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Barley Koji: Exploring the Effects of Steeping and Fermentation on Malt Quality Parameters

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ABSTRACT

Koji has long been used in Japan to supply enzymes, flavors, and nutrients for traditional fermented food and beverages. Its application to brewing offers a unique, versatile, and innovative alternative to specialty malts. This study assessed the brewing suitability of kilned barley koji produced with a barley-koji strain of *Aspergillus oryzae*—here termed barley koji malts (BKM)—by evaluating the effects of steeping times (6, 7, and 8 h) and solid-state fermentation (SSF) durations (40, 44, and 48 h) on malt quality and metabolomic profiles. BKM exhibited desirable malting quality parameters, including extract levels comparable to standard base malts, rapid production times, and high color formation under a base malt kilning profile. Shorter steeping times yielded improved enzymatic and technological performance, while variation in SSF duration produced distinct color development. This study demonstrates the potential of BKM as a versatile and innovative alternative for specialty malts in craft brewing applications.

KEYWORDS

Aspergillus oryzae; metabolomics; solid-state fermentation; specialty malts

Introduction

Many brewpubs and small breweries rely on the development of innovative beers with unique flavors to attract customers. Their business models often feature a rotation of seasonal or experimental beers alongside a core range of standard offerings. The search for novel beer flavors has driven an explosion in hopped beer varieties and the development of new hop cultivars and products. However, brewers have since sought further differentiation by blending elements of other fermented beverages into beer, such as sake, wine, or champagne yeast; wine grapes; spirits such as tequila; and wood barrels that have been previously used in the production of other alcoholic beverages; as well as sake intermediates like koji.[1–4]

Koji refers to grains such as soy, rice, barley, or wheat fermented with specific filamentous fungi, including *Aspergillus oryzae*, *A. sojae*, and *A. luchuensis*, deemed the National Fungi of Japan.[5] Koji has been used for over a thousand years in Japan to produce fermented foods and beverages such as rice wine (sake), soy sauce (shoyu), protein paste (miso), and spirits (shochu), and is intrinsic to Japanese cultural heritage, shaping the country's traditional fermented foods and their characteristic flavor profiles.[5]

During solid-state fermentation (SSF), koji molds can produce more than 50 types of enzymes, including amylases, proteases, lipases, cellulases, and phytases. Notably, amylases from *Aspergillus oryzae* can saccharify starch at a ratio of 2000:1 by weight, highlighting their exceptional value for the enzyme industry.[6] These hydrolytic enzymes break down complex substrates such as starch and proteins into smaller molecules, including sugars, peptides, and amino acids, which serve as nutrients for subsequent microbial fermentation. In addition, they drive the formation of diverse bioactive compounds—such as ferulic acid, biotin, and kojic acid—with potential benefits for gut, cardiovascular, and immune health.[7] Furthermore, koji fermentation produces a wide range of flavor-active compounds, including esters, aldehydes, alcohols, phenols, and furans, imparting notes ranging from floral and herbal to mushroom-like, depending on the fungal strain and fermentation conditions.[5,8]

Koji's role in Japanese fermented beverages mirrors that of malt in beer production, providing enzymes, flavor, color, and body to the final product—hence the common translation of rice koji as "rice malt." The primary difference between malt and barley koji lies in the origin of enzymatic activity: in koji, enzymes are produced through mold metabolism, while in malt, they are synthesized and activated during grain germination. Furthermore, because the grain hull acts as a physical barrier, only cooked pearled (polished) or hulless (naked) grain varieties are suitable for koji production, as these forms facilitate fungal penetration into

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starchy endosperm. Alternatively, processes such as puffing (e.g., popcorn), and toasting and rolling expose the grain's endosperm, making it accessible to the mold.[9]

The koji production process involves rinsing and soaking the grains, followed by draining and steaming until the grains are soft inside but firm outside. These steps ensure gelatinization without stickiness, reducing clumping and potential bacterial contamination. After cooling to inoculation temperature (35–38 °C), koji spores are evenly distributed over the grains. The inoculated grains are then fermented in a warm, humid environment for up to 24 h, until the koji begins generating heat. At this point, mixing, spreading the grains in a thinner layer, and regulating humidity are implemented. Regular mixing ensures adequate oxygenation, temperature control, and even mold distribution. SSF typically lasts 37–72 h, depending on the koji type.[5,10]

Koji can be produced more quickly than traditional malt (approximately 3 days vs 5–7 days) and potentially with less water and energy use.[11] Additionally, the selection of mold species, strains, and substrate grains offers a wide range of enzymatic and flavor profiles.

Malt production, defined as a controlled germination process halted by thermal drying, also begins with rinsing the barley (or other grains) to remove dirt and reduce microbial load. This is followed by steeping (1–2 days), which raises the grain's moisture content to 42–46%, creating optimal conditions for germination. Germination occurs under controlled temperature (12-16 °C), moisture, and ventilation, to optimize enzyme production and grain modification.[12] Its duration (typically 4–5 days) depends mostly on the desired malt specification but is also influenced by the malt house conditions and barley variety. Longer germination times lead to further endosperm degradation and increased transfer of substrate to the developing rootlets and acrospire. When the desired level of modification is achieved, the green malt is dried through kilning—or roasting for some special malts—with process conditions determining defining the final malt color and flavor profile.[12,13,14]

Malts are broadly classified as base or specialty malts. Base malts are rich in enzymes, and provide extract, free amino nitrogen (FAN), and foundational flavors. Specialty malts, however, offer unique colors, flavors, and functionalities, such as enhancing foam, body, or acidity. These can be produced from barley or alternative grains using traditional or specialized methods.[14]

The steeping or soaking phase is critical for both koji and malting processes. Proper hydration ensures adequate gelatinization for koji production or germination for malt. Homogeneous water distribution is essential for consistent results and the moisture of the steeped grain (cast moisture) influences enzyme production and the final properties of both products. In high-quality sake production, the Sake Master can detect even a 2% change in rice moisture by touch, determining when soaking should end.[10,15,16]

Similarly, the temperature profile and duration of koji fermentation and barley germination strongly influence enzyme production. In malt, higher temperatures (19 °C) and extended germination times favor β-glucan degradation but also lead to greater malt losses and reduced extract yields.[15] In koji, Ito et al.[17] reported that enzyme production generally begins around 24 h of fermentation, following distinct developmental patterns: some enzymes reach a plateau (e.g., α-amylase), others peak and decline (e.g., cellulases), while some continue increasing throughout fermentation (e.g., β-glucosidase). Overall, the enzyme profile is strongly influenced by the fungal strain and environmental conditions.

