



Title	Metabolic Profiling and Mass Spectrometry Imaging of Rice Koji During the Extended Koji-Making Process
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22 During sake brewing, rice *koji* produces saccharification enzymes that play crucial roles in flavor
23 development. In this study, we analyzed the metabolic changes in rice *koji* across different *koji*-making
24 durations and clarified the characteristics of *hine koji* prepared via a longer *koji*-making period than
25 usual. Profiling of hydrophilic low-molecular-weight components using gas chromatography/mass
26 spectrometry (GC/MS) revealed that several components, including erythritol, increased with
27 extended *koji*-making time. Quantitative analyses via high-performance liquid chromatography
28 (HPLC) showed that glucose levels plateaued after a certain point, whereas erythritol levels increased
29 continuously. Furthermore, mass spectrometry imaging (MSI) revealed a uniform distribution of
30 glucose throughout rice *koji*, whereas erythritol was localized at the periphery and slightly inside the
31 rice *koji*. This study provides significant insights into the *koji-making* process, offering a valuable tool
32 for optimizing *koji*-making procedures and improving sake quality.

33

34 **Keywords:** metabolic profiling, HPLC, mass spectrometry imaging, rice *koji*, erythritol

35

36 **1. Introduction**

37 Sake is a traditional Japanese alcoholic beverage produced via a parallel double fermentation process,
38 in which enzymes produced by *Aspergillus oryzae* (*A. oryzae*) saccharify steamed white rice, and yeast
39 (*Saccharomyces cerevisiae*, *S. cerevisiae*) carries out alcoholic fermentation¹. The inclusion of
40 Japanese cuisine in the UNESCO Intangible Cultural Heritage list in 2013 triggered a global boom in
41 Japanese food, which in turn increased international interest in sake². Furthermore, on December 4,
42 2024, "Traditional knowledge and skills of sake-making with *koji* mold in Japan" was registered as a
43 UNESCO Intangible Cultural Heritage. Hence, the global demand for sake is expected to continue
44 growing.

45 The brewing process for sake begins with rice polishing to remove the outer layer of brown rice,
46 followed by washing, soaking, and steaming of the resulting white rice. The steamed rice is then
47 inoculated with *koji* mold to produce rice *koji*. This is then mixed with steamed rice, water, lactic acid,
48 and yeast to prepare a starter mash. After the fermentation of the starter mash, additional water, rice
49 *koji*, and steamed rice are added in three stages via a process called "three-step preparation for
50 fermentation mash". Fermentation continues for several weeks, after which the fermented mash is
51 pressed and separated into sake and sake cakes. The resulting sake is subjected to sedimentation,
52 filtration, and pasteurization prior to shipping^{3, 4}.

53 In sake brewing, rice *koji* is an important element that determines the quality of sake, as reflected

54 in the saying: "The first step is making *koji*; the second is making starter mash, and the third is brewing
55 (mash)"⁴. The main functions of rice *koji* include promoting yeast fermentation by producing enzymes
56 that break down starch into sugars and enhancing the sake flavor and bioactivity by generating low-
57 molecular-weight compounds through enzymatic action^{4, 5}. A key visible feature of rice *koji* is *haze*,
58 which is the extension of *koji* mold mycelia across the surface and interior of rice. The extend of
59 internal penetration of *koji* mold into steamed rice is called *haze-komi*^{6, 7}. Rice *koji* is typically prepared
60 over 40–45 h⁸. However, cultivated for longer durations under high humidity promotes excessive *koji*
61 propagation, producing *hine koji*⁹. While several studies have examined the effects of *koji*-making
62 temperature and time on the enzymatic activity of rice *koji*^{10,11}, the compositional characteristics of
63 *hine koji* remain poorly understood. Therefore, clarifying the relationship between *koji*-making time
64 and the composition of rice *koji*—including that of *hine koji*—can contribute to diversified and
65 controllable sake flavor profiles.

66 In this study, rice *koji* samples produced over different *koji*-making periods, including *hine koji*,
67 were subjected to a multifaceted analysis encompassing fungal growth, enzymatic activities, and the
68 dynamics of flavor components, including their spatial distribution. Through this approach, we aimed
69 to obtain insights that would help elucidate the characteristics of *hine koji* and deepen the
70 understanding of the *koji*-making process.

71 Three analytical approaches were employed in this study: gas chromatography/mass

72 spectrometry (GC/MS) for profiling the hydrophilic low-molecular-weight components, high-
73 performance liquid chromatography (HPLC) for quantification of metabolites, and mass spectrometry
74 imaging (MSI) for visualization of metabolite distribution.

