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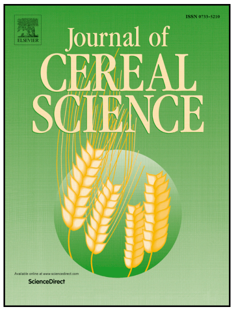

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PII: S0733-5210(17)30887-1

DOI: 10.1016/j.jcs.2018.05.003

Reference: YJCRS 2568

To appear in: *Journal of Cereal Science*

Received Date: 3 November 2017

Revised Date: 19 April 2018

Accepted Date: 8 May 2018

Please cite this article as: Zhang, Y., Yang, N., Fray, R.G., Fisk, I., Liu, C., Li, H., Han, Y., Characterization of volatile aroma compounds after in-vial cooking of foxtail millet porridge with gas chromatography-mass spectrometry, *Journal of Cereal Science* (2018), doi: 10.1016/j.jcs.2018.05.003.

Characterization of Volatile Aroma Compounds after in-vial Cooking of Foxtail Millet Porridge with Gas Chromatography -Mass Spectrometry

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Short version of title: Foxtail Millet Aroma Compounds

ABSTRACT: Foxtail millet has become popular over recent years for its nutritional value and ecological functions. The aroma of foxtail millet is not well characterized, which is critical for its eating quality and understanding the biochemistry and genetics of aroma is important for molecular breeding of millets rich in aroma. In this study, the volatile aroma compounds of the elite millet variety Jingu 21 were investigated at different cooking times, pH, processing methods, and compared with 3 other varieties. An in-vial cooking method was developed which combined solid phase micro-extraction and gas chromatography-mass spectrometry for the detection and identification of volatile compounds. The main findings were: a) Twelve aroma compounds were identified during cooking, which were hexanal, heptanal, octanal, (E)-2-heptenal, nonanal, trans-2-octenal, trans-2-nonenal, 2,4-nonadienal, (E,E)-2,4-decadienal, 1-octen-3-ol, 2-pentylfuran and 6-methyl-5-hepten-2-one. b) Longer cooking times produced higher concentrations of aroma compounds. c) Variations in cooking pH (from 6 to 8) had no obvious impact on the aroma of the millet porridge. d) More volatile compounds were released from millet flour compared to millet grain. e) There were significant differences among varieties and Jingu 21 millet showed the highest abundance of most aroma compounds, explaining partly why it is strongly favored by consumers for decades.

Keywords: Foxtail Millet; Volatile Aroma Compounds; In-vial Cooking; Gas Chromatography-Mass Spectrometry

1. Introduction

Foxtail millet (*Setaria italica*), one of the oldest cereal crops, has been grown in

China for thousands of years, and is now planted in India, North Korea, North Africa, the United States and elsewhere (Lata et al., 2013). It is a drought-tolerant crop adapted to arid or semi-arid areas and has a wide distribution in the northern region of China. Foxtail millet has high utilization value, as seeds can be used for food, bran for stock feed, and stalks for fuels or materials (Pantet al., 2016).

Millet food products such as millet wine, vinegar, crisps, porridge and infant formula powder, are high in protein, vitamins, fatty acids, amino acids and some vital nutrients which also have potential function to restrain type 2 diabetes (Song and Gao, 2005; Ren et al., 2016). Millet flour and bran are also rich in antioxidant components that help to prevent oxidative stress and may reduce free radical damage in humans (Suma and Urooj, 2012). For these reasons, it can be expected that consumer demand for millet will increase, and cultivation of high quality varieties of foxtail millet will likely become a new and growing trend in agriculture.

The quality of foxtail millet is measured in three ways: nutritional quality, physical appearance and eating quality (He et al., 2015). In Chinese traditional cooking, millet is mainly used to make porridge. The flavour, colour, stickiness and viscosity of porridge are the key features for evaluating the quality of foxtail millet. Among these, aroma is critical for consumers and volatile compounds are vital for the flavour characteristics of millet porridge. However, the millet aroma is quite subtle, and not easy to measure and quantify during cooking, and there have been only a few published studies addressing the flavour of millet porridge.

With gas chromatography-mass spectrometry (GC-MS) now in common use, detection and analysis of volatiles produced upon cooking foxtail millet is possible. An alternative way to detect volatile compounds in foxtail millet porridge is to use simultaneous distillation extraction (SDE) combined with GC-MS (Liu et al., 2012), but aroma compounds generated during cooking are very volatile and difficult to capture for accurate GC-MS analysis, and solvent extraction methods used for sampling flavour might not represent the volatile aroma compounds generated during cooking. Furthermore, it is challenging to prepare homogeneous replicates in small batch sizes.

In this study, foxtail millet from Jingu 21 was selected as the test material for its high quality and popularity in China (Shi et al., 2001). The aim of this study was 1) to develop an in-vial cooking method as a novel approach for multiple small volume sampling of large batches to minimize variation in sample processing; 2) to compare volatile aroma profile in the elite foxtail millet variety, Jingu 21 millet, with other three varieties, Jingu 36, Daqinggu and Zhishenggu (Zhang et al., 2016); 3) to investigate the impact of cooking times, pH of cooking water and processing method (whole millet grain vs. millet flour) on twelve important volatile aroma compounds contributing to aroma in plant foods (Table 1). This work will provide a foundation for future research to develop foxtail millet varieties with improved cooking quality.