In this manuscript, we opt for the denomination barley koji malts (BKM) to describe kilned barley koji, acknowledging that while it does not undergo germination, it exhibits functional properties analogous to specialty malts. Like malt, barley koji produces enzymes that will contribute to the grain modification and the development of flavor and color. From an industry perspective, using the term BKM facilitates communication with brewers, enabling them to understand its role as an alternative specialty malt in brewing applications. At the same time, it emphasizes koji's millennial traditional process and Japanese heritage, and its potential unique characteristics.

This study evaluates the production of kilned barley koji as an alternative to barley specialty malts. The effects of three steeping times (6, 7, or 8 h) and three SSF durations (40, 44, or 48 h) on malt quality and the metabolomic profile of BKM are assessed. We recognize that barley koji has a rich and millennia-long tradition in Japanese culture, which we deeply admire. Here, we take koji a step further by kilning it to achieve new flavors and colors—a process not common in traditional koji making—thereby enhancing its applicability in craft malting.

Methodology

Koji malts preparation

The koji-making process followed established methods.[5,10,18,19,20] Barley of the Buzz variety (Barley Program, Montana State University, MT, U.S.A.) was pearled by Montana Milling, Inc. (Great Falls, MT, U.S.A.), removing the hull (2% of grain weight) and part of the bran (10–15% of grain weight). A total of 2 × 500 g of the pearled barley was rinsed 4–5 times and steeped at 20 °C for 6, 7, or 8 h, then drained for 3 h. Steeping times were determined based on Chapon Tests[16] and performed in 2 Mason Ball jars (1/2 gallon, ~1.9 L) with a water-to-barley ratio of 2:1, incubated in a BOD incubator at 20 °C.

Steeped grains were steam-cooked in a pressure cooker (15 psi) for 10 min. After cooling to 35–38 °C, barley koji spores (*Aspergillus oryzae*, fermentationculture.eu, LUVI Fermente KG, Austria – Barley koji variety) were inoculated following the manufacturer's instructions. The inoculated barley was divided into nine sterilized (1/2 gallon, ~1.9 L) Mason Ball jars, incubated in a proofing cabinet (Avantco HPI-1812, Avantco Equipment, USA) at 31.5–33 °C with lids replaced by cheesecloth for air exchange and moisture retention. Koji was mixed at 12, 19, 23, 27, and 33 h and at the end of fermentation (40, 44, or 48 h). Koji internal temperature was measured by a NutriChef Pro BBQ digital thermometer with two probes, placed in the grain bed of two of the jars sitting

Table 1. Design of experiment.

Steeping time	SSF time
6 h	40 h
7 h	44 h
8 h	48 h

on opposite sides of the chamber, while the chamber internal temperature and relative moisture was monitored with a Taylor Digital Thermometer. Additional mixing was performed if the internal temperature exceeded 38 °C.

After fermentation, the jars were transferred to a cold chamber for 12 h. The koji barley were kilned in a CLP kiln (Steep/Germ Combi Kiln, CLP, United Kingdom) using a standard lager malt profile. Experiments were conducted in triplicate, with the design of experiments shown in Table 1.

Malt Quality Analysis (MQA)

MQA were conducted at the Barley, Malt, and Beer Quality Control Lab at Montana State University, following official methods of the American Society of Brewing Chemists (ASBC). Parameters assessed included moisture (%) (ASBC Malt 3), extract (FG db, %) (ASBC Malt-4), β-glucan content (mg/L) (ASBC Wort-18), FAN (mg/L) (ASBC Wort-12), soluble protein (%) (ASBC Wort-17), diastatic power (DP; °L) (ASBC Malt-6), alpha-amylase (DU) (ASBC Malt-7), color (°SRM) (ASBC Wort-9), filtration time (160 mL, minutes), turbidity, and pH (ASBC Wort-8). Analyses were performed using a 1:1 mixture of koji malts and quality control (QC) base malts, with all results converted back to show only the contribution of the koji barley samples, similarly to caramel malt analysis (Malt-9, ASBC).

Koji Malts Metabolomics

Following the MQA, steeping time was fixed at 6 h to evaluate the influence of SSF duration on the metabolomic profile of koji malts.

Sample Extraction: A 100% methanol monophase extraction was performed. Milled samples (100 mg ± 0.5 mg) were vortexed with 1,800 µL methanol, shaken at 4 °C for 1 h, and centrifuged at 3,500 rpm at 4 °C for 20 min. Supernatants were collected and stored at −20 °C.

Untargeted Metabolomics: Analyzes were conducted at Montana State Mass Spectrometry Facility, using a Waters Synapt-XS Q-IMS-TOF coupled with a Waters I-Class UHPLC. Separation utilized a BEH HILIC column (2.1 × 100 mm, 130 Å, 1.7 µm) with a flow rate of 0.4 mL/min. The gradient included a shift from 100% B (acetonitrile, 0.1% formic acid) to 30% B over 18 min. Mass spectrometry data were collected in positive ion mode (50–1,200 m/z, 5 Hz) with lock mass correction. MS^E tandem mass spectrometry used a collision energy ramp of 20–50 V.

Metabolites identification

Metabolomics data were processed using Progenesis QI (v3.0.7927.47290). Metabolite identification was performed using the PlantCyc and Waters "Biomolecules" databases, with precursor and fragmentation tolerances of 10 ppm and 20 ppm, respectively. Compounds with scores >40 were tentatively identified by comparison with the literature and external databases, including NIST, PlantCyc, YMDB, HMDB, FooDB, and KEGG pathway maps for *A. oryzae*.