75 GC/MS is an excellent analytical method for the separation, identification, and quantification of
76 volatile and semi-volatile compounds. After sample evaporation and separation, ionization,
77 fragmentation, and detection were performed using a mass spectrometer, and the detected compounds
78 were identified by comparison with a library¹². GC/MS, which widely used in food science, enables
79 semi-quantitative and simultaneous analysis of various hydrophilic low-molecular-weight compounds
80 such as sugars, sugar alcohols, amino acids, and organic acids (flavor components of foods) via
81 derivatization^{1, 13-16}. MSI offers visualization of the two-dimensional distribution of compounds by
82 directly ionizing molecules in tissue sections and obtaining molecular information using MS¹⁷⁻¹⁹. MSI
83 does not require labeling or staining reactions; it allows chemoselective, multiplex imaging. Previous
84 MSI studies have revealed the distribution of sugars and sugar alcohols in rice *koji*²⁰. In this study, we
85 used surface-assisted laser desorption/ionization (SALDI)-MSI, in which inorganic nanoparticles
86 absorb laser energy and indirectly desorb or ionize sample molecules²¹. High-performance liquid
87 chromatography (HPLC) is an analytical technique that uses a liquid as the mobile phase to efficiently
88 separate target compounds. HPLC can be used in combination with various detectors for quantitative
89 analysis²².

90 In this study, we measured the mycelial content and enzymatic activity in various rice *koji*
91 samples, including *hine koji*, prepared via various *koji*-making times, and profiled the hydrophilic low-
92 molecular-weight components via GC/MS. Furthermore, the component that increased with increasing
93 *koji*-making time was quantitatively analyzed by HPLC, and the distribution of the component was
94 visualized by SALDI-MSI.

95 **2. Materials and methods**

96 **2.1. Samples**

97 In this study, *Nipponbare* white rice (2023 harvest, Yamaguchi Prefecture) with a polishing rate of
98 75% was used. Seed *koji* for sake (*A. oryzae*, for machine) was purchased from Biok Co., Ltd. (Aichi,
99 Japan).

100 **2.2. Chemicals**

101 Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dodecahydrate, potassium
102 tetraborate tetrahydrate, hydrochloric acid, *p*-dimethylaminobenzaldehyde, *N*-acetyl-D-glucosamine,
103 acetic acid, sodium acetate trihydrate, sodium chloride, methanol (guaranteed reagent), ribitol,
104 distilled water, pyridine, and d-glucose were purchased from FUJIFILM Wako Pure Chemical
105 Corporation (Osaka, Japan). Yatalase was purchased from Takara Bio Inc. (Shiga, Japan). α -amylase
106 and glucose-forming activity assay kits were purchased from Kikkoman Biochemifa Company (Tokyo,
107 Japan). Chloroform and methoxyamine hydrochloride were purchased from Merck Ltd. (Tokyo,
108 Japan). *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) was purchased from GL Sciences Inc.

(Tokyo, Japan). The alkane mixtures (C8-C20 and C21-C40) used for the retention index calculations were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Meso-erythritol and sodium hydrogen carbonate (NaHCO_3), sodium carbonate trihydrate (Na_2CO_3), and sodium acetate (CH_3COONa) used for mass spectrometry imaging were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Acetonitrile (HPLC grade) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Methanol and chloroform were purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). Ultrapure water was prepared using an ultrapure water production equipment (GenPure UV-TOC $\times$ CAD PLUS, Thermo Fisher Scientific, Waltham, MA, US).

2.3. *Koji*-making test

White rice was washed for 2 min and then soaked in water at 10 °C for 2 h to absorb water. The white rice had a water absorption of 26.3%. The rice was then steamed for 45 min and cooled to 37 °C. The steamed rice (100 g) was inoculated with 20 mg of seed *koji* mold. The water absorption of the steamed rice was 31.1%. *Koji* production was performed in a constant-temperature/humidity apparatus (SH-221, ESPEC Corp., Osaka, Japan) without airflow, as described by Ito *et al.*²³, under the conditions described in Table S1. Samples with a *koji*-making time of 45 h were used as controls; *hine koji* was prepared at a temperature of 42 °C and a humidity of 90% by extending the *koji*-making time to 55 and 65 h. *Kirikaeshi* (rebreaking up) was performed 22 h after the start of *koji*-making. Rice *koji* was further stirred at 27 and 31 h to make it homogeneous. In addition to the rice *koji* produced at 45, 55,

127 and 65 h, samples were also taken at intermediate stages (22, 27, and 31 h). The prepared rice *koji* was
128 stored at -80°C until analysis.

129 **2.4. Measurement of mycelial content**

130 The mycelial content in the rice *koji* was measured following the method described by Wisman *et al.*⁶,
131 with reference to Fujii *et al.*²⁴. The mycelial content was calculated based on the assumption that 1 mg
132 of mycelium contains 139 μg of *N*-acetyl-D-glucosamine.