2. Materials and Methods

2.1 Samples preparation

Four varieties of foxtail millet (Jingu 21, Jingu36, Daqinggu, Zhishenggu) were harvested in 2014, and dehusked using a rice huller (JLGJ-45, Hangzhou HR, China). Jingu 21 millet grains were milled using a grinder (DēLonghi, KG49, Germany). All samples were stored at 4 ℃.

Millet (1.5 g), ultra-pure water (Pur1te 'Select' DI Water System, UK) (7.5 ml) and 20 µl of internal standard (5 mg) 3-heptanone (Sigma, Saint Louis, USA) in 100 ml methanol (Laboratory reagent grade, Fisher Scientific, UK) were added to a sample vial (20 ml, 75.5 x 22.5 mm, Fisherbrand). Prepared samples were heated in the GC incubator at 100 ℃. Standard cooking time was 20 min, but additional tests were done at 0, 10, 20, 30, 40 min. The pH of the water was adjusted to pH6, pH7, pH8 by 0.1M HCl and 0.1M NaOH; Two sizes of processed millet (millet grain, millet flour) were compared.

2.2 Identification and quantification of volatile aroma compounds by GC -MS

All the samples were extracted using solid phase micro extraction fiber (50/30μm, DVB/ CAR/ PDMS). Before extracting, SPME was heated at 250 ℃ for 1 hour, then desorbed for 10 min for analysis. A GC-MS (Thermo Fisher TRACE 1300, USA) with a ZB-WAX Capillary GC Column (length 30 m, inner diameter 0.25 mm, film thickness 1 μm; Phenomenex Inc., Macclesfield, UK) was utilized for analysing and identifying the extracted compounds. The oven program was set as follows: Initial temperature was 40 ℃ for 2 min, then increased to 250 ℃ at a rate of 6 ℃ per min and held for 3 min. The inlet temperature was set at 250 ℃ in splitless mode. The mass spectrometer settings were: Ionization mode EI, 70 eV; Filament emission current 200 μA; Temperature ion source was 200 ℃; Interface temperature was 250℃. Full scan mode was used to detect the volatile compounds (mass range from m/z 20 to 300).

Volatile compounds were identified and selected by matching the mass spectra with the spectra of reference compounds in the NIST/EPA/NIH mass spectral library version 2.0 (Faircom Corporation, U.S.) using retention time. 3-heptanone was used for standard calibration for quantitative analysis.

2.3 Statistical analysis

Millet processing was conducted in triplicate, and the data were analysed by ANOVA SPSS 17.0 package. Significant differences in measurements were determined by Tukey's HSD test (α=0.05). Principal component analysis (*The Unscrambler® X*) was performed to understand factors affecting the change of volatile components under different treatments.

3. Results and Discussion

3.1 Identification and quantification of volatile aroma compounds

Twelve volatile aroma compounds were identified in Jingu 21 millet after 20 min in-vial cooking, using SPME-GC-MS. Compared to traditional in-pot cooking, which involves high water loss, is time consuming and produces a large amount of waste materials, the new in-vial method was fast and reproducible, involved minimal human handling, reduced working time and allowed the automatic running of larger batches of samples. Furthermore, the coefficient of variation of the proposed method was less than 10% for most volatile aroma compounds in millet porridge (Table 1).

The 12 volatile compounds generated a unique profile (Table 1) and were predicted to play an important contribution to the flavour of the millet porridge. Their production is known to be closely related to the enzymatic activity of the fatty acid metabolism pathway, and lipoxygenase activities (Liavonchanka and Feussner, 2006; Xu and Barringer, 2009), but their relative abundance and release during cooking has not previously been studied in these millet varieties. The fatty acid composition of foxtail millet has previously been reported to include linoleic acid, oleic acid, palmitic acid, stearic acid and arachidic acid. Among these, linoleic acid is the major fatty acid. Hexanal, 2-pentylfuran, (E)-2-heptenal, (E,E)-2,4-decadienal and

1-octen-3-ol are commonly derived from linoleic acid; heptanal, octanal and nonanal were formed by the hydroperoxide degradation of oleic acid. 6-methyl -5-heptene-2-one (Table 1) was probably derived from the oxidative cleavage of acyclic carotenoid, as shown to be important in cooked rice (Widjaja et al., 1996). The result indicate that aldehydes may be important in the formation of the aroma of millet porridge (Liu et al., 2012). This finding could help to study further on the implications of fatty acid metabolism and eating quality in foxtail millet.

3.2 Impact of different millet varieties

Foxtail millet germplasm resources are rich and diverse in China with more than 26,670 landraces of foxtail millet collected to date (Wang et al., 2012). We selected four varieties of foxtail millet that are commonly accepted as being of good eating quality, Jingu 21, Jingu 36, Zhishenggu and Daqinggu. Jingu 21 millet is one of the most popular varieties in China, due to its high eating quality and good nutritional value.

