Master's Thesis of Science in Agriculture

Development of Brewing Functional Beers and Studies on the Biochemical Properties

기능성 맥주의 제조와 그 생화학적 특성에 대한 연구

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Development of Brewing Functional Beers and Studies on the Biochemical Properties

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Abstract

Buckwheat has been used as an ingredient for brewing beer in some studies. However, there are few studies focused on preserving rutin content while brewing beer. This research found the presence of rutin-degrading enzyme (RDE) was identified as a key factor to decrease the rutin content in the final beer brewed by normal process. Improved process to decrease activity of RDE increased rutin content 60 times than normal brewing method. Total flavonoid content was also 1.99 times higher in beer brewed by improved method. Antioxidant capacity test targeted on different types of beer proved improved brewing process is more adequate method to keep oxidative stability. Beer containing tartary buckwheat did not have excessive amount of undesirable compounds like diacetyl and acetaldehyde. However, some aroma compounds associated with fruit-like flavor increased according to the proportion of tartary buckwheat and mashing method. Buckwheat proportion up to 40% did not have any bad impact on beer quality attributes such as alcohol content and wort sugar content. Overall, using some proportion of tartary buckwheat malt as a replacement of barley malt was adequate in regard to the main beer quality attributes, flavor, and taste as well as increasing functional properties.

Keywords : Buckwheat, rutin, rutin degrading enzyme, antioxidant capacity, physicochemical properties

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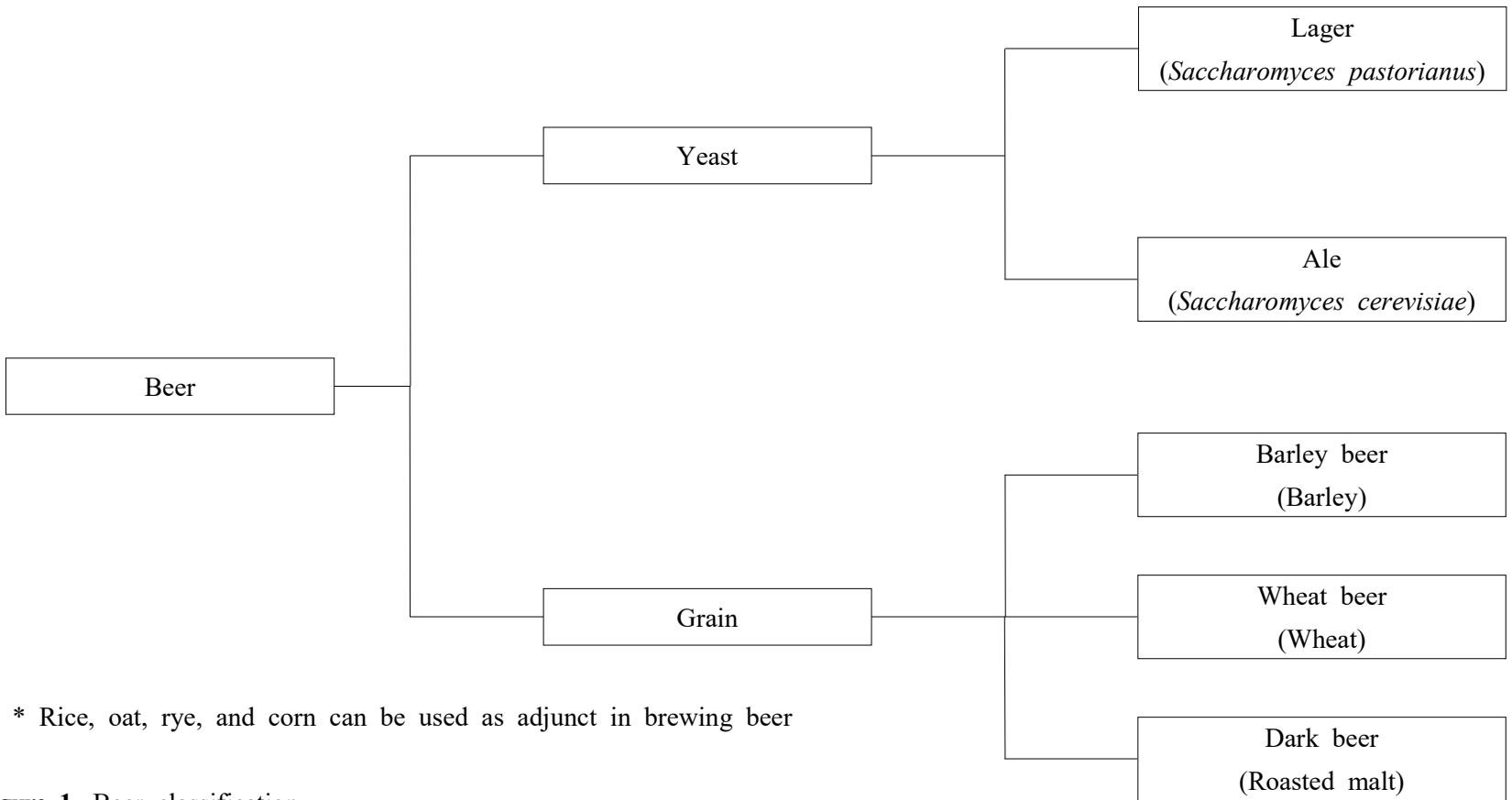
Literature Review

1. Lager beer

Beer is immutable microbial product, which every production step involves microbial activity [1]. Barley malt is most commonly used in brewing, and other grain materials like wheat and rice can be also used [2, 3]. Hop containing essential oils is also an important factor to give bitterness and distinctive flavor in beer production. It also contains polyphenols, so it affects the antioxidant properties in the beer [4]. Owing to the characteristics of used materials, beer involves many nutrients like carbohydrates, phenolic compounds, and vitamins. These nutrients commonly originated from malt and hops, and also contribute to color, aroma, flavor, and stability of the beer [3]. Instead of diversely used yeast strain "*Saccharomyces cerevisiae*", lager beer is fermented by "*S. pastorianus*". Evolved from the iteration of *S. carlsbergensis* and *S. cerevisiae*, *S. pastorianus* is known for bottom-fermenting yeast not rising to the surface during fermentation [1, 5]. *S. pastorianus* works at colder temperature (usually 8-15 °C) than ale yeasts, and ferments slowly by utilizing more wort sugars. This makes light and crisp tastes in the final beer [6]. Historically, beer has been brewed and consumed since ancient egypt, but its benefit is not well-known. Recently, there are many researches focusing on benefits of drinking beer. Surely, moderate consumption of beer on a regular basis associated with appropriate diet and lifestyle is required to be beneficial on the health [7]. Besides, numerous research shows another effects on the body such as bone mineral density, recovery after sports activity,

and cholesterol metabolism in the heart [7, 8].



* Rice, oat, rye, and corn can be used as adjunct in brewing beer

Figure 1. Beer classification

2. Tartary buckwheat

Buckwheat (*Fagopyrum spp.*) is a dicotyledonous crop which includes 25 species. Buckwheat has been cultivated as a economical food crop in Asia and Europe owing to the advantages like short growth period, good adaptability, high survival rate, and high productivity [9]. Nowadays, buckwheat is considered a functional food for patients with gluten intolerance (celiac disease) as it is a gluten-free pseudo-cereal [10, 11]. Common buckwheat (*Fagopyrum esculentum*) is widely used in food and even in pharmaceuticals because of high bioactive compounds like rutin and quercetin. Tartary buckwheat (*F. tartaricum*), which is usually cultivated in East and South Asia, contains about 100 times higher rutin and quercetin than common buckwheat [12]. Furthermore, it also includes polyphenols essential for protection against ultraviolet radiation and pathogens [13].

Figure 2. Germination of tartary buckwheat
A: Tartary buckwheat seed
B: Sprouted tartary buckwheat

3. Rutin and quercetin

Rutin, also known as rutoside and quercetin-3-rutinoside, is composed of flavonolic aglycone quercetin and rutinose. The name “rutin” came from *Ruta graveolens*, and passion flower, onion, apple, and buckwheat are known for containing rutin [14, 15]. As a bioactive compound, rutin is reported to include therapeutic effects including strong antioxidant capacity, anti-inflammatory, and cardioprotective effects. Furthermore, current studies considerably focus on rutin as a potential pharmacological compound against neurodegenerative diseases [14, 16]. Quercetin (3,3,4,5,7-pentahydroxyflavone) is a flavonoid aglycone found in mulberry, apple, kale, and buckwheat [17]. Quercetin has been also reported to possess diverse pharmacological properties such as antioxidant, anticancer, anti-carciogenic, and anti-inflammatory activities [18]. Even though quercetin has a lot of functional effects on human body, its bioavailability is very poor (less than 2%) due to its low absorption with rapid metabolism [19].