Progenesis QI provides a detailed file with the m/z, compound ID (based on the Human Metabolome Database [HMDB] or PlantCyc), adducts, molecular formula, score, fragmentation score, mass error (ppm), isotope similarity, and theoretical isotope distribution. The scores, ranging 0–100, are used to evaluate the quality of the identification and comprises of 5 categories with equal weights: (a) mass similarity; (b) isotope similarity; (c) retention time similarity; (d) collision cross-section (CCS) similarity; and (e) fragmentation score, which represents the match between the fragmentation data and the search. Based on previous experiments using this equipment, a minimum score of 40 was set as an acceptable confidence in identification.[21,22]

Statistical analysis

Malt quality data were analyzed using XLSTAT (2024.2.2, Addinsoft), with two-way analysis of variance (ANOVA) (factors: steeping and SSF time) and post hoc Tukey and Dunnett tests. Pearson correlation analysis, principal component analysis (PCA), and heatmaps (RStudio) were used to visualize correlations. Comparisons between koji and QC malts were also conducted.

Metabolomic data analysis employed MetaboAnalyst v6.0 (Xia Lab, McGill University, Canada). Features with >45% missing values were excluded and remaining missing values were estimated using K-nearest neighbors (KNN) imputation. Annotated compounds were filtered for interquartile range (IQR), leaving 211 features from 694 peaks. Data were normalized by sum, log-transformed (base 10), and pareto-scaled for statistical analysis. Identified compounds were auto scaled.

PCA, PLS-DA (partial least squares discriminant analysis), and hierarchical clustering heatmaps with Euclidean-Ward clustering were used to analyze the experimental results. Volcano plots (false discovery rate [FDR] < 0.1, fold change (FC) 2.0) and Orthogonal Partial Least Squares (oPLS-DA) compared treatment pairs (e.g., control vs. koji, SSF times). Orthogonal projection to latent structures (OPLS) modelling in SIMCA regressed QC malt quality data against metabolomic profiles to identify relationships between technological (response) and metabolomic features (predictors).

Results and discussion

Malt Quality Analysis (MQA)

Initially, we discussed the kilned BKM quality parameters compared to the literature, to understand their overall malt properties and their potential uses in brewing. Next, we focused on how variations in steeping and SSF durations affected those parameters. Lastly, we compared the results

with those of the base malt used as QC during the analyses. The malt samples are coded as Steeping time*SSF time.

Table 2 presents the least squares (LS) means summary for malt quality parameters of BKM and the QC malt. Pearson correlation coefficients are shown in Figure 1. These analyses provide insights into the impact of SSF time and steeping conditions on malt characteristics and offer a foundation for optimizing the process to improve quality and adapt it for brewing innovations.

Malt quality characterization

Moisture: The average moisture content of the BKM was 5.44%, below the 6% threshold at which malt may become slack and susceptible to mold growth.[23,24] While base malts typically target 3.8–4.6% moisture,[25] specialty malts can reach up to 6.5% depending on the process (e.g., caramelization) and the grain used (e.g., wheat malts).[26] Moisture values within this range are acceptable, particularly for experimental specialty malts such as BKM.

Fine Grind Extract Dry Basis (FGdb): FGdb, an indicator of soluble solids released during mashing, ranged from 83.93% to 85.90% in BKM These values exceed the minimum standard of 81% recommended by AMBA[27] for distilling and brewing malts. Hulless barley, as used for BKM, typically provides higher extract yields due to the absence of the hull, which otherwise reduces extract potential.[28,29] Recent studies on Canadian hulless barley lines, for example,

Table 2. Summary of malt quality analysis LS means.

Category	Moisture	Extract	Color	β-Glucan	S. Protein	FAN	Diastatic Power	α-Amylase	Filtration Time	pH
	g/100g	FG db, g/100g	°SRM	mg/L	g/100g of malt, db	mg/L	°L	D.U.	160ml, min	
QC Base Malt	4.354 ± 0.090	84.05 ± 0.52	2.95 ± 0.07	54.08 ± 6.2	5.34 ± 0.9	207.78 ± 3.2	115.2 ± 2.9	81.87 ± 1.71	38 ± 6	5.74 ± 0.02
Bk 6*40	5.683 ± 0.276	85.63 ± 0.86	6.31 ± 1.05	2043.3 ± 61	5.37 ± 0.32	164.30 ± 8.5	63.4 ± 4.7	27.06 ± 4.14	132 ± 6	5.05 ± 0.08
Bk 6*44	5.892 ± 0.444	85.90 ± 0.78	9.56 ± 0.50	1927.1 ± 91	5.78 ± 0.32	155.00 ± 15.3	64.2 ± 4.8	20.39 ± 4.38	143 ± 36	4.97 ± 0.07
Bk 6*48	5.974 ± 0.403	84.77 ± 0.75	15.11 ± 1.85	1809.0 ± 27	6.14 ± 0.14	138.60 ± 4.5	65.6 ± 4.9	15.47 ± 1.18	133 ± 14	4.83 ± 0.08
Bk 7*40	5.172 ± 0.243	84.67 ± 0.86	7.56 ± 0.64	2061.0 ± 96	5.09 ± 0.58	141.80 ± 15.8	62.5 ± 5.5	18.13 ± 3.58	137 ± 30	5.05 ± 0.12
Bk 7*44	5.298 ± 0.437	84.76 ± 1.01	9.50 ± 1.77	2007.7 ± 93	5.65 ± 0.13	145.50 ± 15.3	62.8 ± 4.7	14.41 ± 3.56	145 ± 33	4.98 ± 0.09
Bk 7*48	5.604 ± 0.403	84.58 ± 0.71	12.62 ± 4.38	1877.1 ± 185	6.00 ± 0.80	133.60 ± 6.8	63.1 ± 4.4	10.65 ± 2.50	136 ± 6	4.83 ± 0.14
Bk 8*40	5.035 ± 0.314	84.78 ± 0.42	5.57 ± 0.94	2245.4 ± 23	4.75 ± 0.36	142.70 ± 10.3	59.9 ± 4.5	20.92 ± 4.13	142 ± 12	5.07 ± 0.09
Bk 8*44	5.120 ± 0.431	84.38 ± 0.37	6.41 ± 0.19	2181.5 ± 59	4.88 ± 0.24	145.60 ± 0.7	60.5 ± 4.5	18.80 ± 1.49	143 ± 5	5.02 ± 0.09
Bk 8*48	5.215 ± 0.172	83.93 ± 0.31	7.86 ± 0.16	2051.8 ± 45	5.26 ± 0.10	142.40 ± 8.3	61.4 ± 5.9	17.37 ± 2.14	147 ± 32	4.94 ± 0.07
Buzz	4.053 ± 0.049	82.13 ± 0.49	2.64 ± 0.39	85.3 ± 20	5.91 ± 0.29	284.30 ± 29.5	154.0 ± 7.0	126.30 ± 20.79	Normal	5.73 ± 0.04