The volatile aroma compounds produced in different varieties of millet were compared using a principal component analysis (PCA). The PCA results showed that a total of 80% of the variance could be explained in the first two dimensions (Fig. 1a). Along PC1, the 4 varieties were separated into different groups. Jingu 21 millet was clustered on the right side with the highest level of most aldehydes: hexanal, 2-pentylfuran, (E)-2-heptenal, 6-methyl-5-heptene-2-one, trans-2-octenal, 1-octen-3 -ol, trans-2-nonenal, 2,4-nonadienal and (E,E)-2,4-decadienal, and the Daqinggu millet was clustered on the left side and was associated with octanal, nonanal and heptanal. Jinggu 36 and Zhishenggu millets were clustered together and located in the middle. Along PC2, Daqinggu was clustered on the top left quadrant, whereas

Jingu 21 clustered in the bottom right.

The results showed that Jingu 21 millet had more abundant volatile aroma compounds than the other varieties tested. Most of the aroma compounds in Jingu 21 were present at a higher level than the other three varieties with no significant difference ($p < 0.05$) except for trans-2-octenal, 6-methyl-5-hepten-2-one (Figure. 1b). Trans-2-octenal in Jingu 21 was significantly higher than Daqinggu and Jingu 36 ($p<0.05$). 6-methyl-5-hepten-2-one in Jingu 21 millet was present at a higher concentration compared to the other three varieties ($p<0.05$) (Fig. 1b). Octenal, nananal, heptanal in Daqinggu were in a higher level than other varieties. ANOVA showed that octenal in Daqinggu was significant difference with Jingu 36 ($p<0.05$), but not significant difference with Jingu 21 and Zhishenggu ($p<0.05$). Nonanal in Daqinggu was both higher and significant difference than other varieties ($p<0.05$). Heptanal in Daqinggu was higher and significant difference than Jingu 36 and Zhishenggu, but not significant difference with Jingu 21 (Fig. 1b).

Jingu 21 produced more aroma compounds than the other three varieties, whereas Daqinggu contained a higher level of heptenal, octanal and nonanal. The difference in the profiles may arise from their different contents of protein, fatty acid and amino acids, which under heat-treatment may undergo maillard reaction, stecker degration and lipid oxidation, thereby producing different levels of heterocyclic compounds, aldehydes, pyrazine, hydrocarbons and other aroma compounds (Smith and Barringer, 2014). Based on data for Jingu 21, Jingu 36 and Daqinggu (Zhishenggu not available) from the Chinese Crop Germplasm Resources Information System (CCGRIS), Jingu 21 does show higher protein (15.12%) and fat (5.76%) contents than the other varieties (Jingu 36 13.38%, 4.29% and Daqinggu 10.74%, 4.92%). The study showed the flavour of millet may not be determined by a single aroma compound but is rather the result of a combination of several compounds, similar to the findings with rice aroma by Widjaja et al. (1996). The flavour of millet can also be influenced by other factors, such as the ecological environment, the weather and the soil quality where the crop is grown (Minet al., 2005). In this study, we only chose four varieties of foxtail millet for primary testing, but the in-vial method will make it possible to carry out extensive further research by which the effect of different genetic backgrounds and geographical conditions on aroma profiles could be assessed.

3.3 Impact of cooking time

Cooking quality is an important index for assessing foxtail millet. It is impacted by the cooking time, the gelatinization temperature of millet starch, the content of amylase and amylopectin (He et al., 2015). Usually, cooking time influences the stickiness and viscosity of millet porridge, but there is no information on the relationship between volatile aroma compounds and cooking time. A range of cooking times (0, 10, 20, 30, 40 min) was tested for aroma profiles, with the traditional cooking time (20min), which is the standard residential and commercial productions and preparation in catering establishments.

In order to better understand the changes in aroma compounds under different cooking time, a PCA chart was used, which showed 93% of the variance could be explained in the first two dimensions (Fig. 2a). Along PC1, different cooking times were separated into 5 groups. The longer cooking times, 20, 30 and 40 min, were clustered on the right side, whereas the shorter times of 0 and 10 min were clearly clustered on the left side.

The radar chart (Figure 2b) showed hexanal, heptenal, 2-pentylfuran, octanal, (E)-2-heptenal, nonanal, trans-2-octenal, 1-octen-3-ol, trans-2-nonenal, 2,4-nonadienal and (E,E)-2,4-decadienal increased significantly in concentration from 0 to 40 min. Conentrations of most of the volatile aroma compounds were significantly different after different cooking times ($p<0.05$), with the exception of 6-methyl-5-heptene-2-one (Fig. 2b).

The higher concentrations of volatile compounds found after extended cooking times maybe due to extend chemical reactions such as lipid oxidation and maillard reactions. It should be noted that the millet samples were prepared in a sealed vial from which the gas could not easily escape to the atmosphere, and with a longer cooking time, the concentration of volatile compounds in the vial was an accurate reflection of the total volatiles produced. These results were similar to those for rice, with longer cooking time releasing more flavour and increasing stickyness (Champagne et al.,2008).