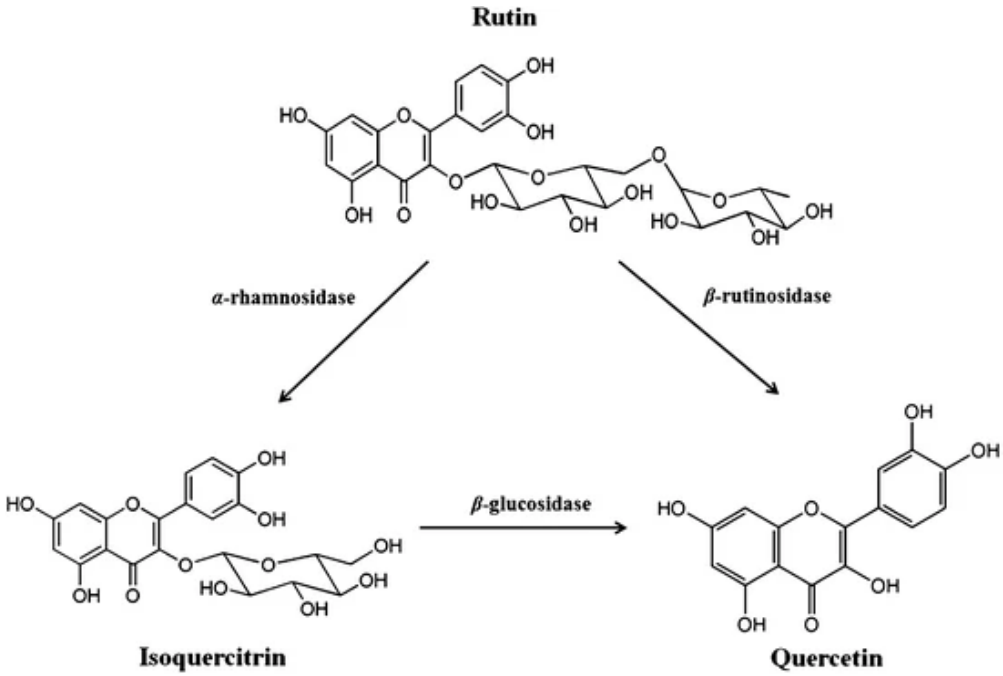


Figure 3. The structure of rutin and quercetin [17]

4. Volatile compounds in the beer

Volatile compounds are key factors to give special aroma and flavor in the beer. Yeasts can make a diversity of secondary products during fermentation, and higher alcohols, diacetyl, esters, aldehyde, and ketone are important contributors for beer quality evaluation [20, 21]. However, high concentration of buttery, alcohol-like aroma can make off-flavor in the beer, so consumers feel unpleasant to drink. In case of diacetyl, Likewise, excess amount of higher alcohol including propanol, isobutanol, isoamyl alcohol, benzyl alcohol, and phenetyl alcohol can provide unpleasant aroma into the beer [21]. Furthermore, more than 20% of isobutanol among propanol, isobutanol, and isoamyl alcohol causes undesirable effects [22]. In regard to esters, which includes ethyl hexanoate, isoamyl acetate, and ethyl acetate, fruity flavor could provide rich and fresh flavors in the beer [23]. Surely, these compounds should be kept at proper concentration for beer quality. In this research, different proportion of buckwheat malt was used as a replacement of common barley malt. By comparing volatile compounds in the final beer product containing buckwheat malt with 100% barley malt beer, it could be possible to evaluate whether buckwheat malt is adequate to be an adjunct in brewing beer.

Table 1. Description and threshold of flavor compounds in the beer

Classification	Properties	Flavor description	Threshold (mg/L)
Carbonyl compound	Diacetyl	Buttery, butterscotch [21, 24]	0.1-0.15 [21, 24]
	Acetaldehyde	Green apple, grassy [21, 25]	10-20 [21]
Ester	Ethyl acetate	Fruity, sweetish [21, 23]	25-30 [21, 23]
	Isoamyl acetate	Banana, sweet [23]	1.2-2.0 [21, 23]
	Ethyl hexanoate	Apple, fruity [23]	0.2-0.21 [21, 23]
	Ethyl caprylate	Sour [26]	0.9-1.0 [21, 23]
Higher alcohol	Propanol	Alcohol [21]	600-800 [21, 23]
	Isobutanol	Alcohol [21]	100-200 [21, 23]
	Isoamyl alcohol	Alcohol, medicinal [21, 26]	50-70 [21, 23]

5. Oxidative stability

Oxidation of beer after packaging is responsible for deteriorative beer by degrading bitter acids such as α-acids, β-acids and iso-α-acids from hops [27]. Furthermore, unpleasant flavor compound like trans-2-nonenal (T2N) and 3-methylbutanol could happen in the beer oxidized beer even though its flavor does not have any harm on human health [28-30]. Therefore, it needs to evaluate oxidative stability of beer product, especially focusing on the functional effect. Many researches using buckwheat as a replacement for barley malt focused on sensory characteristics such as tastes, aroma, and flavors from buckwheat [31]. However, only a few studies conducted research on rutin amount and oxidative stability in the buckwheat beer [32]. Antioxidant capacity is defined two mechanisms of action: single electron transfer (SET) and hydrogen atom transfer (HAT) [33]. To determine oxidative stability of different beers, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which mechanism is related to both SET and HAT, was conducted. DPPH assay is also adequate to assess radical scavengering activity of material [34]. Rutin is also known for strong ability in radical scavengering [35]. The purpose of this research is preserving rutin content in the beer, so this method can also assess oxidative stability of rutin beer. Ferric ion reducing antioxidant power (FRAP), which mechanism of action is HAT, was also performed to determine antioxidant capacity of different beers [36].

6. Purpose of study

Tartary buckwheat includes much higher amount of rutin and quercetin than common buckwheat and barley malt. Rutin, which is known for its bioactive properties including strong antioxidant capacity and anti-inflammatory effect, is main target of this research. Specifically, preserving rutin content in the final beer by removing factors to lessen the rutin content during brewing process is the purpose of this study. The high amount of rutin would provide functional factors like oxidative stability in the final beer. In addition to functional effects of tartary buckwheat beer, we verified qualities of the final beer such as alcohol content, bitterness, and flavor compounds. Development of rutin-enriched beer would be a good way to provide information how tartary buckwheat can be used in brewing beers and it affects the qualities of the final beer.

Materials and methods

1. Materials

Tartary buckwheat and common buckwheat were acquired from a local market (Pyeongchang, Korea). The barley malt for pilsner was obtained from Weyermann Specialty Malts (Bamberg, Germany). Lager yeasts (*S. pastorianus*) were purchased from Fermentis Ltd. (Marcq-en-Baroeul, France). Hop pellets were acquired from Lupex GmbH (Hallertau, Germany). Aluminium chloride, 2,2-diphenyl-1-picrylhydrazyl reagent, 2,4,6-tris(2-pyridyl)-s-triazine, and quercetin standard for were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rutin standard and formic acid was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). LC-grade methyl alcohol, ethyl alcohol, water, and acetonitrile were acquired from Honeywell International Inc. (Muskegon, MI, USA).

2. Brewing process

Malting process for tartary buckwheat and common buckwheat included three steps: steeping at 20 ºC for 8 h, germination at 20 ºC for 88 h, and drying at 60 ºC for 22 h) [37]. A total of 4 kg of grains were used to brew beer and its proportion was 100%, 80%, 60% barely and 0%, 20%, and 40% tartary buckwheat. Milling process for brewing lager beer was conducted before starting mashing process with a two-roller grist mill (Frensdorf, Germany) at 0.2 mm. Mashing process was conducted by two different ways illustrated in **Figure** 5. Mashing method A, a widely used brewing procedure, included four successive processes at different temperatures. The water and the milled malt were mixed in the proportion of 4:1 and mashed at 50 ºC for 20 minutes. Then, the mash was heated to 64 ºC and kept for 70 min. Afterwards, it was heated to 72 ºC and left for 20 min. The last temperature was set at 78 ºC and maintained for 10 min. Method B, which is an improved mashing method to decrease the activity of rutin-degrading enzyme, includes five successive processes. Before starting conventional mashing process, the milled buckwheat malt was mixed with heated water at 80 ºC, maintained for 20 min. Then, the mash was cooled under 50 ºC. After that, the milled barley malt transferred into the mashing bath containing buckwheat and heated at 50 ºC for 20 min. The proportion of the water and the mash was set 4:1. Left three successive processes were conducted same to mashing method A. The beer using 100% barley malt as a control was only brewed by mashing method A. After successive processes, the used malt was removed from the kettle and the mashed wort was transferred to the kettle again. The sediment was washed twice with 70 ºC water as a lautering process. The clear liquid wort was boiled for 60 min. During this process, 5 g

of cascade hops were added at 5 min and 3 g of cascade hops were added at 40 min. After boiling process, the final wort was filtered by muslin hop boiling bag. The final hopped wort (around 11 °Bx) was cooled to 15 ºC using stainless wort chiller and immediately moved into 25 L steel tanks (Duisburg, Germany) for fermentation. 16 g of the Saflager S-23 (*S. pastorianus*) were prepared in 200 mL of water and the final wort mixture and added into the final wort in the 25 L steel tanks. The fermentation was conducted by two steps: the first fermentation was maintained at 10°C for 8 days and the second was at 12 °C for 4 days. Afterwards, the final beer was filtered by centrifugation at 6,850 x*g* for 15 min. Finally, the beers were conditioned in the amber glass bottle at 4°C in the dark for 14 days before starting experiments. Scheme for brewing different types of beers was illustrated in **Figure** 4.