Sample names are expressed as BK Steeping time*SSF time ± standard deviation. Abbreviations: LS, least squares; QC, quality control; SSF, solid-state fermentation; BK, barley koji; FG, fine ground; db, dry basis; S. Protein, soluble protein; FAN, free amino acids; D.U., dextrinizing units.

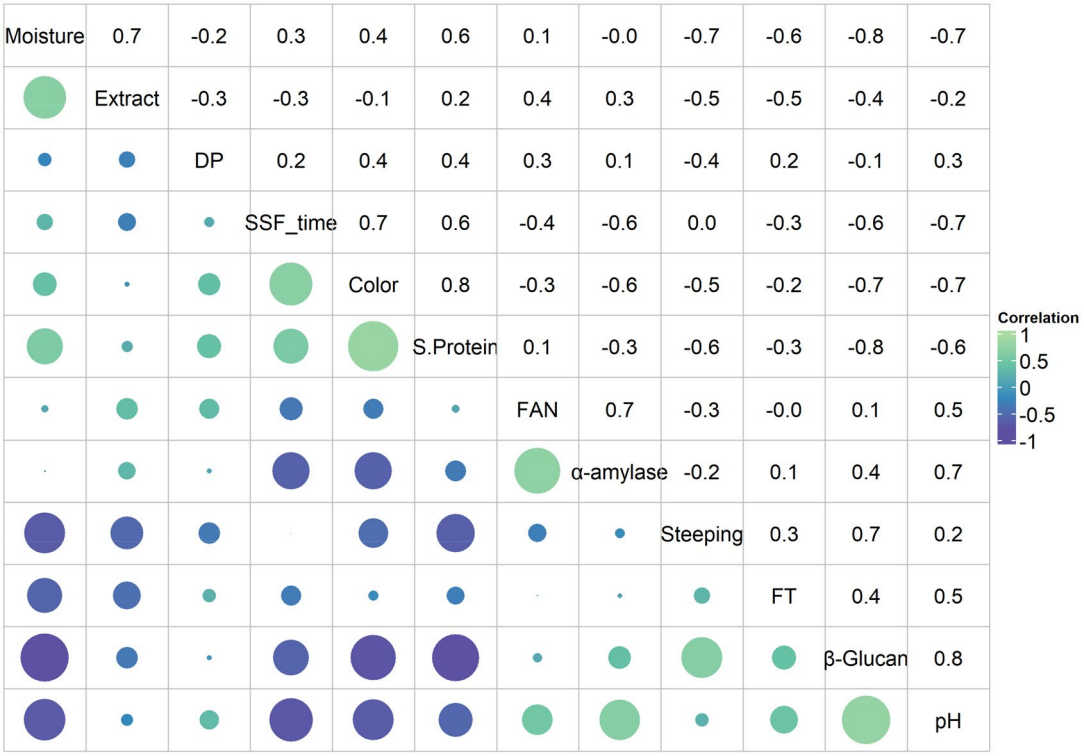

Figure 1. Correlation heatmap displaying the Pearson correlation between dependent and independent variables. The size of the circles corresponds to the magnitude of the correlation, while the colors to its value: green colors correspond to positive correlation, while purple to negative one. Abbreviations: Extract, extract fine grind dry basis; SSF_time, solid-state fermentation time; S. Protein, soluble protein; FAN, free amino nitrogen; DP, diastatic power; FT, filtration time.

reported fine extract values averaging 87.5%, compared to 81.4% in hulled varieties.[29]

Diastatic Power (DP): DP measures the activity of saccharifying enzymes responsible for starch hydrolysis. BKM averaged 62.16°L, comparable to some light Munich malts.[26] While AMBA recommends a minimum DP of 110°ASBC for all-malt brewing, craft brewers often prefer lower DP malts (~60°L) to enable longer and more consistent conversion times.[12,27,30,31] This highlights the potential compatibility of BKM with craft brewing processes.

α-Amylase Activity (aA): α-Amylase, the primary enzyme for starch breakdown during brewing, ranged from 10.7 to 27.1 D.U. AMBA[27] guidelines recommend 40–70 D.U. for all-malt brewing, while European standards recommend >30 D.U. for base malts.[32] Similarly to DP, craft brewers often prefer lower levels (~30 D. U.) for better conversion control.[12,31] The low amylase activity in BKM, despite their high extract values, suggests that most starch hydrolysis occurred during SSF, leaving limited enzyme activity in the final malt, or that the method followed for aA in malts needs adaptation and optimization for barley koji.

