standard stock solution was prepared and diluted to 5 different concentrations (y=0.0033x-0.0601 and \( R^2 = 0.996 \)). Then 28 mM Na₃HPO₄.12H₂O solution, 0.6 M H₂SO₄ solution, 4 mM ammonium molybdate solutions were prepared and 25 mL of them were mixed and used as the reagent solution. 0.3 mL of garlic extract was taken into a tube and 3 mL of the reagent solution was added. The tubes were mixed well and kept at 95 °C for 90 minutes. The absorbance of the solutions was then measured at 695 nm. All these procedures were performed for ascorbic acid which is used as standard antioxidant. Antioxidant activity was calculated as ascorbic acid equivalent (mg AE/g garlic or black garlic) (Prieto, Pineda, & Aguilar, 1999).

**HMF Analysis**

The samples were thoroughly ground and homogenized. Weigh 2.5 g of sample into 50 mL flasks. The samples weighed 50 mL with ultrapure water. After shaking, dissolution in ultrasonic bath for 30 sec was achieved. Filtered through coarse filter. The filtrate was filtered through the 25 µm microfilter and collected in the vial. Analysis was carried out by HPLC belonging to Kastamonu University Central Research Laboratory Application and Research Center. Results were calculated to be mg HMF in kg garage or black garlic. The HPLC conditions were applied according to the method of Kalábóvá and Večerek, (2006).

**Total Flavanoid Analysis**

Quercetin stock solution was prepared at a concentration of 200 mg/L and five different concentrations were obtained by dilution from this concentration (y=0.0349x+0.0295 and \( R^2 = 1 \)). Black garlic extracts (1 mL) were mixed with 2% AlCl₃ solution of 300mM acetate buffer (3.1 g of C₂H₃NaO₂.7H₂O, pH 3.6, 10mM TPTZ (2,4,6-trisrydyl-triazine) in 40mM HCl and 20mM FeSO₄. The fresh working solution was prepared by mixing 1: 1: 10 TPTZ solution, FeSO₄.7H₂O solution and acetate buffer and heated at 37 °C for 25 minutes before use. Plant extract or reference was allowed to react with FRAP solution in dark conditions for 30 min. Measurements of the colored product (iron tripliridridiazine complex) were then performed at 593 nm. Results were expressed as mM FeSO₄.7H₂O equivalent in mg garlic or...
black garlic. The equation and correlation coefficient of the calibration graph were as $y=1.9558x-0.0024$ and $R^2 = 0.999$, respectively.

**Results and Discussion**

**The Evaluation of Odour, Taste and Colour**

Blacking changes in different fermentation days of has been observed garlic by Reis Tarim Urnuleri San Tic. A.S. Black garlic samples were observed at the 20th, 25th, 30th and 35th days. The pre-frozen and non-frozen samples were compared at 20th day and it was found that pre-frozen garlic was darker and drier and the odour of pre-frozen and non-frozen samples was disturbingly sharp. When the 25th day samples were examined, it was determined that the taste bitter and water decreased. In the 30th day samples, it was found that the color of the frozen samples was darker and these samples have more hard structure and the better taste compared to the non-frozen samples. When the samples were examined on the 35th day, it was observed that the frozen samples were black, bitter and dry; the non-frozen samples were black, bitter and hard.

**The Evaluation of DPPH Radical Scavenging Activities, Total Phenolic Compound, Total Antioxidant Capacity, Total Flavanoid Content and FRAP Analysis**

The DPPH radical scavenging activities, total phenolic content, total antioxidant capacity, total flavanoid and FRAP analysis of frozen and non-frozen black garlic species were evaluated at different storage times in this study. The results are presented in Table 1. All of the results of analysis indicated antioxidant properties of black garlic were found that higher than those of white garlic. Similarly, black garlic has proven its benefits to human health, due to its high content of antioxidants and phenolic substances compared to fresh garlic (Akan, 2014). Purev et al. (2012) showed that black garlic and fresh garlic were directly related to anti-carcinogenic and antioxidant activity in their study on immune system cells. Kim et. al., (2012) studied the change in total phenolic and flavanoid content of black garlic produced in different moisture, temperature and storage media. Studies have shown that garlic is a plant with antitumor and antioxidant properties besides regulating blood pressure, lowering cholesterol, bacterial infections and strengthening the immune system (Ayaz and Alpsoy 2007). The inhibition (%) values in the concentration range of 0.33-2.66 mg/mL as compared to the storage times for black garlic samples were shown in Figure 1.

![Figure 1. Free radical scavenging capacities (measured by the DPPH test) of different concentrations of frozen and non-frozen black garlic extract kept at different storage times.](image-url)
The % inhibition values obtained DPPH analysis of all garlic samples were changed in proportion to the increase in concentration. Similarly, when different storage periods were taken into consideration, the inhibition values increased with storage times. Frozen samples were found to have higher inhibition at all concentrations compared to non-frozen black garlic samples. Accordingly, frozen black garlic has been shown to increase the phenolic and flavanoid contents in optimum conditions compared to non-frozen black garlic. The IC50 values were inversely proportional to the storage time taken into consideration, the inhibition values increased with the storage period (Table 1). However, it is seen that total antioxidant capacity, the total phenolic compound and total flavanoid content values increase with the storage period (Table 1). Moreover, the FRAP value of frozen and non-frozen black garlic was almost never increased with storage time. So when these results are evaluated for black garlic samples, the reasons of high antioxidant capacity and good health effects is thought to be related to high free radical scavenging activity, total phenolic and flavanoid content. Similarly, Sato et al. (2006) explained that the shorter time fermenting activity, total phenolic and flavanoid content. Similarly, Sato et al. (2006) explained that the shorter time fermentation than the activities of non-frozen garlic samples were changed in proportion to the increase in concentration.