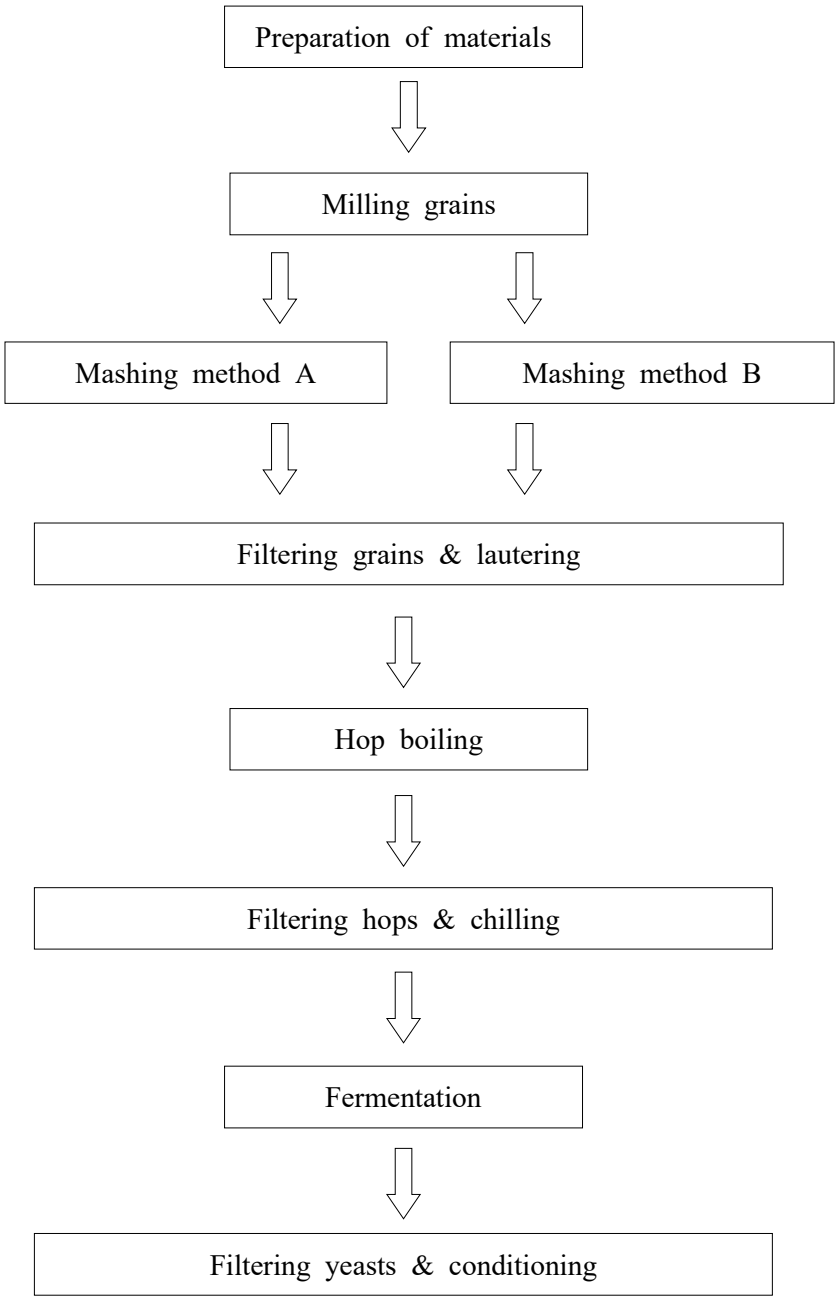


Figure 4. Brewing process

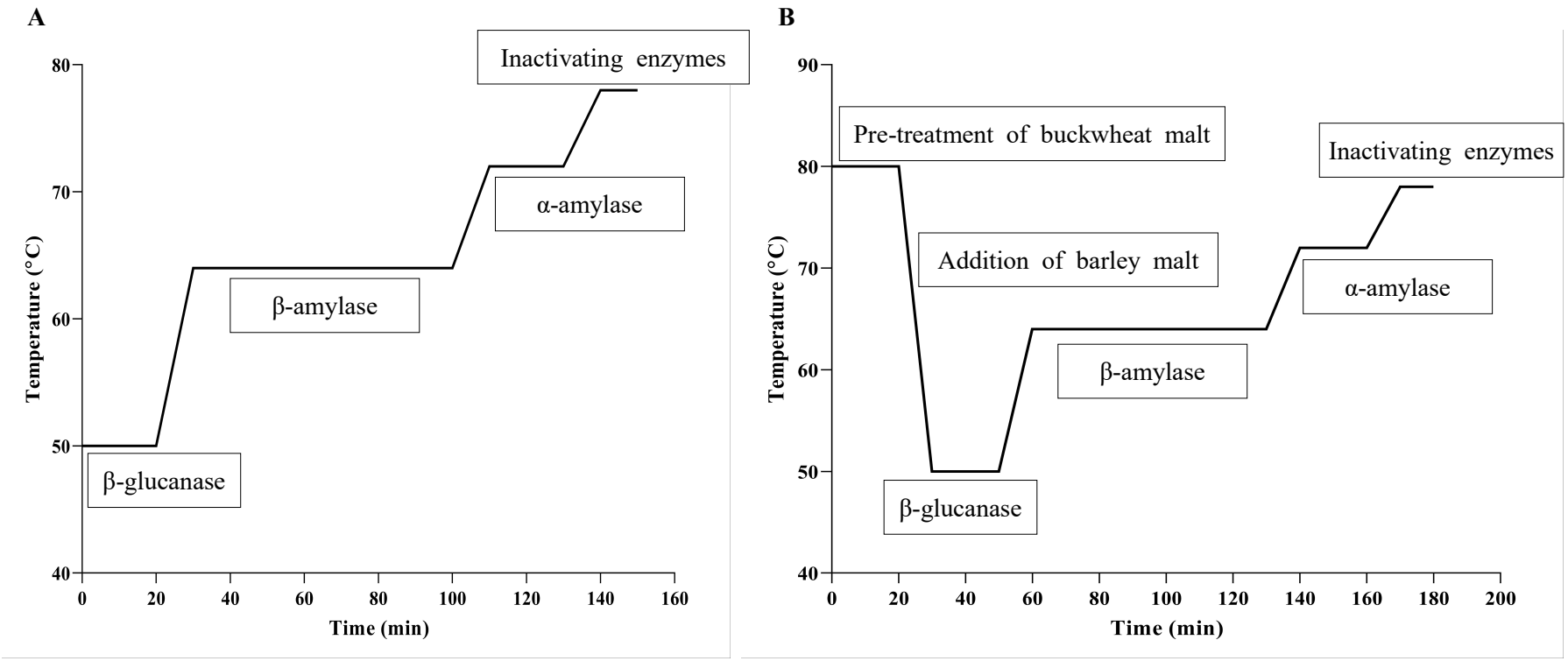


Figure 5. Mashing process

A: Common method

B: Modified method

3. Determination of rutin and quercetin in the final beer

One gram of tartary buckwheat and common buckwheat were extracted with 10 mL of 70% (v/v) ethanol for 60 min and diluted by LC-grade methanol. Final beers were mixed with the same volume of distilled water and diluted by LC-grade methanol. All samples were filtered using a Merck millipore 0.2 μm membrane syringe filter (Burlington, MA, USA). Then 1 μL of each sample was injected into UPLC-MS, Waters H-class equipped with QDa detector (Milford, MA, USA) on a 1.7-μm BEH C_{18} column (2.1 mm × 150 mm) to detect rutin and quercetin as described in our previous report [38] Mobile phase was progressed with solvent A (100% acetonitrile with 0.1% formic acid) and solvent B (100% triple distilled water with 0.1% formic acid). Waters QDa detector was used to quantify rutin and quercetin. Conditions for rutin and quercetin were set as electrospray ionization (ESI) positive for quercetin and negative for rutin. Capillary energy was 1.3 kV, and 10 V of cone voltage for quercetin and 20 V for rutin were prepared. The mass value of rutin and quercetin was 609.00 m/z and 303.00 m/z. The full scan of ions was ranged from 100 to 800 m/z in the positive and negative ion modes. Calibration curve for rutin and quercetin was set from 0.02 to 2.0 μg/mL (r^2 > 0.99). The following elution gradient was applied for rutin and quercetin analyses: the initial elution gradient was 5% A, and increased to 10% A at 0.5 min, 15% A at 2.1 min, 23% A at 10.0 min, 50% A at 12.0 min, 100.0% A at 15.1 min, back to the first gradient 5% A at 16.1 min and maintained until 20.0 min for equilibrium step.