3.4 Impact of different pH

The pH of tap water in different areas of China varies and might influence the cooking quality of millet. Normally, alkalizing agents are used to increase the pH and retain the colour of certain foods (Andrés-Bello et al., 2013) and sodium bicarbonate, is commonly added to the pot during the cooking process for millet porridge by local people to shorten the cooking time and to promote a good texture of millet porridge. However, it is not known whether or not the alkaline condition of water influences the generation of flavour aroma compounds. Thus, in this study, we set a pH range as 6, 7 and 8.

The PCA chart showed that 88% of the variance could be explained in the first two dimensions (Fig. 3a). However, the pH zone and the volatile compounds were not clustered clearly in dimensions in the PCA chart. Also, the radar chart showed no general trends and ANOVA showed no major significant difference for the 12 volatile aroma compounds tested (Fig. 3b), which suggests that the pH of the cooking water had little or no impact on the production of volatile aroma compounds.

It is known, however, that the pH value can influences the colour and texture of cooked millet during cooking, and alkaline conditions (pH>8) could help to prevent colour and texture loss due to its effect on the protein properties of food, enzymatic activities, gelification, and possibly chemistry (Shen et al., 2015).

3.5 Impact of whole grain and ground flour

Millet flour is important for making and developing processed food products, and flavour is critical for the quality assessment of millet because it closely affects consumer preference. Thus, we studied the extractability of flavour components from flour and grain of Jingu 21 millet after cooking for 20 min and observed the volatile changes.

The radar chart (Fig. 4) clearly shows higher production of flavour components from flour compared with grain. Almost every flavour components was significantly different comparing grain and flour ($p<0.05$), and hexanal, heptanal, 2-pentylfuran, octanal, (E)-2-heptenal, 6-methyl-5-heptene-2-one, trans-2-octenal, 1-octen-3-ol, 2,4-nonadienal, but not nonanal, trans-2-nonenal, (E,E)-2,4-decadienal, were higher using flour. It is assumed that this is related to fact that the particle size of millet flour is substantially smaller than millet grains and the increased surface area of millet leads to enhanced production or release of volatile compounds from flour. Similar results were found in a previous study by Bhumiratana et al. (2011) in coffee, where the ground coffee released more aroma compounds than coffee beans due to the processing of grinding, which helped to expose and release the volatile aroma compounds.

4. Conclusion

In this study, we developed an in-vial cooking method for the automatic cooking and detection of volatile aroma compounds in the foxtail millet, obtained a preliminary understanding of the volatile aroma compounds in millet porridge by observing the changes of volatiles with different treatment methods. Among the 12 volatile aroma compounds selected, aldehydes played an important role in the aroma profile of the millet varieties analysed. During extended cooking (40min), a higher concentration of aroma compounds was produced. The pH of cooking water had no impact on the volatile compounds. When millet grain was milled to flour, higher concentrations of aroma compounds were released than for the whole grain. Comparisons among four varieties of millet showed most of the aroma compounds were at a higher level in Jingu 21. Secondary products from lipid oxidation were elevated in the Daqinggu variety. Additional research on sensory evaluation will be helpful to improve the quality of foxtail millet in the future. Improved understanding and facile measurement of the aroma components should prove useful in breeding programs to improve these attributes.

Acknowledgments

This work was supported by National Nature Science Foundation of China (grant number 31371693, 31471556)

The authors thank Sharon Lim Mui Ting, Rob Linforth for experimental operation and guidance, thank Professor Donald Grierson for help with the manuscript, and thank Food Sciences at the University of Nottingham for providing access to instruments and facilities.

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Table 1. Volatile aroma compounds identified from Jingu 21 millet porridge

RT	Compound and+Cas#	Odor Description	References	Coefficient of Variation in Present in-vial Experiments
	Alkanals			
4.45	Hexanal（66-25-1）	Green, grass-like	Lei & Boatright, 2008	7.1%
6.5	Heptanal (111-71-1)	Fruity, fatty	Lee, Xiao, Zhang, Ebeler & Mitchell, 2014	5.9%
8.82	Octanal (124-13-0)	Green, citrus-like	Park & Drake, 2016	3.9%
9.56	(E)-2-Heptenal (18829-55-5)	Herbaceous	Globisch, Schindler, Kress-ler & Henle, 2014	5.9%
11.2	Nonanal (124-19-6)	Soapy, citrus-like	Franklin, Chapman, King, Mau, Huang & Mitchell, 2017.	4.0%

11.95	Trans-2-octenal (2548-87-0)	Green, fatty	Shi, Li, Wang, Zhang, Qiu, Han, Wang, Chang & Guo, 2015.	8.6%
14.29	Trans-2-nonenal (18829-56-6)	Fatty, tallowy	Kaneko, Kumazawa & Nishimura，2001	10.3%
17.72	2,4-Nonadienal (6750-03-4)	Fatty, beany	Park & Drake, 2016	18.3%
19.84	(E,E)-2,4-Decadienal (25152-84-5)	Fatty, waxy	Pan, Huang, Hsu, Lee, Liu, Cheng, Tsai, Shen & Lin，2014.	22.0%
	Phenols and Alcohols			
12.55	1-Octen-3-ol (3391-86-4)	Green, beany	Sugawara, Ito, Odagiri, Kubota & Kobayashi, 2014	5.2%
	Heterocycles			
7.54	2-Pentylfuran (3777-69-3)	Beany	Pripdeevech, Moonggoot, Popluechai & Chukeatirote, 2014	7.0%
9.93	6-Methyl-5-hepten-2-one (110-93-0)	Banana-like, floral	Christensen, Edelenbos & Kreutzmann, 2007	3.9%

Volatile compounds were identified after 20 min cooking by GC-MS retention time (RT) compared with internal standard compounds.