133 **2.5. Measurement of α -amylase and glucose-forming activity**

134 A portion of a 10-mM acetate buffer (50 mL, pH 5.0) containing 0.5% sodium chloride was added to
135 10 g of rice *koji*, followed by leaching at room temperature ($12-20^{\circ}\text{C}$) for 3 h with occasional shaking.
136 The obtained extract was then filtered to obtain the final extract, and the α -amylase and glucose-
137 forming activities were measured using an α -amylase assay kit and a glucose-forming activity assay
138 kit, respectively. The α -amylase and glucose-forming activities were converted to values according to
139 the 4th Revised Official Analytical Method by the National Tax Agency of Japan^{25, 26}.

140 **2.6. Sample pretreatment for GC/MS analysis**

141 Rice *koji* was freeze-dried for 24 h in a freeze-dryer (FDU-2100; Tokyo Rikakikai Co., Ltd., Tokyo,
142 Japan), followed by grinding at 2000 rpm for 30 s using a multi-bead shocker (Yasui Scientific
143 Instruments Co., Ltd., Osaka, Japan). Each sample (30 mg) was placed into a 2.0-mL microtube
144 containing 1000 μL of a methanol/water/chloroform mixture = 5:2:2 (volume ratio) and mixed well

145 with a vortex mixer. A ribitol solution (60 μ L) prepared at 0.2 mg/mL was added as an internal standard,
146 followed by vortexing and shaking at 37 °C and 1200 rpm for 30 min using a heated shaker
147 (Thermomixer comfort, Eppendorf). The samples were then centrifuged at 12,300 $\times$ g for 3 min. The
148 supernatant (900 μ L) was mixed with 400 μ L of distilled water and centrifuged at 12,300 $\times$ g for 3 min.
149 The aqueous layer of the supernatant (400 μ L) was concentrated via centrifugation for approximately
150 2 h using a centrifugal concentrator EC-57CS (Sakuma Seisakusho Co., Ltd., Tokyo, Japan) and
151 lyophilized overnight.

152 The lyophilized sample was mixed with 100 μ L of methoxyamine hydrochloride solution
153 dissolved in 20 mg/mL pyridine, and methoxylation was performed by shaking at 30 °C, 1200 rpm for
154 90 min in a heated shaker. Trimethylsilylation was performed by adding MSTFA upon mixing and
155 shaking in a heated shaker at 37 °C and 1200 rpm for 30 min. Upon completion of the reaction, the
156 entire solution was transferred to a vial for GC/MS analysis.

157 **2.7. GC/MS analysis**

158 The GC/MS analysis was performed using a GCMS-QP2010 Ultra instrument (Shimadzu Corporation,
159 Kyoto, Japan) under the analytical conditions presented in Table S2.

160 **2.8. Data processing and analysis of GC/MS**

161 The raw data (AIA format) obtained from the GC/MS analysis (ver. 2.72, Shimadzu Corporation) was
162 submitted to MetAlign for peak detection and alignment²⁷, and the output files in the comma-separated

163 value (CSV) format were submitted to AIOutput. The retention index (RI) and mass spectrum
164 information of the detected peaks were matched with the information in the in-house library to
165 annotate the compounds for each peak²⁸, apart from α -D-glucosylglycerol, which was annotated by
166 reference to literature^{1,29}. The ionic intensity of the quantitative m/z of each compound was divided
167 by the ionic intensity of the quantitative m/z of the internal standard ribitol to obtain the relative
168 quantitative value.

169 An SIMCA-P Ver. 13 instrument (Umetrics AB, Umeå, Sweden) was used for a principal
170 component analysis (PCA) and an orthogonal partial least squares (OPLS) regression analysis, without
171 data transformation. Auto-scaling was used as the scaling method. In the OPLS regression analysis,
172 an OPLS regression model was constructed using the profiles of hydrophilic low-molecular-weight
173 components in rice *koji* as explanatory variables, with *koji*-making time as the response variable.
174 Components that varied with the *koji*-making time were extracted using the variable importance in
175 projection (VIP) values, which indicate the magnitude of the contribution to the predictive model
176 construction.

177 **2.9. Sample pretreatment for HPLC analysis**

178 Rice *koji* was frozen in liquid nitrogen and crushed several times at 2500 rpm for 5 s using a multi-
179 bead shocker. Each sample (30 mg) was transferred to a 2.0-mL tube, and 560 μ L of acetonitrile
180 together with 240 μ L of ultrapure water were added, followed by vortexing for 10 s, sonication for 30

181 min, and centrifugation at 9390 ×g, 4 °C for 10 min. In a new tube, 360 µL of ultrapure water, 720 µL
182 of the supernatant, and 720 µL of chloroform were added sequentially, followed by vortexing for 10 s
183 and centrifugation at 9390 ×g for 10 min at 4 °C. The supernatant (500 µL) was collected and
184 centrifuged in a 0.2-µm Syringe Driven Filter Unit (Merck Ltd., Darmstadt, Germany); 100 µL of the
185 final supernatant was transferred to a vial.