Table 2. Determination of UPLC-QDa condition for rutin and quercetin

Compound	Concentration range (μg/mL)	M/Z	Corn voltage (V)	Linearity ($R^{2)}$	Regression equation
Rutin	0.02-2.0	609.00	20	0.996390	Y = 261000X-11000
Quercetin	0.02-2.0	303.00	10	0.997081	Y = 877000X-27900

4. Rutin-degrading enzyme activity

The activity of the rutin-degrading enzyme was evaluated by measuring produced quercetin by rutin-degrading enzyme in the tartary buckwheat [39]. The heat-treated tartary buckwheat powder (2 g) at 80°C for 20 min were extracted in 30 mL of 0.2 M acetate buffer (pH 4.0) at 4 °C for 3 h. After centrifugation at 9,605 x*g* for 15 min at 4 °C, the supernatant of each sample was collected and stored at 4 °C. Then, 1.6 mL of rutin solution (100 μg/mL) and 0.4 mL of the supernatant were mixed and incubated at 50 °C for 3 min. The enzymatic reaction was stopped by 8 mL of methanol. The produced quercetin content was used to check the activity of rutin-degrading enzyme. The quercetin content was measured using UPLC in the same method described above.

5. Total flavonoid contents

Total flavonoid content was measured by the aluminium chloride colorimetric assay [40]. 1 mL of beer samples or standard solutions of quercetin was mixed with 4 mL of distilled water. After that, 0.3 mL of 5% $NaNO_2$ was added and left for 5 minutes. Then, 0.3 mL of 10% $AlCl_3$ was added. After five minutes, 2 mL of 1 M NaOH solution was transferred into the mixture and the final volume (10 mL) was filled with distilled water. After 15 min, the final result was measured at 510 nm by spectramax M3. The total flavonoid content was evaluated as quercetin quivalent (QE) mg/L beer.

6. Determination of beer quality attributes

The original sugar content, alcohol content in the final beer, pH, and color of the degassed beers were measured using a DMA 4500 Density Analyzer and Alcolyzer Plus (Anton Paar, Austria). Bitterness of the final beer was measured by using International bitter units (IBU) described by Geisler and Weiß [41]. Briefly, degassed beer was mixed with isooctane and 6 N hydrochloric acid in the proportion of 20:1:40. The mixture was shaken at 150 rpm at 20 ℃ for 15 min and centrifuged at 1,351 x*g* for 3 min. The 100 μL of supernatant was transferred to 96-well plate and the absorbance was measured at 275 nm using spectramax M3. The final IBU was calculated by multiplying the measured absorbance with 50.

7. Determination of flavor compounds in the beer

Headspace gas chromatography (HS-GC) was carried out to measure diacetyl, acetaldehyde, ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl caprylate, propanol, isobutanol, and isoamyl alcohol and using the method described by Dong, et al. [42]. Briefly, flavor compounds in the beer was measured using a gas chromatograph (Clarus 580, GC System, Perkin Elmer, USA) equipped with a head-space sampler (HS 40, Perkin Elmer, USA). The detector was chosen a flame ionization detector (FID). 5 mL of beer samples were prepared in 20 mL HS-GC vials and heated in the head-space sampler at 60 ℃ for 15 min. The temperature of injection port was set 150 ℃ and transfer line was set 110°C each. The original oven temperature was maintained at 50°C for 3 min, and increased until 180 °C at a rate of 30°C/min. and then remained unchanged for 3 min. FID temperature was fixed at 250 ℃. For the analysis of diacetyl, electron capture detector (ECD) was used. 10 mL of beer samples were prepared in 20 mL HS-GC vials and heated in the head-space sampler at 68 ℃ for 20 min. The temperature of injection port and transfer line was set 110°C. The original oven temperature was maintained at 50°C for 3 min, and increased until 90°C at a rate of 45°C/min. Then, the temperature remained unchanged for 3 min. ECD temperature was fixed at 150 ℃.

8. Evaluation of oxidative stability of different types of beer

Evaluation of oxidative stability was conducted at forced-aging condition. The forced-aging condition was prepared at 40 ℃ in the thermodynamically controlled dark room. Determination of oxidative stability was performed by antioxidant capacity tests, DPPH and FRAP assay. DPPH assay was conducted using 2,2-diphenyl-1-picrylhydrazyl reagent described by Zhao et al. [43]. In short, 0.3 mL of each beer was mixed with 2.7 mL of 100 μM 2,2-diphenyl-1-picrylhydrazyl ethanol solution for 60 min at 25 ℃ in the dark room. After that, 100 μL of each sample was transferred to 96-well plate and the absorbance was measured at 517 nm using spectrophotometer. Trolox was used as a standard to determine antioxidant capacity of each sample. FRAP assay was performed according to the method described on He et al. [44]. Briefly, FRAP working solution was prepared by adding 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40mM HCl. 2.5 mL of TPTZ solution, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of 0.3 M acetate buffer (pH 3.6) were mixed before checking the antioxidant capacity of beer. After that, 0.1 mL of sample was added into the mixture solution and incubated for 10 min at 37 ℃. Finally, 100 μL of each sample was transferred to 96-well plate and the absorbance was measured at 593 nm using spectrophotometer. FRAP values were expressed as 1 mM Fe (II) solution for beer samples.

9. Statistical analysis

Result values are presented as mean ± standard deviation of three independent replicates. Statistical comparisons were made by one-way ANOVA using Tukey's comparison test and t-test. Results were considered to be significant when $p<0.05$

Results and discussion

1. Comparison of quercetin amount in differently treated tartary buckwheat malt

The activity of rutin-degrading enzyme was measured by using 100 μg/mL rutin solution and tartary buckwheat extracts. The amount of produced quercetin by rutin-degrading enzyme was determined by adding rutin-degrading enzyme into the rutin solution. Heated tartary buckwheat at the temperature of 80 ℃, which conventional mashing process starts, the amount of produced quercetin was 83.7% lower than untreated one. This result indicates the activity of rutin-degrading enzyme decreased at 80 ℃, so advanced mashing method B includes one more pre-treatment before beginning the mashing process at 50 ℃. The result was verified using UPLC-MS (**Figure 6**).

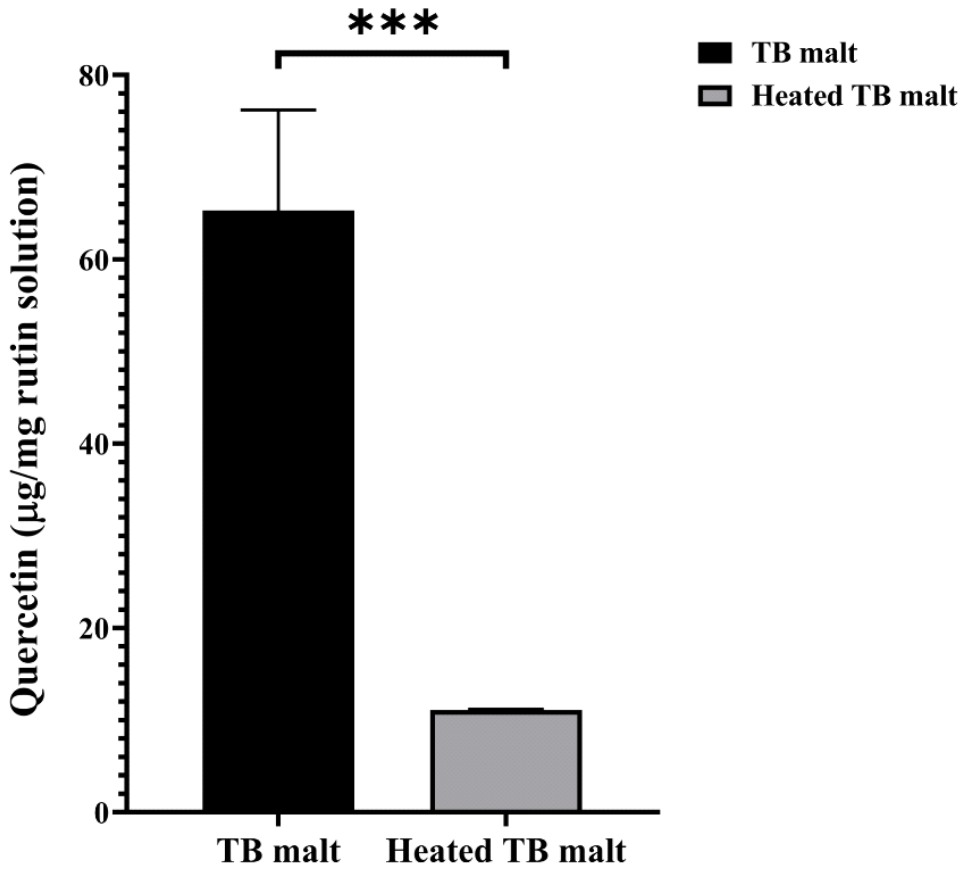


Figure 6. Comparison of quercetin amount in differently treated tartary buckwheat malt