### The Evaluation of HMF Formation

The HMF causing irritation to upper respiratory tract, eyes, skin and mucous membranes, and is cytotoxic at high concentrations. In addition, also determined an oral LD50 to be 3.1 g/kg body weight for rats (Ulbricht, et al., 1984; Matic, et al., 2009). Carcinogenic potential of HMF has been researched in investigation on rodents. HMF has been demonstrated to promote and induce preneoplastic lesions as aberrant crypt foci (ACP) in rat colon (Archer et al.,1992; Bruce et al.,1993; Zhang et al., 1993) Recently, HMF has been shown that a weak carcinogen in multiple intestinal neoplasia (min/+ ) mice and significantly increasing the number of small intestine adenomas (Svendsen, Husøy, Glatt, Paulsen, & Alexander, 2009). Lastly, HMF has been reported that produces mutagenic effects by transforming into 5-sulfoxymethylfurfural (SMF), a highly reactive intermediate that can react with DNA and other macromolecules. (Bakhiya et al., 2009; Córdovaa et al., 2019).

<table>
<thead>
<tr>
<th>Total Phenolic (mg GA/g)</th>
<th>0</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH IC50 (mg/mL)</td>
<td>2.68</td>
<td>84.61</td>
<td>69.72</td>
<td>170.28</td>
<td>278.29</td>
<td>86.48</td>
<td>140.48</td>
<td>181.45</td>
<td>378.85</td>
</tr>
<tr>
<td>Total Antioxidant Capacity Analysis (mg AE/g)</td>
<td>6.21</td>
<td>6.45</td>
<td>7.26</td>
<td>7.94</td>
<td>8.49</td>
<td>8.33</td>
<td>9.56</td>
<td>10.40</td>
<td>10.85</td>
</tr>
<tr>
<td>Total Flavanoid (mg QE/g)</td>
<td>0.26</td>
<td>0.31</td>
<td>0.34</td>
<td>0.36</td>
<td>0.59</td>
<td>0.38</td>
<td>0.45</td>
<td>0.62</td>
<td>0.85</td>
</tr>
<tr>
<td>FRAP Analysis (mMFeSO4.7H2O/g)</td>
<td>0.09</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
<td>0.24</td>
<td>0.23</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>HMF (mg/kg)</td>
<td>0.18</td>
<td>2.00</td>
<td>6.40</td>
<td>19.80</td>
<td>34.00</td>
<td>3.20</td>
<td>9.80</td>
<td>27.60</td>
<td>110.00</td>
</tr>
</tbody>
</table>

Table 1. The Result of Total Phenolic, DPPH IC50, Total Antioxidant Capacity, Total Flavanoid, FRAP and HMF Analysis at different storage times under fermentation conditions for frozen and non-frozen black garlic samples.
The HMF analysis results show that a very high and risky HMF formation on the 35th day in the frozen sample at 110 mg/kg, and show that a very low HMF level at 9.80 mg/kg on the 25th day. Even on the 30th day, the frozen black garlic has a relatively low HMF level (34.00 mg/kg), the level of HMF of the black garlic produced without freezing was lower on the 35th day (27.60 mg/kg). However, since the fermentation time of black garlíc produced from frozen garlics is almost completed at the range of 25-30th days of fermentation according to data obtained from our study, it is clear that the consumption pre-frozen black garlic does not pose any risk for HMF toxicity at 25th and 30th days of fermentation.

**Conclusion**

In the production of black garlic, it is seen that pre-freezing before fermentation will shorten the production process and thus the costs will decrease. It has been found that the use of frozen samples results in an increase in total phenolic substances and antioxidant capacity in black garlic contents compared to non-frozen products. It has also been found that the formation of HMF which has toxic properties occurs in low amounts at the 25th and 30th day as the process completion time of the fermentation of frozen samples. Hence, no present any danger in terms of health hazards of HMF occurred in frozen black garlic at 30th day fermentation. The results of this study show that the use of frozen garlies in black garlic production will be more efficient in respect of antioxidant properties and time and cost saving.

**Compliance with Ethical Standard**

**Conflict of interests:** The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

**Financial disclosure:** This study was supported by Kastamonu University Scientific Research Coordination Unit. Project Number KÜ-BAP06/2017-1.

**Ethics committee approval:** No ethics committee approval is needed.

**Acknowledgement:** We thank the Kastamonu University Scientific Research Studies Project Management Coordination. The authors are also grateful to Kastamonu University Central Research Laboratory Application and Research Center. Special thanks for Reis Gıda Company supplied to material.

**References**