186 **2.10. HPLC analysis**

187 Glucose and erythritol were detected using an ELSD-LT III evaporative light-scattering detector
188 (Shimadzu Corporation), and their contents were determined from the peak area values using a Nexera
189 XS inert HPLC system (Shimadzu Corporation) equipped with a HILICpak VG-25 4D column (150
190 mm × 4.6 mm I.D., 2.5 µm). A quantitative analysis was performed using an external standard. The
191 analytical conditions are listed in Table S3. The injection volume was 10 µL, and the column
192 temperature was maintained at 40 °C. The mobile phase consisted of 70% acetonitrile at a flow rate of
193 0.4 mL/min under isocratic elution.

194 **2.11. Sample preparation of rice *koji* for mass spectrometry imaging**

195 For sectioning, rice *koji* was embedded in a 4% carboxymethylcellulose (CMC) solution and frozen
196 at −80 °C for 20 min. Frozen sections were then prepared at −20 °C using a cryomicrotome (CM1950,
197 Leica Biosystems, Germany) at thicknesses of 20 µm; the sections were then collected with cryofilm
198 (SECTION LAB, Yokohama, Japan). The collected sections were fixed on an indium tin oxide (ITO)-

199 coated glass (Matsunami Glass Ind., Ltd., Osaka, Japan) using a double-sided conductive tape (3M,
200 St. Paul, USA).

201 **2.12. Sample pretreatment for SALDI-MSI**

202 A carbonate buffer (85 mM, pH 10.3) was prepared using 10.6 mM sodium bicarbonate and 74.4 mM
203 sodium carbonate. The carbonate buffer was mixed with 1 M sodium acetate at a 1:1 ratio.
204 Approximately 15 μ L of this mixture was sprayed evenly per rice *koji* section using an airbrush
205 (Procon BOY SQ, Creos, Tokyo, Japan), following a previously reported method³⁰.

206 A nanoconductive platinum coating (approximately 20 nm) was then applied to the surface of the
207 sections by irradiation with an ION SPUTTER E-1045 (Hitachi, Ltd., Tokyo, Japan) operated at 25
208 mA for 120 s as a pretreatment for SALDI-MSI analysis.

209 **2.13. SALDI-MSI analysis**

210 A quadrupole time-of-flight mass spectrometer (iMScope QT, Shimadzu Corporation) equipped with
211 an optical microscope and an atmospheric pressure Nd:YAG (neodymium: yttrium aluminum garnet)
212 laser ion source ($\lambda = 355$ nm, 1 kHz) was used for SALDI-MSI analysis. The analytical conditions are
213 listed in Table S4.

214 **2.14. SALDI-MSI data analysis**

215 Data analysis was performed using IMAGEREVEAL MS analysis software (version 1.30.0.12570;
216 Shimadzu Corporation), and images of glucose (m/z 203.05) and erythritol (m/z 145.04) in rice *koji* at

217 each *koji*-making time were generated from each m/z peak.

218 **2.15. Observation of *haze-komi* using a digital microscope**

219 Rice *koji*, which was embedded and cut during section preparation for MSI analysis pretreatment, was
220 removed by dissolving the CMC solution at room temperature. The resulting sample was then placed
221 on a glass slide, and *haze-komi* was observed at $\times 50$ magnification using a VHX-X1 (Keyence
222 Corporation, Osaka, Japan).

223 **3. Results and discussion**

224 **3.1. Mycelial content, α -amylase activity, glucose-forming activity, and GC/MS analysis**

225 Figures S1A, S1B, and S1C show the mycelial content, α -amylase activity, and glucose-forming
226 activity of rice *koji*, respectively, at each *koji*-making time (22, 27, 31, 45, 55, and 65 h). α -Amylase,
227 a starch-degrading enzyme, is essential for the dissolution of steamed rice²⁵. The glucose-forming
228 activity, representing the sum of the enzymatic activities of glucoamylase and α -glucosidase, is an
229 indicator of glucose production during fermentation²⁶. These activities play critical roles in sake
230 fermentation, highlighting the importance of understanding their temporal profiles during *koji*-
231 making^{25,26}. The mycelial content, α -amylase activity, and glucose-forming activity increased with the
232 *koji*-making time up to 65 h, suggesting that extending the *koji*-making time from 45 to 55 and 65 h
233 promotes further mycelial growth and enzymatic activity. However, the changes in the mycelial
234 content and enzymatic activities observed between 55 and 65 h were smaller than those observed

235 between 45 and 55 h, indicating that mycelial growth and enzymatic activities will gradually slow and
236 reach a plateau with prolonged cultivation.