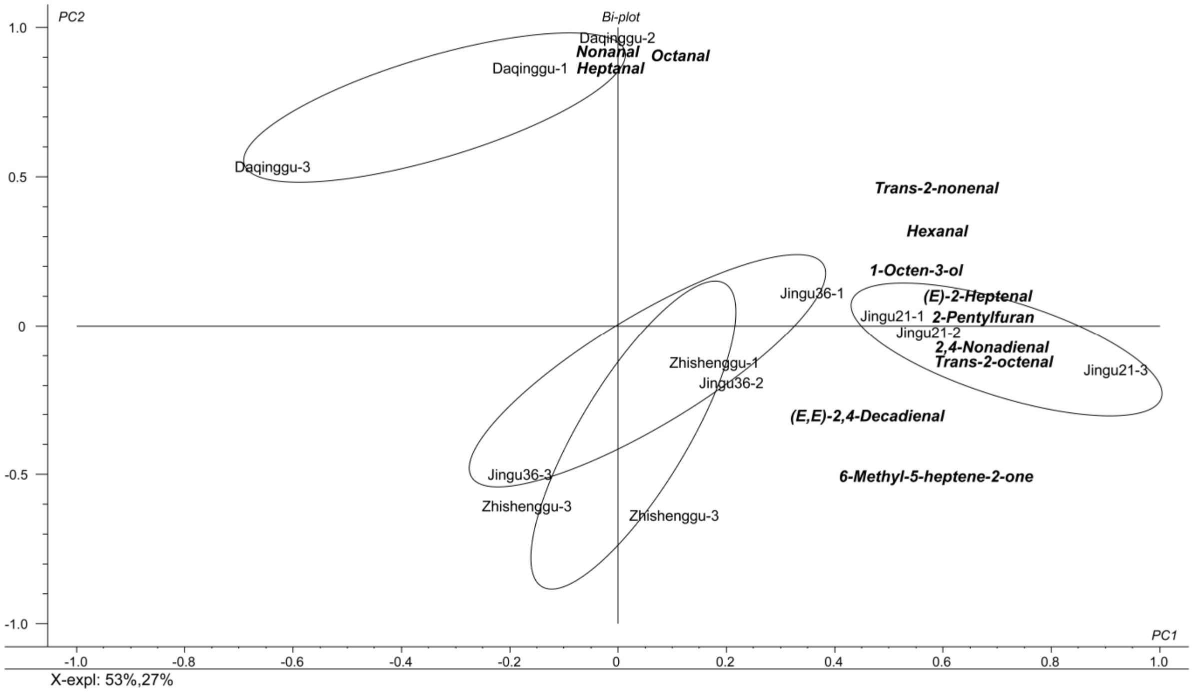

Fig. 1a. PCA analysis of aroma components for 4 varieties of millet

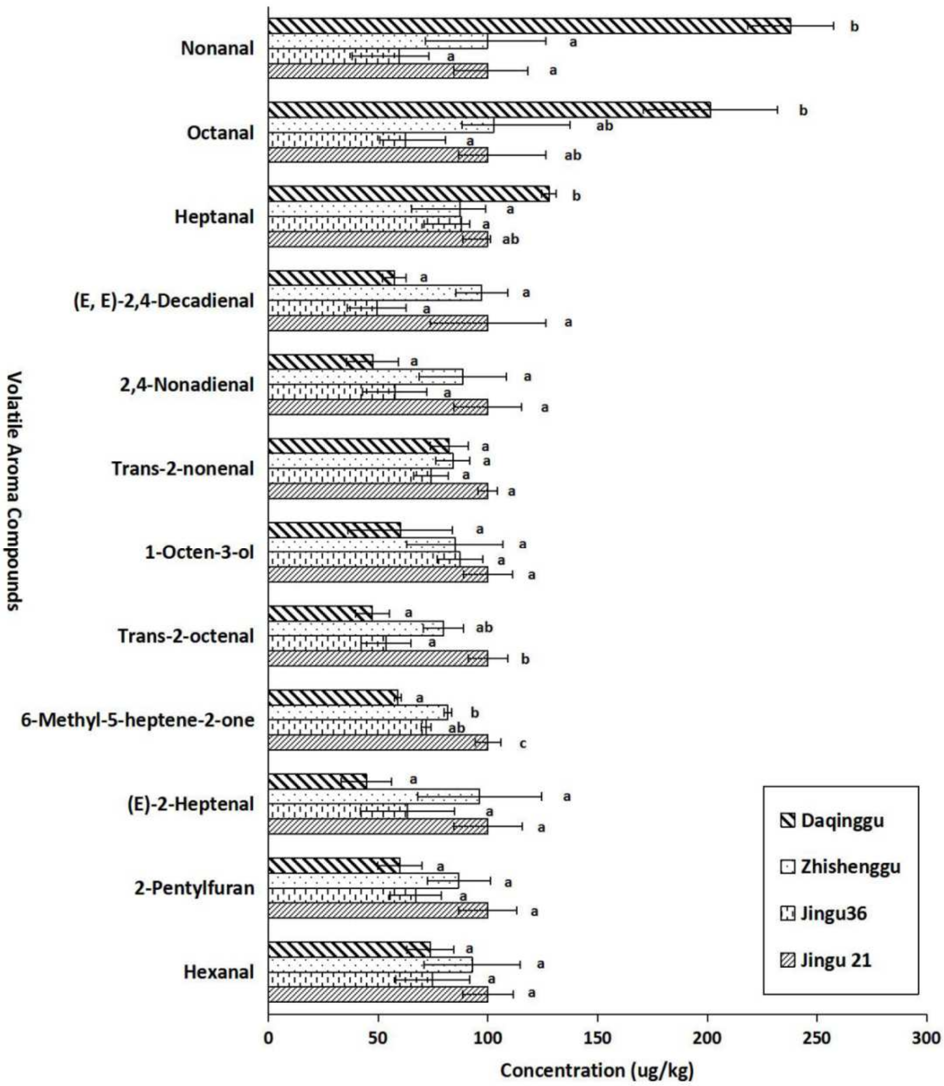

Fig. 1b. Comparison of volatile aroma compounds from four varieties of millet. Cooking time was 20 minutes. Different