* TB: Tartary buckwheat

2. Determination of rutin, quercetin, and total flavonoid content in different types of buckwheat and the final beers

The amount of rutin and quercetin was determined by UPLC-MS with QDa detector at m/z and m/z, respectively. Tartary buckwheat seed contained 51.9 times rutin and 16 times higher amount of quercetin than common buckwheat. After germination process, similar pattern was determined in rutin while quercetin content increased a lot. Specifically, tartary buckwheat malt contained 51.5 times higher amount of rutin and 256 times higher quercetin than common buckwheat (**Table** 3). This result is similar to our previous study by Lee et al. [38]. However, rutin content in 20% and 40% final buckwheat beer brewed by mashing method was lower than the final beer brewed with 100% barley malt, but quercetin content in both 20% and 40% buckwheat beer was 16.5 and 19.4 times higher than 100% barely malt beer (**Table** 4). This result indicates there was some changes in rutin content of used tartary buckwheat. Specifically, the activity of rutin-degrading enzyme is very strong at 50 ℃, which temperature is the first stage in common mashing process. Therefore, improved mashing method B involved one more pre-treatment to decrease rutin-degrading enzyme by heating tartary buckwheat 80 ℃ before starting common mashing process. The amount of rutin in 20% and 40% buckwheat wort brewed by mashing method B 23.4 mg/100 mL and 41.4 mg/100 mL, which is 68.8 and 63.7 times higher than worts brewed by mashing method B. Its amount in the final beer decreased to 21.0 mg/100 mL and 30.8 mg/100 mL during fermentation, but still 61.8 and 56.0 times higher than the final beer by mashing method A. Total flavonoid content determined using the aluminium chloride calorimetric assay also showed decreasing the activity of rutin-degrading enzyme is a key factor to

increase flavonoid content in the beer as well as preserving rutin [40]. Specifically, measured total flavonoid content in 20% and 40% buckwheat beer brewed by mashing method B 1,180.2 mg QE/L and 1,704.7 mg QE/L, respectively. This value is 2.23 and 1.99 times higher than 20% and 40% buckwheat beer using mashing method A. Overall, heating tartary buckwheat malt at 80 ℃ before beginning mashing process increased rutin content a lot and this also contributed to increase flavonoid content in the final beer.

Table 3. Contents of the content of rutin and quercetin in the grains

Samples	Rutin (mg/g)	Quercetin (mg/g)
Common buckwheat	0.21±0.001[a]	0.13±0.07[a]
Tartary buckwheat	10.9±0.11[c]	2.08±0.52[b]
Common buckwheat malt	0.13±0.07[a]	0.02±0.01[a]
Tartary buckwheat malt	6.70±0.03[b]	5.12±0.05[c]

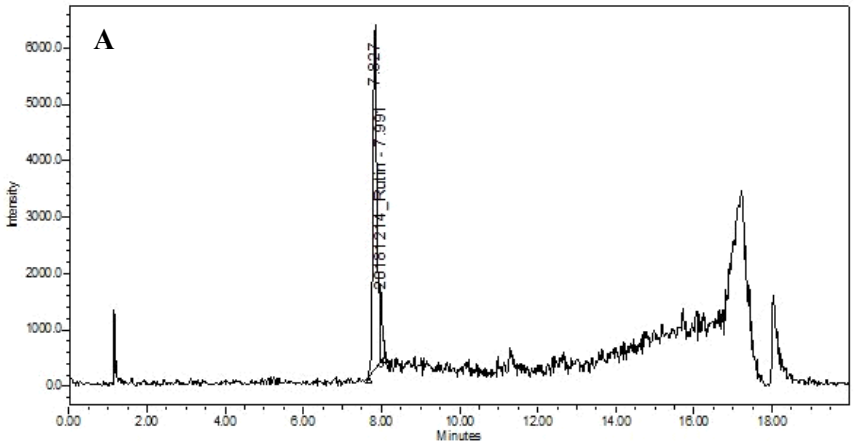
*: Results are the means ± standard deviations (n=3). Different letters following the numbers on the same line indicate means separation at $p < 0.05$.

Table 4. Rutin, quercetin, and total flavonoid contents in the buckwheat lager beer

Samples	Mashing conditions	Rutin (mg/100mL)	Quercetin (mg/100mL)	Total flavonoid (mg QE/L)
Control beer wort		0.58±0.02[a]	0.15±0.01[a]	294.75±15.42[a]
Control beer (final)		1.60±1.39[a]	0.13±0.01[a]	303.69±20.14[a]
20% buckwheat beer wort	Method A	0.34±0.02[a]	4.93±0.06[i]	516.75±28.19[b]
20% buckwheat beer (final)		0.34±0.01[a]	2.47±0.06[c]	530.75±19.13[b]
40% buckwheat beer wort		0.65±0.02[a]	4.39±0.06[h]	876.75±30.44[c]
40% buckwheat beer (final)		0.55±0.01[a]	2.62±0.04[d]	855.30±21.95[c]
20% buckwheat beer wort	Method B	23.40±0.10[c]	2.97±0.04[e]	1096.45±40.55[d]
20% buckwheat beer (final)		21.00±0.10[b]	1.82±0.04[b]	1180.23±34.06[d]
40% buckwheat beer wort		41.40±1.20[e]	3.91±0.05[g]	1758.46±38.98[e]
40% buckwheat beer (final)		30.80±0.50[d]	3.36±0.06[f]	1704.68±40.49[e]

*: Results are the means ± standard deviations (n=3). Different letters following the numbers on the same line indicate means separation at $p < 0.05$.