237 A GC/MS was performed to profile the hydrophilic low-molecular-weight components of rice
238 *koji* at various stages of production, revealing 42 compounds. Table S5 presents the annotated
239 compounds, quantitative *m/z*, and relative quantitative values for each *koji*-making time. Figure 1
240 shows the score plots (Figure 1A) obtained by subjecting the profiles of the 42 annotated compounds
241 to PCA and by loading the first principal components (Figure 1B). The first and second principal
242 components contributed 69.2% and 20.7%, respectively, and the plot positions shifted toward the
243 positive end of the first principal component with increasing *koji*-making time. The loading of the first
244 principal component showed that the negative component decreased while the positive component
245 increased as the *koji*-making time increased. These results demonstrate that the flavor component
246 profile of rice *koji* changes dynamically with *koji*-making time, suggesting that using *hine koji*
247 produced with extended cultivation can impart a distinct taste profile to the sake compared to rice *koji*
248 produced with the standard *koji*-making time.

249

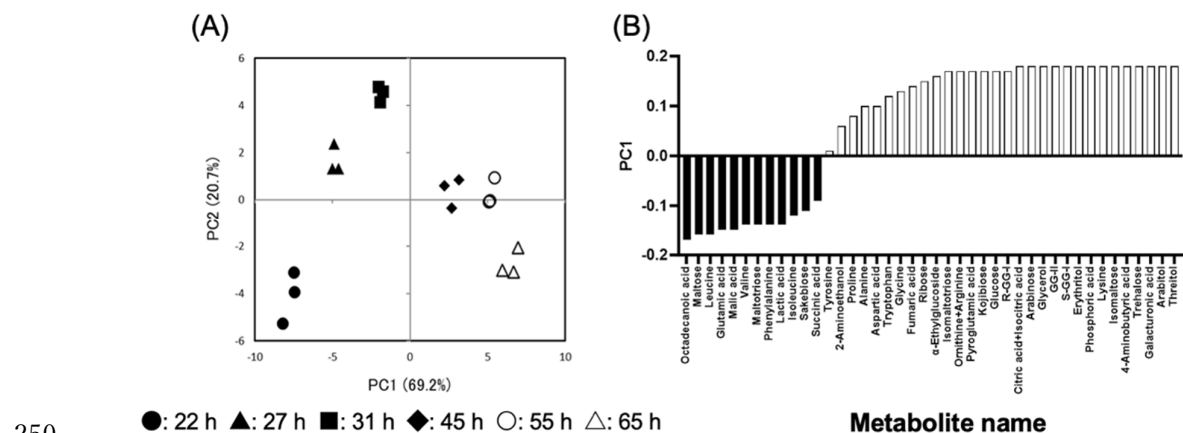
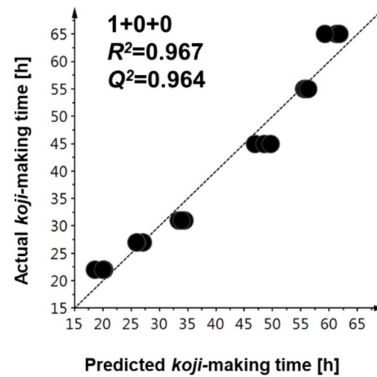


Figure 1. PCA results of the component profiles of rice *koji* at each *koji*-making time obtained via GC/MS. (A) Score plot and (B) loading for PC1. Each *koji*-making time is analyzed in triplicate ($n = 3$).

OPLS regression analysis generated a predictive model (Figure 2) correlating the *koji*-making time (response variable) with the compound profiles obtained from rice *koji* via GC/MS (explanatory variable). The accuracy of the model was evaluated using R^2 (linearity) and Q^2 (robustness), with values of 0.6 or higher being considered good³¹. The model achieved high accuracy (R^2 and Q^2 of 0.967 and 0.964, respectively), demonstrating robust predictive capability. In the composition of the latent variables in OPLS regression analysis, the first component indicates the predictive element of the response variable, the second is orthogonal to the response variable, and the third is the residual component of the objective variable³². Thus, this model has only one major predictive component in the compound profile (X) that explains the *koji*-making time (Y). No irrelevant orthogonal or residual components of X were extracted, indicating that a clear and simple model could be constructed.



264
 265 **Figure 2. Predictive model constructed via OPLS regression analysis using *koji*-making time as**
 266 **the response variable.**

267
 268 Table 1 shows the top five components with the highest VIP values, representing their
 269 contribution to model construction. Trehalose, arabinose, erythritol, kojibiose, and isomaltose were
 270 identified as metabolites whose contents varied significantly with *koji*-making time. Because these
 271 five compounds showed a positive coefficient of effectiveness, their contents are assumed to increase
 272 with extended *koji*-making durations.