Soluble Protein and FAN: Soluble protein, an indicator of protein breakdown during malting, ranged from 4.75 to 6.14 g/100 g, averaging 5.43 g/100 g. This aligns with typical values for malting barley,[27,32] although slightly lower than the Buzz variety's standard malting results (~5.91 g/100 g). FAN, which is crucial for yeast nutrition and beer color, foam, body, and flavor,[14] ranged from 133 to 164 mg/L, meeting AMBA[27] guidelines (140–190 mg/L for all-malt brewing). These values are sufficient to support healthy yeast fermentation while avoiding excess FAN, which can negatively impact beer flavor stability.[30]

Color: BKM displayed a wide range of color values (5.57 to 15.11°SRM), comparable to Munich and caramel malts.[14] Color was highly correlated with soluble protein ($r = 0.818$) and negatively correlated with β-glucan ($r = -0.737$). Malt color is a combined result from amino acids and reducing sugars availability from malt modification (steeping and germination), and the kilning, roasting, or caramelization parameters.[14] For the BKM, the differences in color are likely due to varied starch and protein modifications during koji fermentation in different conditions, yielding different extents of *Maillard* reactions during kilning.

β-Glucan Content: BKM had very high β-glucan levels (1,809–2,245 mg/L), significantly exceeding that achieved by Buzz malts 85.3 mg/L. When compared with Buzz pearled barley (2.4 g/100 g), it is notable the limited β-glucan hydrolyzing power of the *A. oryzae* strain used. High β-glucan levels, especially of high molecular weight fractions (>120 kDa), can cause lautering and filtration challenges due to increased wort viscosity, potentially leading to longer brewing times and reduced efficiency.[33] This could potentially be mitigated using exogenous beta-glucanase.

Some filamentous fungi are known to produce β-glucanase and other cellulolytic enzymes.[34,35] However, for *Aspergillus oryzae*, this activity is strain-specific.[36] Even though we selected a barley-koji strain, it shows low β-glucanase activity. A broad screening of commercial *Aspergillus oryzae* spores for koji production would be recommended to identify strains capable of reducing barley's β-glucan content.

Nevertheless, β-glucan is not entirely undesirable. It is considered a dietary fiber with potential health benefits, including immunomodulatory and cholesterol reducing properties, colonic and cardiovascular health improvements.[37,38] Additionally, *Aspergillus* spp. produces -(1→3)-glucans as part of their cell wall, which are recognized as bioactive compounds.[7] Despite these potential benefits, from a brewing perspective, the high β-glucan levels in BKM necessitate either strain selection for enhanced β-glucanase activity or the use of exogenous enzymes to mitigate filtration issues.

Filtration Time and Turbidity: Filtration times of 160 mL (FT) for BKM were slow, ranging from 123.3 to 152.6 min, likely due to high β-glucan content and large molecular weight fractions. Hazy filtered extracts further support this hypothesis, as high molecular weight β-glucans have been linked to haze formation in wort and beer.[39] The use of exogenous enzymes, such as β-glucanases, is therefore recommended when using BKM in brewing to improve efficiency.

However, FT showed a weak, though significant, correlation (0.4) with β-glucan content, as can be seen in Figures 1 and 2. That suggests other compounds playing a role in FT, in addition to β-glucan. Other non-starchy polysaccharides (NSP), like arabinoxylans (AX) and polymeric arabinoxylan (PAX), as well as high molecular weight nitrogen are also known to impact wort filterability.[40,41]

pH: The mash pH of BKM, which can affect enzyme activity and colloidal stability, ranged from 4.83 to 5.07, lower than typical base malt pH (5.6–6.0).[42] This reduction is consistent with the production of organic acids, such as kojic and malic acids, by *A. oryzae* during fermentation.[43,44] The darker color of BKM further correlates with their lower pH values, as acidic compounds are liberated during Maillard reactions.[45]

Additionally, some bacterial contamination can occur during koji production, particularly at the early stages before full mold development. The optimal temperature and humidity for koji molds also favor bacterial growth, and the relationship between koji and its associated bacterial community—and its impact on sake flavor and koji characteristics—has been investigated for decades. Therefore, although standard food hygiene procedures were followed, it is possible that part of the observed pH decline resulted from bacterial growth.[5,46]

These findings demonstrate that BKM possess a distinctive malt profile, characterized by high extract potential, unique enzymatic activity, and a color range comparable to high-kilned and light caramel malts, making them well-suited for craft brewing applications. Further optimization through strain selection and process adjustments could enhance specific properties, such as reducing β-glucan content, enhancing DP, and improving overall performance in brewing.

Effect of steeping and SSF time on BKM quality analysis

The malt quality results were analyzed using two-factor ANOVA, considering steeping time and SSF time as