*This result was published “Brewing Rutin-Enriched Lager Beer with Buckwheat Malt as Adjuncts (2019)” in Journal of Microbiology and Biotechnology [45].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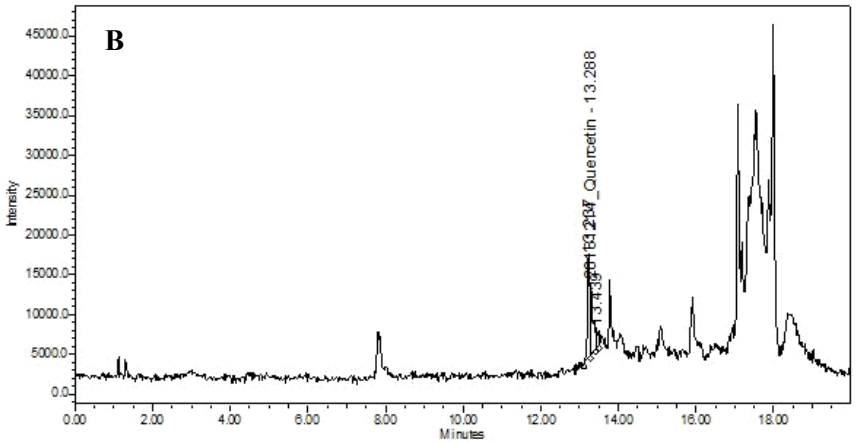
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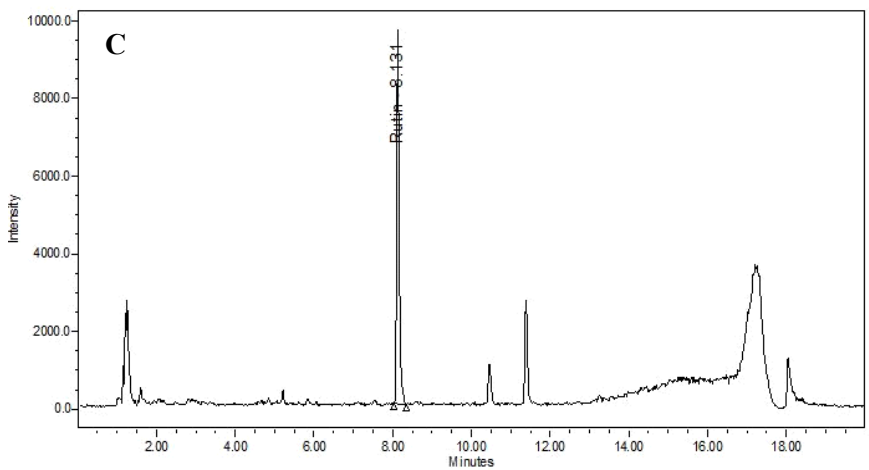
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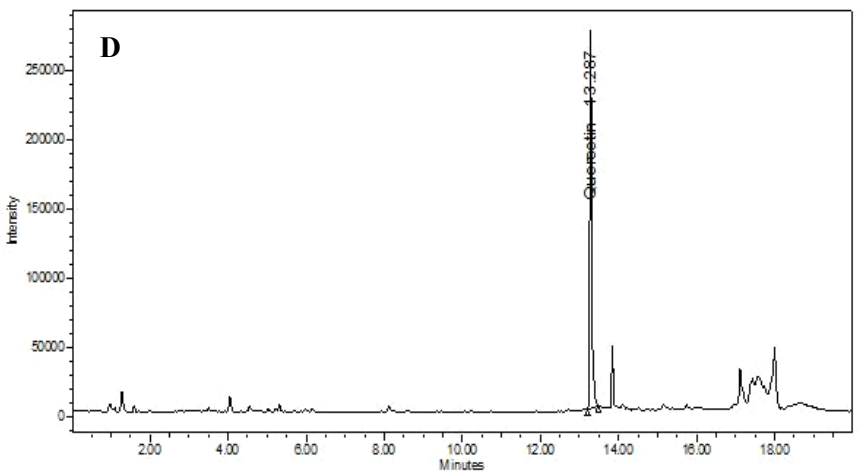
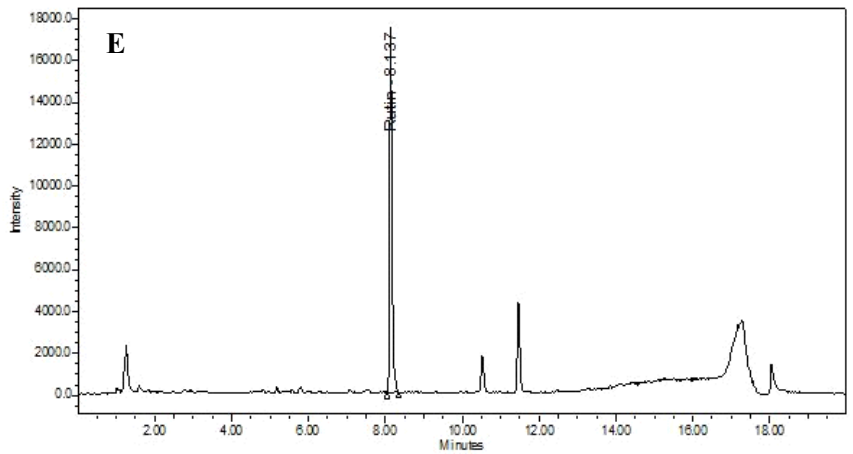
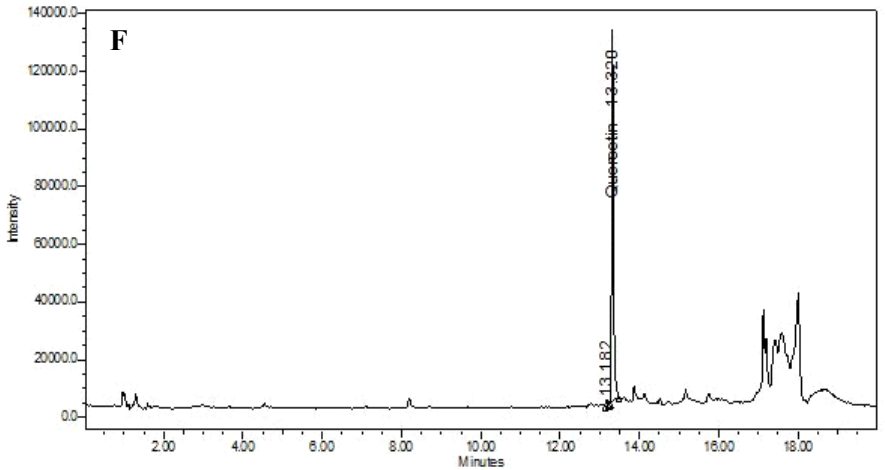
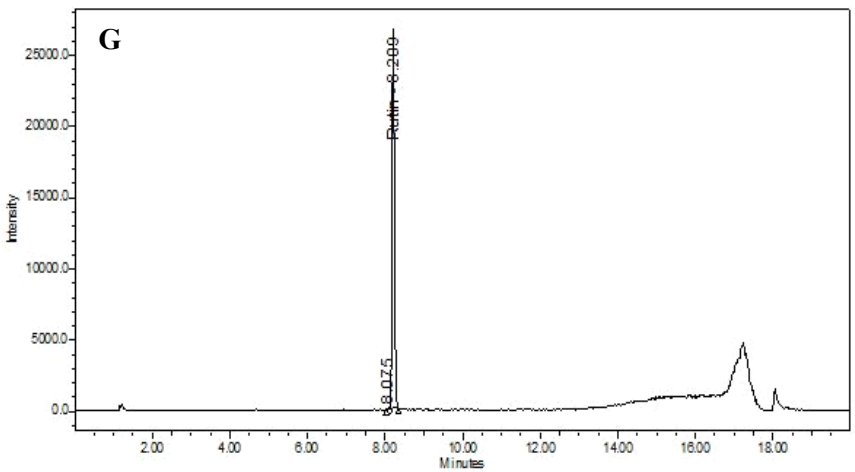
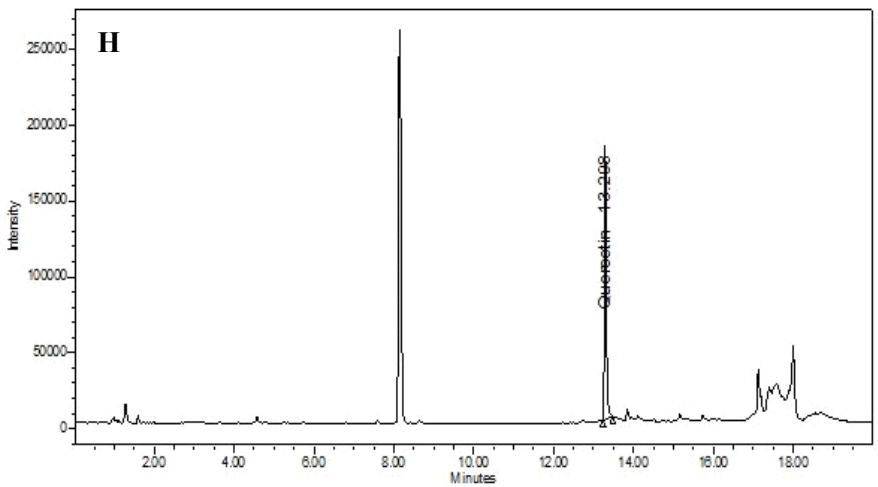
Minutes
Rutin - 8.131


D
Intensity
Minutes
Quercetin - 13.287


E
Rutin 8.137
Intensity
Minutes


F
Quercetin 13.320
13.182
Intensity
Minutes


G
Rutin 8.209
8.075
Intensity
Minutes


H
Quercetin 13.208
Intensity
Minutes

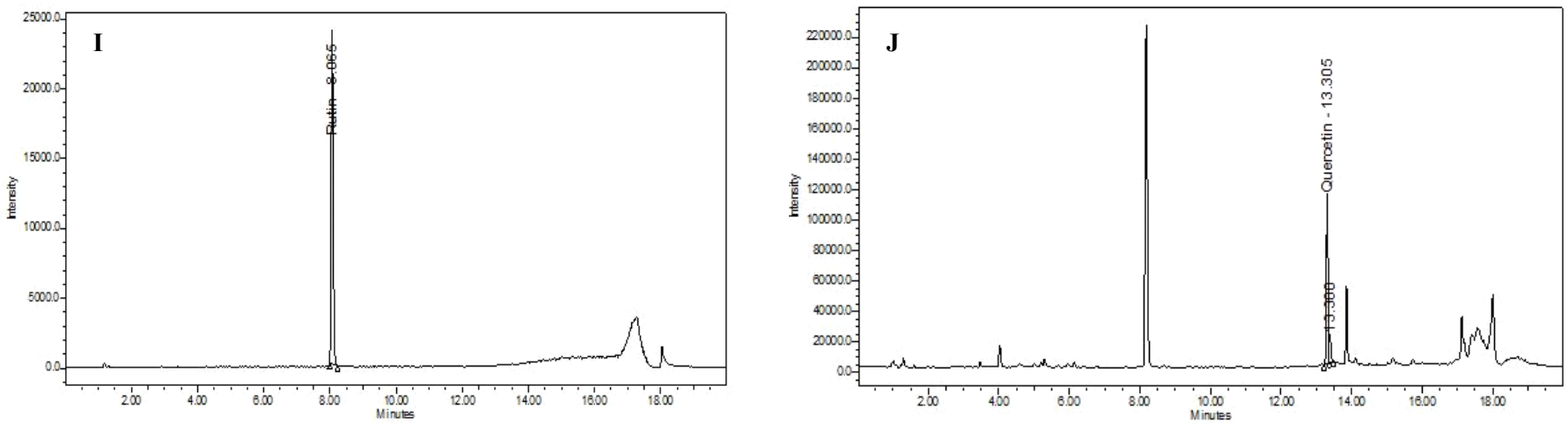


Figure 7. Chromatograms of rutin and quercetin in different types of beer

(A): rutin in 100% barley malt beer; (B): quercetin in 100% barley malt beer;