273 **Table 1. Top 5 components with the highest VIP scores in the OPLS regression analysis using**
 274 ***koji*-making time as the response variable**

Compounds	VIP	Coefficient
Trehalose	1.209	0.038
Arabinose	1.208	0.041

Erythritol	1.208	0.039
Kojibiose	1.204	0.042
Isomaltose	1.203	0.038

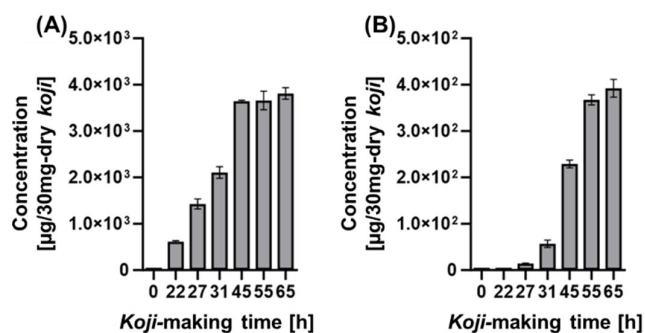
Based on the ionic strength of the quantitative m/z of each component, relative quantification values were calculated. Notably, erythritol was suggested to exhibit a markedly higher abundance among the top five VIP components based on its relative quantification value (Table S5). Erythritol, a tetracarbohydrate sugar alcohol, exhibits approximately 70% of the sweetness intensity of sucrose³³. Unlike glucose, it imparts a sharp, clean sweetness with no aftertaste. It has been reported to be present in rice *koji*³³ and found in sake at concentrations ranging from 0.01 to 0.02 w/v%³⁴. There are no reports on erythritol production by *S. cerevisiae*; the majority of erythritol present in sake originates from rice *koji*³⁵. Therefore, increasing erythritol content in sake requires focusing on rice *koji*. Accordingly, we focused on erythritol and analyzed its content in rice *koji* at various time points during the *koji*-making process, along with glucose, its substrate, and a major rice *koji* component.

3.2. Quantitative analysis of glucose and erythritol via HPLC

The glucose and erythritol contents of rice *koji* at each *koji* making time are presented in Figure 3. The glucose content increased up to 45 h and then remained constant. The glucose concentration in the rice *koji* was approximately 13%. Conversely, the erythritol levels continued to increase over time. After 45 h, erythritol comprised approximately 1–2% of rice *koji*. A high erythritol content was

290 observed in rice *koi*, and the data suggest that erythritol is produced following glucose. Despite the
 291 increase in glucose-forming activity over time (Figure S1C), the glucose content remained almost
 292 constant after 45 h (Figure 3A), indicating that glucose was metabolized, creating a balance between
 293 its production by saccharification and metabolism. However, the erythritol content continued to
 294 increase (Figure 3B), possibly because of an ongoing metabolic reaction that converted glucose to
 295 erythritol via the pentose phosphate pathway.

296



297

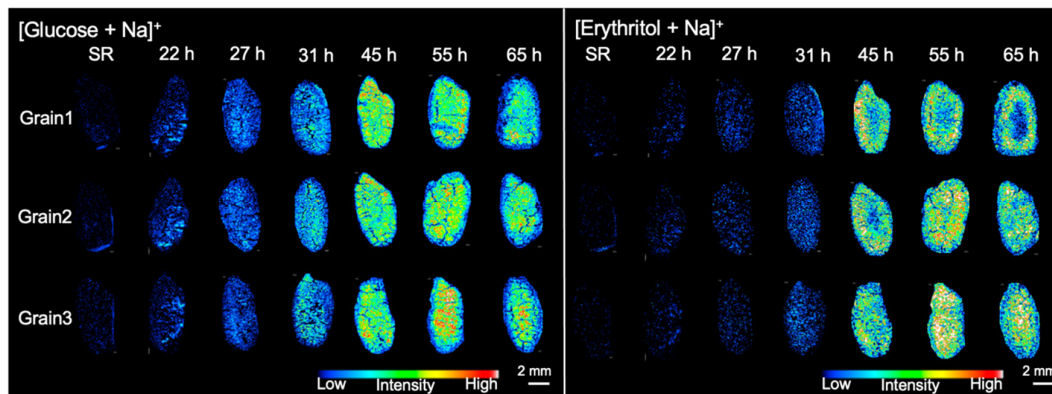
298 **Figure 3. Quantitative analysis of components via HPLC.**

299 (A) Glucose and (B) erythritol (mean ± standard deviation, $n = 3$).

300 **3.3. Distributions of glucose, erythritol, and haze-komi in rice koji**

301 An integrated analysis combining MSI and microscopic observation was performed to elucidate
 302 the relationship between *koi*-making time and the characteristics of rice *koi*, focusing on the spatial
 303 distribution of *A. oryzae* mycelia and their components. The spatial distributions of glucose, the major
 304 component of rice *koi*, and erythritol, a metabolite that markedly increases with the extension of the

305 *koji*-making period, were analyzed using MSI. Figure 4 presents the MSI results showing the
 306 distributions of glucose and erythritol.