letters indicate significant differences between varieties (p<0.05). Jingu 21 millet values were normalized to 100%.

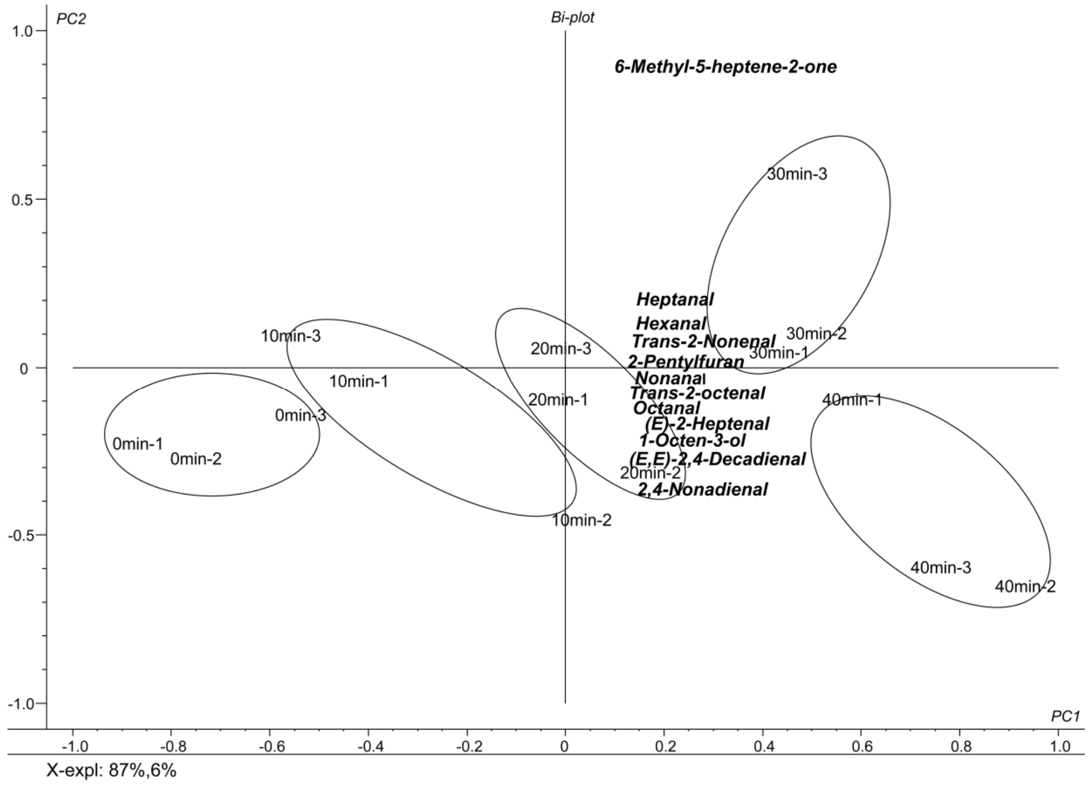

Fig. 2a. PCA analysis of aroma components of millet Jingu 21 produced after different cooking times