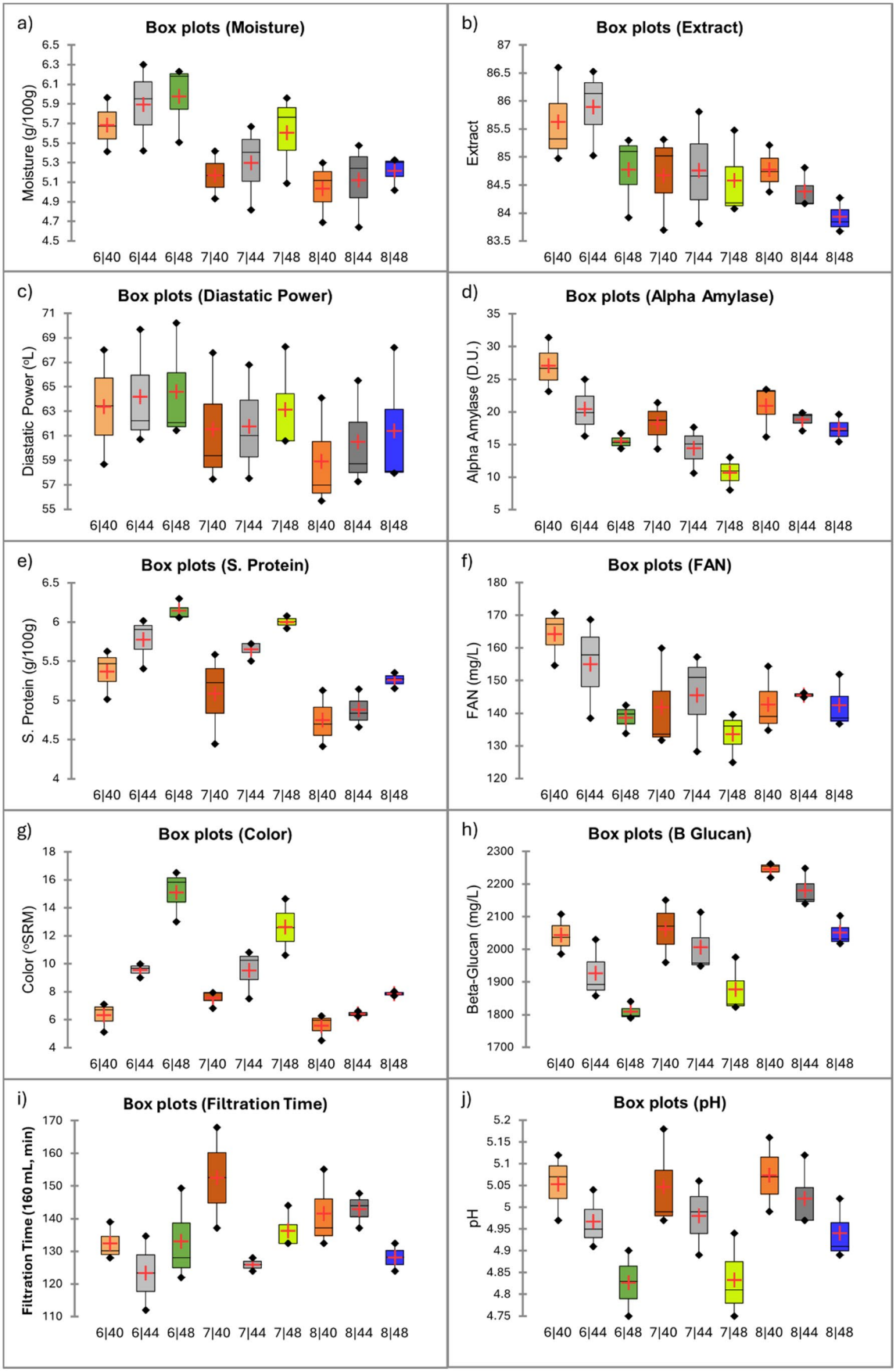

Figure 2. Boxplot representation of the malt quality analysis results for the koji malts. Samples are represented by their steeping time (hours)|SSF time (hours). Abbreviations: Extract, extract fine grind dry basis; S. Protein, soluble protein; FAN, free amino nitrogen; SSF, solid-state fermentation.

Table 3. ANOVA with two-factors of the malt quality analysis.

		Moisture	Extract	Color	β-Glucan	S. Protein	FAN	Diastatic Power	Alpha Amylase	Filtration time	pH
R^2		0.5499	0.4815	0.8998	0.8395	0.7861	0.4881	0.1583	0.7329	0.5513	0.6019
F		2.7492	2.0895	20.2054	11.7730	8.2676	2.1454	0.4232	6.1736	2.7640	3.4018
Pr > F		**0.0360**	0.0930	**<0.0001**	**<0.0001**	**0.0000**	0.0850	0.8920	**0.0010**	**0.0350**	**0.0150**
Steeping	F	9.2070	5.2749	25.0184	27.1642	18.1375	3.1852	1.3491	9.9123	2.2118	1.4855
	Pr > F	**0.0020**	**0.0160**	**<0.0001**	**<0.0001**	**<0.0001**	0.0650	0.2840	**0.0010**	0.1380	0.2530
SSF time	F	1.5237	2.0399	44.7789	19.5500	13.9192	3.1092	0.2952	12.4001	3.6293	11.5495
	Pr > F	0.2450	0.1590	**<0.0001**	**<0.0001**	**0.0000**	0.0690	0.7480	**0.0000**	**0.0470**	**0.0010**
Steeping*SSF time	F	0.1330	0.5217	5.5121	0.1890	0.5068	1.1436	0.0242	1.1909	2.6075	0.2861
	Pr > F	0.9680	0.7210	**0.0040**	0.9410	0.7310	0.3680	0.9990	0.3480	0.0700	0.8830

Data in bold: significant at $p < 0.05$. Abbreviations: ANOVA, analysis of variance; SSF, solid-state fermentation; S. Protein, soluble protein; FAN, free amino acids.