307
 308 **Figure 4. Spatial distribution of glucose and erythritol in steamed rice and rice *koi***
 309 ***ji*-making time, as obtained via mass spectrometry imaging.**

310 SR: Steamed rice; scale bar: 2 mm. Three grains were analyzed for each *koi*-making time.

311 Metabolite distributions were normalized by the total ion chromatogram (TIC) intensity, indicating the
 312 relative percentage of a metabolite of interest in a grain of rice *koi* with a total intensity of 1000.

313 Glucose was uniformly distributed throughout the rice *koi* prepared at 45 h. However, in the rice *koi*

314 prepared at 55 and 65 h, glucose was less abundant in the peripheral regions, possibly because of its

315 metabolization in the periphery, which resulted in a lower strength. Conversely, erythritol was barely

316 detected in the steamed rice or rice *koi* prepared within 31 h of the *koi*-making process. After 45 h,

317 erythritol was distributed at the periphery and slightly inside the rice *koi*. Although there are individual

318 differences among rice grains, erythritol tended to accumulate more toward the outer regions of the

319 rice grain compared to glucose.

Microscopic observations, combined with a correlation analysis of image brightness, revealed that regions containing fungal hyphae generally exhibit brightness values between 170 and 255. Therefore, in this study, areas within this brightness range were defined as *haze-komi* regions (regions invaded by hyphae) and are indicated in red in Figure 5.

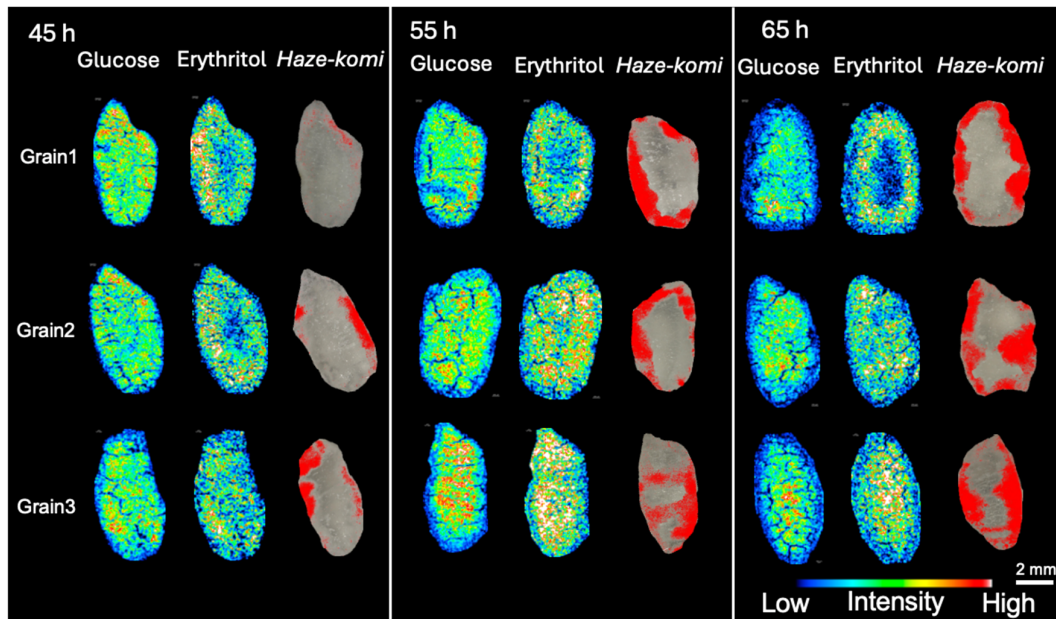


Figure 5. Comparison of the distributions of glucose, erythritol, and *haze-komi* at 45, 55, and 65 h of *koji*-making.

Observations showed that in rice *koji* cultivated up to 31 h, hyphal penetration into the interior of the rice grain was minimal, indicating that the fungal growth occurred mainly on the grain surface. In contrast, after 45 h of cultivation, hyphal invasion into the interior of the grain became apparent, and this tendency became more pronounced at 55 and 65 h. These findings suggest that prolonged *koji*-making promotes internal penetration of *A. oryzae* hyphae, implying that in *hine koji*, the mycelia

332 likely extend deeper into the rice grains.

333 An analysis of the glucose distribution revealed that glucose produced by saccharifying
334 enzymes was widely distributed, even in regions without fungal presence, consistent with the findings
335 reported by Wisman *et al.*⁶. Notably, after 45 h of cultivation, glucose was also detected in the central
336 region of the rice grain, indicating the diffusion of metabolites derived from *A. oryzae* throughout the
337 entire grain.