(C): rutin in 20% b. malt beer (method A); (D): quercetin in 20% b. malt beer (method A); (E): rutin in 40% b. malt beer (method A); (F) quercetin in 40% b. malt beer (method A); (G): rutin in 20% b. malt beer (method B); (H): quercetin in 20% b. malt beer (method B); (I): rutin in 40% b. malt beer (method B); (J): quercetin in 40% b. malt beer (method B)

3. Beer quality attributes in the beer

Beer attributes including sugar content in the wort and the final beer, ethanol content, pH, color, and bitterness were measured using DMA 4500 density analyser and Alcolyzer Plus (**Table** 5). Original sugar content before starting fermentation ranges from 11.05 to 11.10 °Bx. Ethanol content of each beer was also in similar range (4.5-4.7%) regardless of buckwheat proportion and mashing method. Bitterness expressed as international bitterness unit was also similar among five types of beer. The color of beer containing 100% barley malt was measured 3.9 EBC while its value increased up to 5.1 EBC in beer with 40% buckwheat malt, especially its value was highly correlated with rutin and quercetin content of beer [41].

Table 5. Beer quality attributes in the buckwheat lager beer

Properties	Control beer	20% buckwheat beer (method A)	20% buckwheat beer (method B)	40% buckwheat beer (method A)	40% buckwheat beer (method B)
Wort sugar (°Bx)	11.06±0.03[a]	11.10±0.03[a]	11.08±0.02[a]	11.05±0.02[a]	11.09±0.02[a]
Ethanol content (% v/v)	4.6±0.1[a]	4.7±0.1[a]	4.6±0.0[a]	4.5±0.1[a]	4.7±0.0[a]
pH	4.43±0.04[a]	4.46±0.07[a]	4.44±0.05[a]	4.45±0.04[a]	4.46±0.05[a]
Color (EBC)	3.9±0.0[a]	4.5±0.1[b]	4.4±0.1[b]	5.0±0.2[c]	5.1±0.2[c]
Bitterness (IBU)	10.25±0.12[a]	11.33±0.09[b]	11.34±0.11[b]	11.37±0.10[b]	11.42±0.09[b]

[*]: Results are the means ± standard deviations (n=3). Different letters following the numbers on the same line indicate means separation at $p < 0.05$.

*This result was published “Brewing Rutin-Enriched Lager Beer with Buckwheat Malt as Adjuncts (2019)” in Journal of Microbiology and Biotechnology [45].

4. Flavor compounds in different types of beer

The flavor compounds in the final beer were determined using headspace gas chromatography. The flavor threshold of diacetyl, which is known for buttery, is 100-200 μg/L [24]. When its concentration is higher than this standard, it could lower beer quality by providing undesirable buttery flavor to the beer. Diacetyl content in different types of beer brewed by both mashing method A and B was measured from 80.9 μg/L to 86.7 μg/L. This result shows 40% buckwheat malt of total proportion did not affect buttery flavor in the beer. In case of acetaldehyde, its high concentration above 25 mg/L in the beer is known for unpleasant to consumers [25]. In the final beer including 20% and 40% buckwheat malt, any value did not exceed it. Higher alcohol affects the alcohol and solvent-like aroma in the beer. Higher alcohol content including isoamyl alcohol, propanol, and isobutanol of each beer was also similar. Specifically, the sum of higher alcohol of each beer was detected 72.03 mg/L to 75.3 mg/L. More than 20% of isobutanol in sum of propanol, isobutanol, and isoamyl alcohol causes undesirable effects in the beer. In the final beer brewed by both mashing method A, isobutanol proportion was measured 14.02%, 13.45%, and 13.37%. Similarly, 20% and 40% buckwheat beer by using mashing method B also contained 13.44% and 13.40% of isobutanol proportion. This result indicates buckwheat beer did not affect unpleasant aroma related to higher alcohol in the final beer. Esters in the beer mainly include ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl caprylate. Isoamyl acetate, which flavor is banana-like fruity, was increasing while the proportion of tartary buckwheat malt increased. Furthermore, isoamyl acetate content in the 20% and 40% tartary buckwheat beer using mashing method B was measured 1.43 mg/L and 1.58 mg/L, and this was 30% and 41%

higher than 20% and 40% buckwheat beer brewed by mashing method A. Ethyl acetate also increased depending the proportion of tartary buckwheat while other esters were similar in the final beer. Measured ethyl hexanoate was below threshold [21, 23]. Overall, buckwheat malt did not affect undesirable alcohol or solvent flavor in the beer. In the meantime, tartary buckwheat malt can be a good factor to intensify fruity flavor like isoamyl acetate and ethyl acetate in the beer. The result is shown in below (**Table** 6).

Table 6. Flavor compounds in the buckwheat lager beer

Properties	Flavor description	Control beer	20% buckwheat beer (method A)	20% buckwheat beer (method B)	40% buckwheat beer (method A)	40% buckwheat beer (method B)
Diacetyl (μg/L)	Buttery, butterscotch [21, 24]	83.4±2.5[a]	85.7±1.8[a]	80.9±2.1[a]	86.7±1.9[a]	84.4±2.0[a]
Acetaldehyde (mg/L)	Green apple, grassy [21, 25]	6.75±0.54[a]	7.12±0.33[a]	6.42±0.50[a]	7.01±0.46[a]	6.88±0.38[a]
Ethyl acetate (mg/L)	Fruity, sweetish [21, 23]	12.18±0.31[a]	13.90±0.22[b]	14.02±0.34[b]	14.69±0.26[c]	14.73±0.30[c]
Isoamyl acetate (mg/L)	Banana, sweet [23]	0.99±0.15[a]	1.10±0.12[ab]	1.12±0.24[ab]	1.43±0.21[bc]	1.58±0.25[c]
Ethyl hexanoate (mg/L)	Apple, fruity [23]	0.17±0.04[a]	0.13±0.05[a]	0.14±0.06[a]	0.13±0.05[a]	0.12±0.04[a]
Ethyl caprylate (mg/L)	Sour [26]	0.19±0.08[a]	0.16±0.07[a]	0.17±0.09[a]	0.16±0.07[a]	0.15±0.08[a]
Propanol (mg/L)	Alcohol [21]	8.12±0.31[a]	9.15±0.18[b]	8.84±0.19[c]	9.11±0.22[bc]	9.07±0.24[bc]
Isobutanol (mg/L)	Alcohol [21]	10.10±0.15[a]	9.99±0.12[a]	10.05±0.31[a]	10.12±0.10[a]	10.09±0.11[a]
Isoamyl alcohol (mg/L)	Alcohol, medicinal [21, 26]	53.81±0.40[a]	55.12±0.41[b]	56.25±0.40[b]	56.07±0.25[b]	56.10±0.28[b]

*: Results are the means ± standard deviations (n=3). Different letters following the numbers on the same line indicate means separation at $p < 0.05$.

*This result was published “Brewing Rutin-Enriched Lager Beer with Buckwheat Malt as Adjuncts (2019)” in Journal of Microbiology and Biotechnology [45].

5. Determination of oxidative stability of different types of beer

Oxidation of the beer can affect the taste and flavor in the final beer. Undesirable flavor compound like trans-2-nonenal (T2N) could happen in the oxidized beer, and aroma and taste can deteriorated while bitter compounds are degraded [47-49]. Rutin, which is a main component in tartary buckwheat, is well-known as a free radical scavengering activity [50]. In the previous result, we checked different mashing method affected rutin content in the final beer. In addition to rutin content in the final beer, determination of oxidative stability could verify the benefit of tartary buckwheat as an adjunct for brewing beer. Rutin content was quitely different depending on mashing method, so antioxidant capacity tests by DPPH and FRAP assay were conducted to check how tartary buckwheat malt as a replacement for the barley malt and different mashing process affected the oxidative stability of the final beer. Oxidative stability tests were conducted using the final beer day 0, 3, 6, 9, 12, and 15 day. In the forced-aging condition, DPPH result indicated beer containing tartary buckwheat malt has intensified oxidative stability. Furthermore, beer brewed by mashing method B had a higher antioxidant capacity compared to mashing method A. Specifically, DPPH antioxidant capacity of 40% tartary buckwheat beer in 0 day was measured 3.19 mmol TE/L, and this value is 4.83 times higher than that of 100% barley malt beer. Evaluation result showed beers containing buckwheat malt had more intensified antioxidant capacity than reference barley malt beer in all measured section (**Figure** 4). Overall, antioxidant capacity of each beer decreased rapidly until forced-aging 9^{th} day. After forced-aging 12^{th} day, antioxidant capacity was stable. Similar result was determined by FRAP assay.