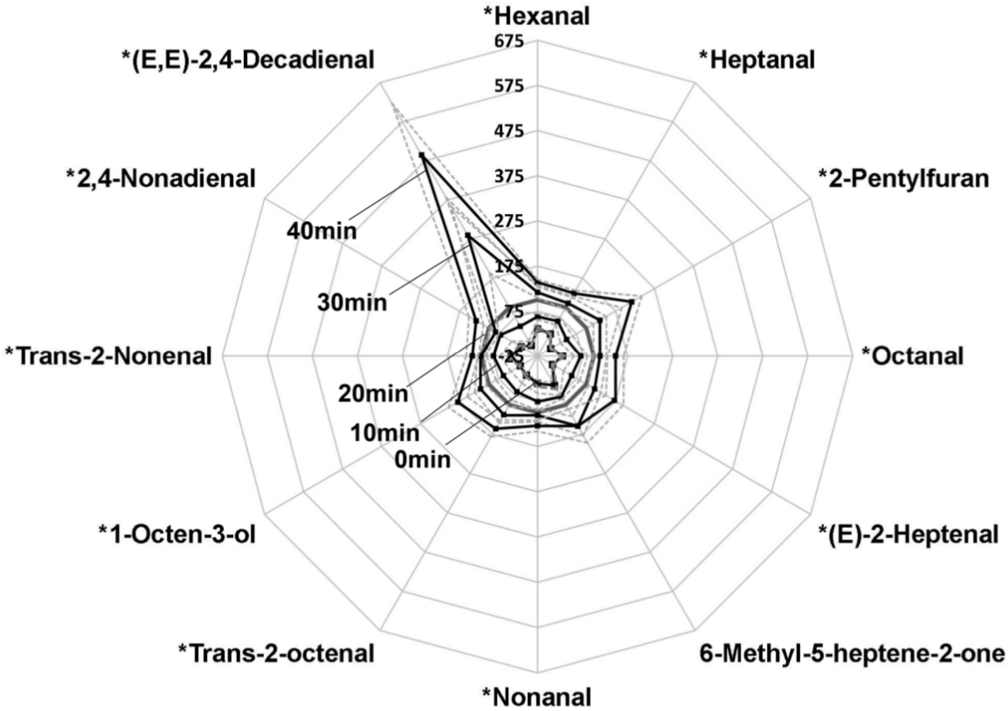

Fig. 2b. Effect of cooking times on volatile production of millet Jingu 21. Aroma profiles generated after 20 minutes (standard cooking time) compared with 0, 10, 20, 30, 40min. with +/- standard errors showed by grey dotted lines. * indicates significant differences for each compound (p=0.05).

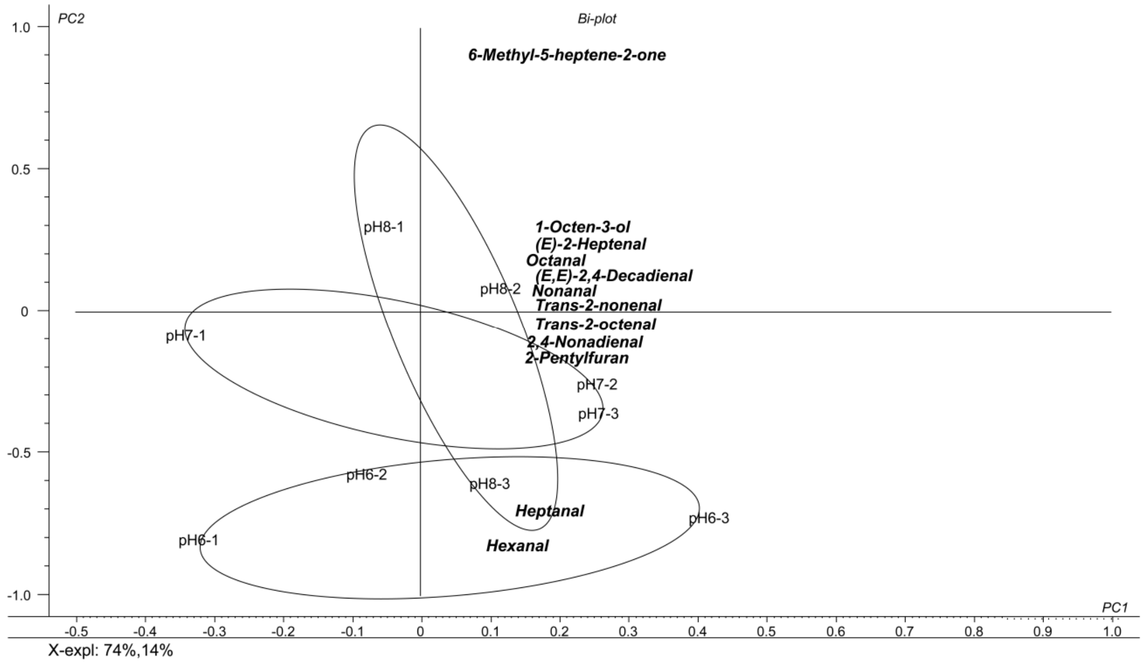

Fig. 3a. PCA analysis showing poor correlation between different cooking pH and aroma compound production of millet Jingu 21.