338 In contrast, erythritol levels markedly increased after 45 h of cultivation, and an MSI
339 analysis confirmed its accumulation within the interior of the rice grains. Under solid-state cultivation,
340 *A. oryzae* exhibits elevated NADP⁺-dependent erythritol dehydrogenase activity under low water
341 activity conditions, leading to the production of erythritol at high concentrations³⁶. Since glucose
342 accumulation is considered a factor contributing to the reduction of water activity³⁷, glucose
343 accumulation around fungal hyphae may reduce water activity, thereby promoting erythritol
344 production.

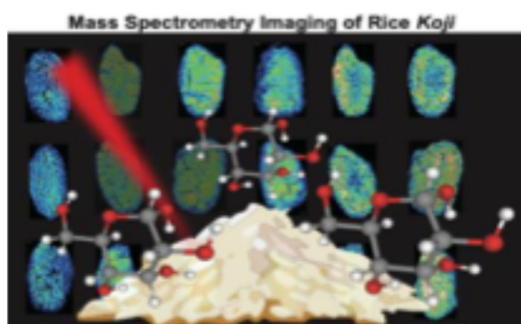
345 Furthermore, similar to glucose, erythritol was observed even in regions lacking fungal
346 growth, suggesting that the produced metabolites diffuse throughout the rice *koji*, beyond the fungal
347 localization sites. This observation implies the possible involvement of metabolite transport via
348 capillary action or moisture migration within the rice matrix, providing an important perspective for
349 optimizing the *koji*-making process and functional evaluation of rice *koji*. Collectively, by integrating

350 microscopic observation of hyphal penetration with MSI, this study provides new spatial insights into
351 the *koji*-making process and the characteristics of *hine koji* from standpoint of fungal growth and
352 metabolite distribution.

353 In this study, we examined the rice *koji* produced at varying *koji*-making times, including
354 *hine koji* prepared with extended cultivation. The mycelial content and enzymatic activities were
355 measured, and the hydrophilic low-molecular-weight components were profiled. The mycelial content,
356 α -amylase activity, and glucose-forming activity increased up to 65 h, and 42 hydrophilic low-
357 molecular-weight components were identified via GC/MS. An OPLS regression analysis revealed that
358 trehalose, arabinose, erythritol, kojibiose, and isomaltose significantly increased with the *koji*-making
359 time. The relative quantitative values of each component indicated high erythritol contents among
360 these five components. Subsequently, we quantified and visualized the distribution of glucose, a major
361 component of rice *koji*, and erythritol, identified as a characteristic component of *hine koji*, across
362 different *koji*-making times. An HPLC analysis showed that the glucose levels increased for up to 45
363 h and remained constant thereafter, whereas the erythritol content continued to increase over time in
364 parallel with *haze-komi* progression. A SALDI-MSI analysis revealed that glucose was relatively
365 uniformly distributed throughout the rice *koji*, whereas erythritol tended to accumulate more around
366 the outer regions of the grains compared to glucose. To the best of our knowledge, this study is the
367 first to employ metabolite profiling for clarifying the relationship between extended *koji*-making times

368 and the production of the characteristic flavor components in *hine koji*, as well as the correlation
369 between the *koji* content and *haze-komi*, including its spatial distribution. Our approach provides a
370 deeper understanding of the rice *koji*-making process, clarifies the characteristics of *hine koji* produced
371 via extended *koji*-making times, and offers valuable guidelines for controlling the *koji*-making
372 conditions and enhancing the yield of flavor components, thus improving sake quality.
373

374 ToC figure (4.45 cm x 8.25 cm)



375

376

377 **ASSOCIATED CONTENT**

378 **Supporting Information**

379 The Supporting Information is available free of charge at <https://pubs.acs.org/doi/xxx/yyy>.

380 Mycelial content, α -amylase activity, and glucose-forming activity of rice *koji* are shown in Figure S1.

381 Other analytical conditions are also shown in the supporting information (PDF).

382 **Author Contributions**

383 K.F. , S.I. and Y.T. performed the measurements, E.F. and S.S. were involved in planning and

384 supervised the work, K.F., S.I., Y.T., and S.S. processed the experimental data, performed the analysis,

385 drafted the manuscript and designed the figures. All authors discussed the results and commented on

386 the manuscript and have read and agreed to the published version of the manuscript.

387 **Conflicts of interest**

388 Y.T. and T.A. are employees of Hakutsuru Sake Brewing Co., Ltd.

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392 **Data Availability**

393 The data that support the findings of this study are available on request from the corresponding author,

394 S.S.

395 **Abbreviations**

396 GC/MS Gas chromatography/mass spectrometry

397 HPLC High-performance liquid chromatography

398 MSI Mass spectrometry imaging

399 MSTFA *N*-methyl-*N*-trimethylsilyl trifluoroacetamide

400 PCA Principal component analysis

401 OPLS Orthogonal partial least squares

402 ITO Indium tin oxide

403 SALDI Surface-assisted laser desorption/ionization

404

405

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