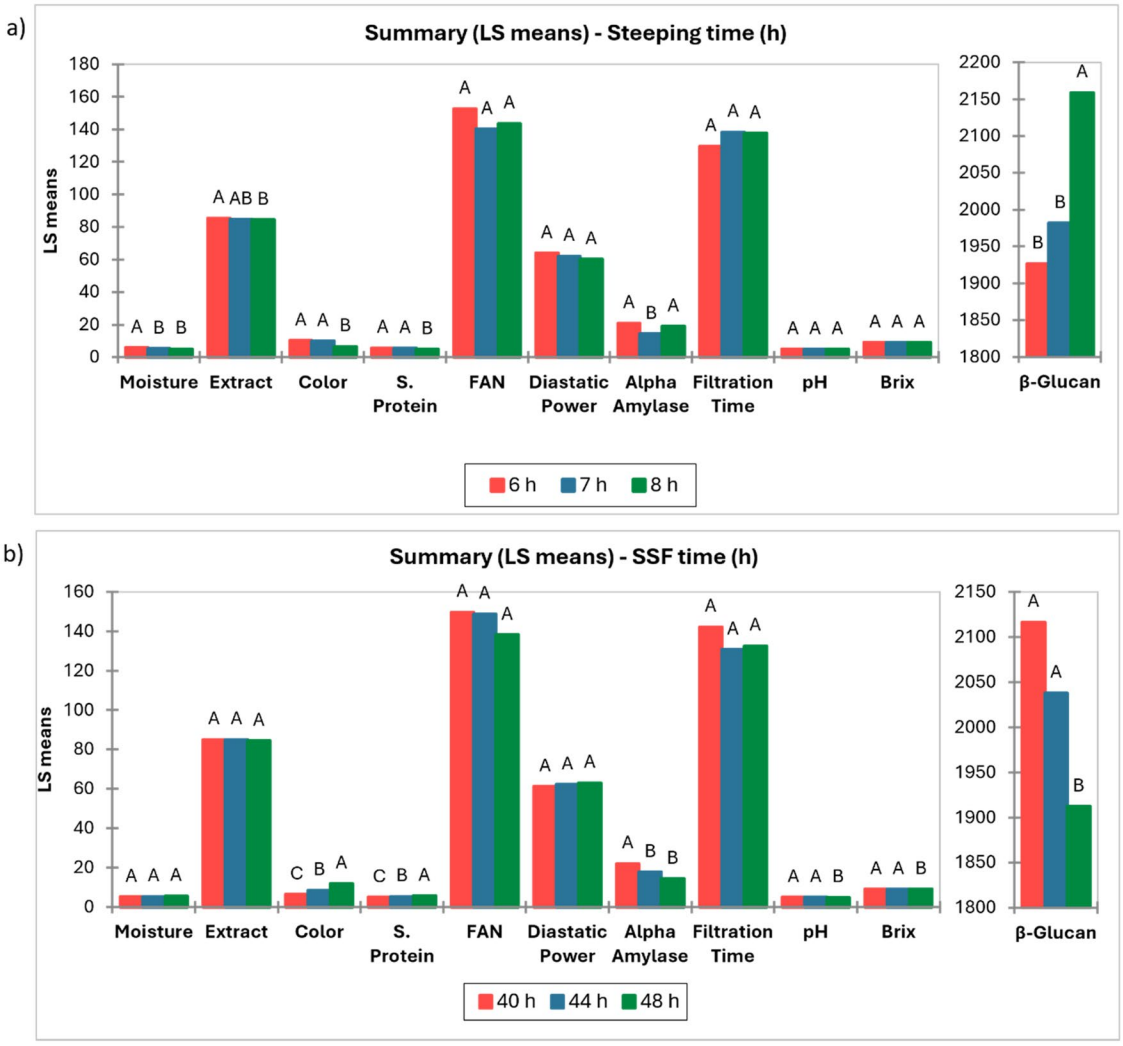

Figure 3. Graphical representation of the koji malt quality analysis statistical results by factor, displaying the results for the post-hoc test Tukey HSD. Different letters for each parameter indicate significant difference ($p < 0.05$). a) Considering steeping time (hours) as independent variable (displaying for each category, from left to right, 6 h, 7 h, 8 h); b) considering SSF time (hours) as independent variable (displaying for each category, from left to right, 40 h, 44 h, 48 h). Abbreviations: HSD, honestly significant difference; SSF, solid-state fermentation; Extract, extract fine grind dry basis; S. Protein, soluble protein; FAN, free amino nitrogen. SSF and steeping time are represented in hours (h).

independent variables and checking for interactions (Steeping Time x SSF time). Figure 2 presents boxplots for an easy visualization of trends, while Table 3 summarizes the ANOVA for malt quality parameters. Figure 3 displays LS means by factor, highlighting statistical differences determined by Tukey honestly significant difference (HSD) tests.

ANOVA revealed that SSF time significantly influenced color, β-glucan, soluble protein, α-amylase, filtration time, pH, and °Brix (°Plato). Prolonged fermentation led to further breakdown of starch, proteins, and cellulosic material, as indicated by a decline in β-glucan and an increase in soluble protein.

The reduced °Brix, stable FAN levels, and increased color at higher SSF times suggest that some sugars and amino acids underwent Maillard reactions, resulting in melanoidin formation. Sugars and carbohydrates are metabolized by the mold, providing energy for mycelial growth and transformation into metabolites such as organic acids, alcohol sugars, and phenolic acids. *A. oryzae* is known to produce organic acids, including TCA cycle intermediates like oxalic, succinic, fumaric, glyceric, malic, kojic, citric, and gluconic acids,[47,48,49] contributing to the pH decrease observed with SSF time.

Filtration time decreased with SSF time and showed a weak but significant correlation with β-glucan ($r = 0.395$), suggesting that other substances, such as xylans and gelling proteins, also influence filtration speed and are broken down during fermentation.[50]

The decline in α-amylase activity can be attributed to the mold entering the stationary growth phase.[48] The activity levels and peak production of koji enzymes are highly dependent on environmental conditions, strain, and substrate composition.[17,51] Additionally, the expression of genes regulating amylolytic enzyme production is repressed in the presence of glucose, regardless of the presence of inducing substances such as isomaltose, a mechanism known as carbon catabolite repression (CCR).[52] Consequently, a decrease in α-amylase and other amylolytic enzymes is expected at advanced SSF stages due to the accumulation of glucose from starch hydrolysis.

Steeping time significantly affected moisture, extract, color, β-glucan, soluble protein, and α-amylase. Longer steeping times appear to saturate the grains with excess water, potentially hindering mold growth. Most enzyme-related parameters (soluble protein, extract, FAN [non-significant], and DP [non-significant]) decreased with longer steeping times, while β-glucan content increased, suggesting slower fungal growth or reduced enzymatic activity.

The lighter malt color observed with extended steeping times supports this hypothesis, indicating less substrate availability for Maillard reactions. The high correlation between soluble protein and color ($r = 0.818$, $R^2 = 0.669$) reinforces this observation. Lower moisture levels in these samples could result from fewer solutes binding water, leaving more free water to evaporate during kilning.

Castro et al.[53] emphasized the importance of substrates with high water-holding capacity (WHC) for SSF with filamentous fungi, as sufficient moisture supports fermentation. However, excessive water absorption can disrupt substrate structure, reducing porosity and oxygen diffusion, which impairs mold growth and enzyme production. WHC in barley varies with the degree of pearling, as outer layers rich in fiber have higher WHC than the starchy endosperm.[54]

These findings suggest that steeping times longer than 6 h, under the experimental conditions, modified grain structure sufficiently to impair mold growth. This could compact the endosperm during steaming, impacting water, and gas dynamics during SSF.