Overall, antioxidant capacity was high in beers containing high rutin

and quercetin content. This result is corresponding to previous research that rutin is a strong radical scavenger [49]. However, buckwheat beer brewed by mashing method A had higher antioxidant capacity value than 100% barley malt beer even though rutin content was lower. This can be related to total flavonoid content difference among beers. According to the previous research by Panche et al., flavonoid acts as a protector for the body against reactive oxygen species [50]. This is possible because flavonoid contains high reactive hydroxyl group and it can react with free radical. Free radical is stabilized by flavonoid and results in more stable and less reactive radical.

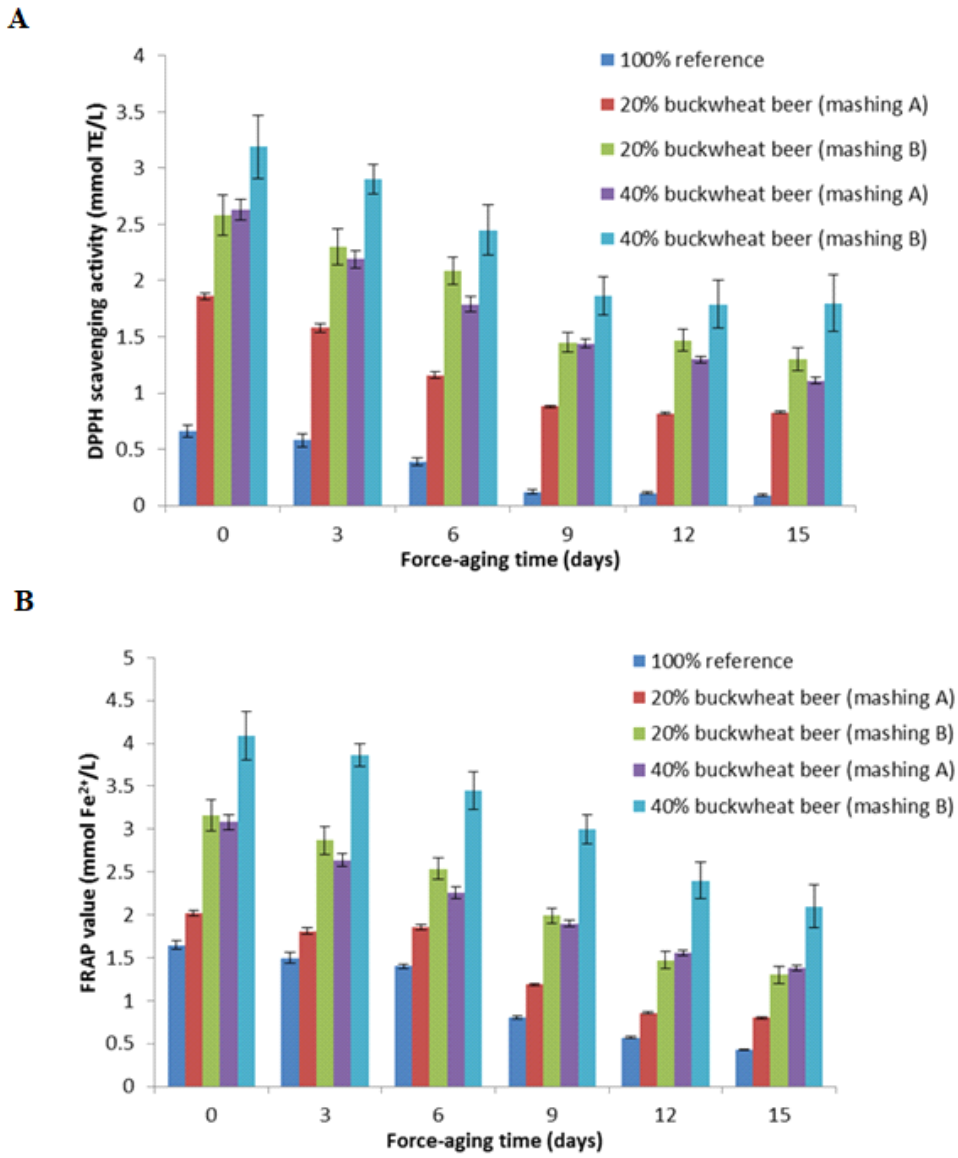


Figure 8. Antioxidant capacity of beer

A: DPPH scavengering activity of each beer;

B: FRAP value of each beer

*This result was published "Brewing Rutin-Enriched Lager Beer with Buckwheat Malt as Adjuncts (2019)" in Journal of Microbiology and Biotechnology [45].

Conclusions

In this research, tartary buckwheat was used in brewing beer as an adjunct with barley malt, specifically 20% and 40% proportion of total used grain. However, common mashing method was not adequate to extract functional compounds (rutin and quercetin) from tartary buckwheat because of the presence of rutin-degrading enzyme. Improved mashing method focused on decreasing the activity of rutin-degrading enzyme, and the final beer using this method increased rutin content 60 times than common method. In addition to increased rutin content, enhanced mashing method was more helpful to make the final beer resistant to oxidative stress. The beer quality attributes including sugar, ethanol, pH, and IBU were stable in beers containing tartary buckwheat. Furthermore, both mashing method did not make undesirable flavor nor unpleasant aroma in the final beer. In the meantime, enhanced brewing method with tartary buckwheat malt can be a good factor to intensify fruity flavor by providing rich aroma compounds in the beer. Consequently, tartary buckwheat in enhanced brewing method could be helpful in functional properties as well as flavor and tastes.

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Abstract in Korean

메밀은 여러 연구에서 맥주를 주조하는 재료로 사용되어왔다. 하지만, 맥주를 주조하는 동안 유실될 수 있는 루틴함량에 초점을 맞춘 연구는 거의 존재하지 않았다. 본 연구는 루틴분해효소가 일반적인 방법에 따라 주조된 메밀맥주에서 루틴함량을 감소시키는 원인임을 확인하였다. 개선된 주조법은 루틴분해효소를 약화시키는 과정을 통해 일반적인 주조법에 비해 맥주 내 루틴함량을 60배만큼 더 증가시켰다. 총 플라보노이드 함량 역시 개선된 방법으로 주조된 맥주에서 1.99배 향상되었음을 확인하였다. 유형별 맥주를 대상으로 한 항산화력 측정역시도 개선된 방법이 산화안정성을 유지하는데 더 적합함을 보여주었다. 메밀을 함유한 맥주는 일정 수준 이상의 함량이 맥주에 포함되었을 때 불쾌한 향을 유발할 수 있는 다이아세틸과 아세트알데히드와 같은 성분이 그 기준치보다 낮게 나타났다. 하지만, 맥주에 풍부한 과일향을 더해줄 수 있는 성분은 메밀의 함량과 담금과정의 차이로 그 함량을 더 증진시킬 수 있었다. 맥주를 주조하는데 있어 40%까지의 맥아를 메밀로 대체하였을 때 맥주의 주요 특성인 알코올 함량과 맥즙의 당도에 어떠한 악영향도 미치지 않았다. 전반적으로, 맥주의 주조에 이용되는 맥아의 일정 비율을 메밀로 대체하는 것은 맥주의 기능적인 부분을 향상시킬 뿐만 아니라 맥주의 맛, 향, 알코올 함량, 당도와 같은 특성 부분에서 모두 수용할 수 있는 수준임을 본 연구를 통해 확인할 수 있었다.

주요어 : 메밀, 루틴, 루틴분해효소, 항산화력, 물리화학적 성질

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Juho Lim,

Upon your request, we will permit the reproduction of "Brewing Rutin-Enriched Lager Beer with Buckwheat Malt as Adjuncts" from Journal of Microbiology and Biotechnology 29(6): 877-886, 2019 in Development of brewing functional beers and studies on the biochemical properties in Graduate School of International Agricultural Technology to be published by Seoul National University.

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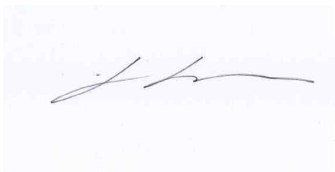

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