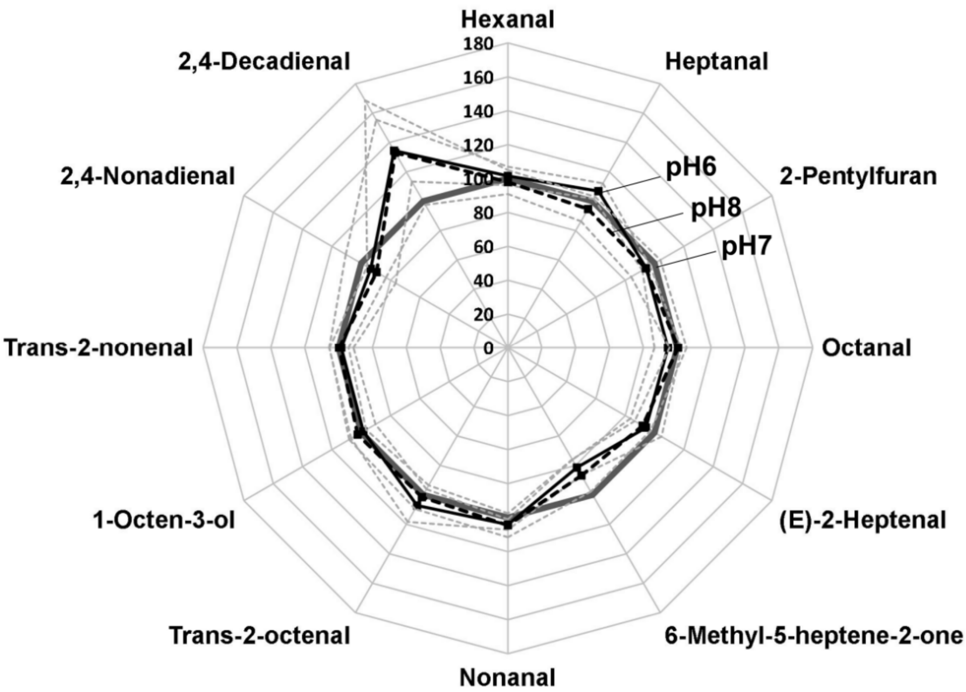

Fig. 3b. Aroma volatiles production of millet Jingu 21 at different pH. Aroma profile produced at the standard pH 7 (gray smooth circle, set at 100%). Average values of volatiles produced at pH 6 (black line), 7 (gray line) and 8 (dashed line) with +/- standard errors showed by grey dotted lines.

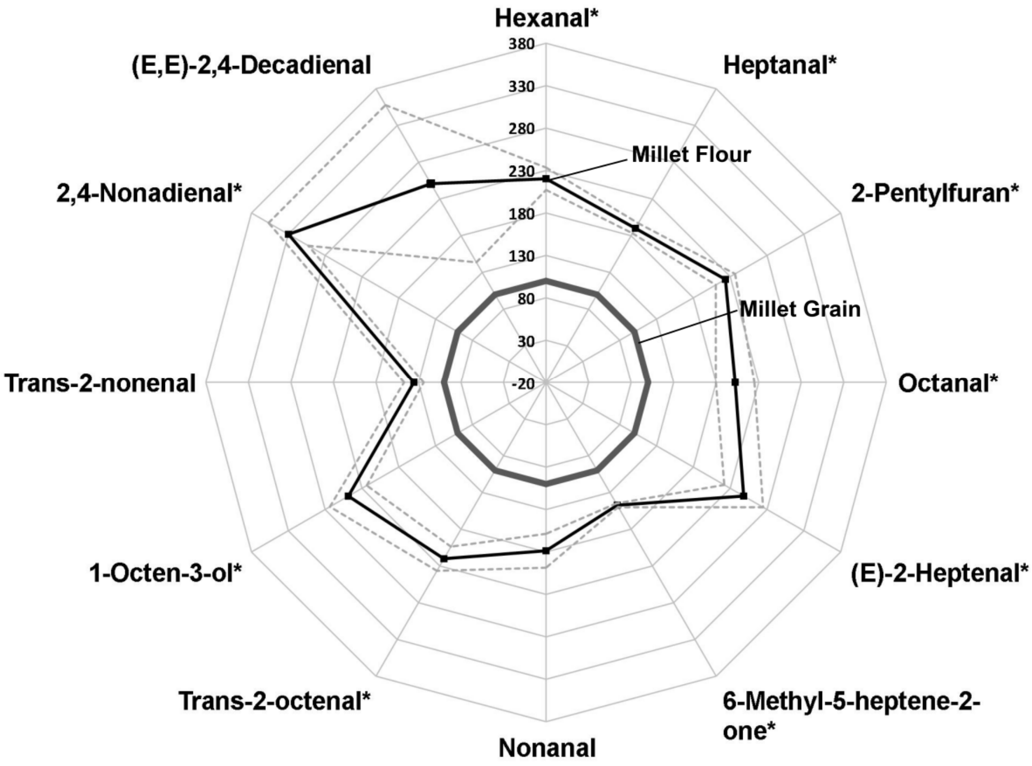

Fig. 4. Comparison of aroma profiles between flour and grain of millet Jingu 21. Aroma profile of the millet grain (smooth circle at 100%), millet flour (average value showed in black solid with square markers, with +/- standard errors showed by grey dotted lines). * indicate significant difference (p=0.05).

Research Highlight

- An in-vial cooking was developed to analyse aroma compounds in foxtail millet
- Within 40 min, longer cooking times result in higher levels of volatiles
- More aroma compounds were released from millet flour than grain during cooking
- Popular variety Jingu21 released higher levels of aroma compounds than the others