In scientific studies, barley is typically steeped to 35% moisture.[51,55] Practical guides by experienced koji makers suggest shorter steeping times of 1–4 h for barley and longer times for rice.[9,18] In this study, the minimum steeping time of 6 h was based on Chapon tests,[16] as shorter times yielded insufficient Steep Index values. Although barley reached 35% moisture at 3 h, longer times ensured full hydration and gelatinization after steaming.

The interaction term (Steeping Time x SSF time) was statistically significant only for color but showed moderate negative correlations with moisture, extract, FAN, α-amylase, and °Brix (Table 3).

Moisture was strongly correlated with other parameters, highlighting its importance in understanding water dynamics during koji growth and malt production. Steeping time significantly influenced moisture, underscoring the need for further research into its effects on pearled barley microstructure and koji-malt quality. Additionally, other parameters like temperature, oxygen concentration, and gas exchange warrant consideration.

The PCA biplot (Figure 4) shows samples categorized by steeping time (a) or SSF time (b) and their correlations

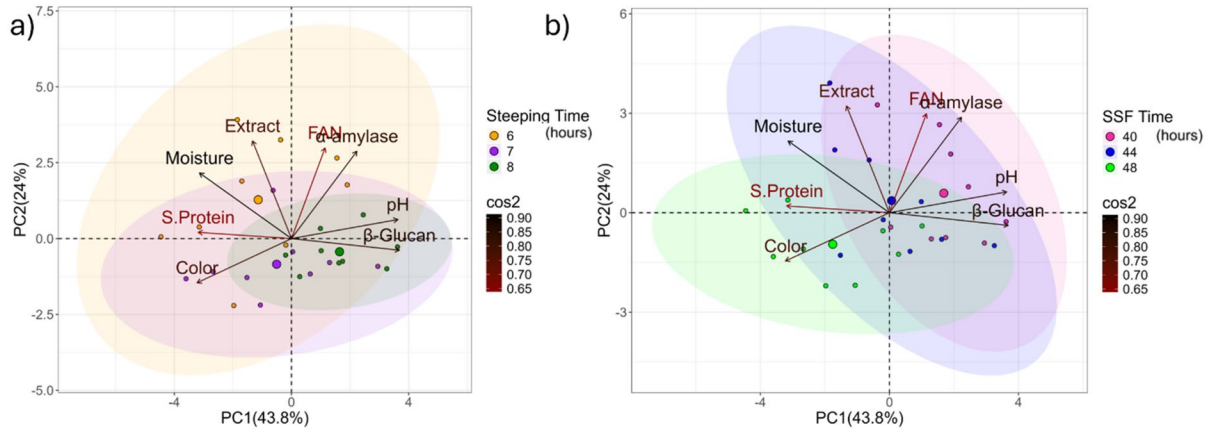

Figure 4. Principal component analysis biplot showing samples categorized by steeping time (hours) (a) or SSF time (hours) (b) with their correspondent confidence ellipses (95%) and their correlation with koji malt quality parameters. Variables are filtered by cos2 > 0.5. Abbreviations: SSF, solid-state fermentation; cos2, cosine squared; Extract, extract fine grind dry basis; S. Protein, soluble protein; FAN, free amino nitrogen; Contrib, variable contribution.

with malt quality parameters. Variables with squared cosine values >0.5 for the first two factors are included in the correlation graph, while poorly represented variables are excluded.

No distinct separation between groups was observed at 95% confidence, though trends emerge, especially around centroids. For 6-h steeping, SSF time had a notable impact on malt characteristics. For longer steeping times, sample differences decreased, as indicated by the smaller confidence ellipse for 8-h steeping.

The centroids suggest that 6-h steeping favors higher extract, moisture, and soluble protein with lower β-glucan, while 8-h steeping correlates with higher β-glucan and lower soluble protein, FG extract, color, and moisture. SSF time trends show a shift towards higher color and soluble protein but lower pH and α-amylase at 48 h, with opposite trends at 40 h.

The correlation heatmap on Figure 1 highlights correlations between malt quality results and categorical variables, showing strong positive correlations between steeping time and β-glucan content and negative correlations with moisture and soluble protein.

Comparison with base malts

Although this study aimed to evaluate the feasibility of BKM for brewing, as alternative specialty malts, their general characteristics are more effectively understood by comparing them to a base malt. For this comparison, the results were analyzed by considering the interaction between each steeping time and SSF duration as independent treatments. The samples are designated according to the Steeping time*SSF time interaction.

BKM from all treatments exhibited overall significantly different results compared to the control malt, as determined by the Dunnett test (two-sided, $p<0.05$). BKM showed significantly higher values (Tukey HSD, $p<0.05$) for color, β-glucan, and filtration time (FT), while significantly lower values (Tukey HSD, $p<0.05$) were observed for FAN, α-amylase, pH, and DP.

The extracts and soluble proteins of the BKM were generally comparable to the control, despite lower levels of amylolytic enzymes and free amino nitrogen (FAN), with a few exceptions. Specifically, BK 6*40 and BK 6*44 exhibited significantly higher extract values ($p<0.05$), while BK 6*48 showed elevated soluble protein content. Although not significantly different, experiments with 8 h of steeping tended to exhibit lower soluble protein content than the control, whereas extended fermentation times resulted in higher values.

As previously discussed, the lower values of FAN, pH, and DP compared to the control would be considered adequate for a base malt in craft brewing applications. However, the α-amylase results do not fully reflect the potential of the koji enzymes when considering the extract values. Given these parameters, brewers could potentially substitute any percentage of the base malt with BKM without impacting extract yield and yeast nutrition. Additionally, the higher color values of BKM would impart a warm distinctive hue to the beer—possibly ranging from honey to amber-brown—likely derived from intensified Maillard and caramelization reactions during kilning of the koji substrate.

The high β-glucan content and increased filtration times associated with BKM would need to be addressed similarly to brewing with other established ingredients high in wort viscosity and β-glucan content, such as oats or rye. This can be managed by applying external β-glucanase enzymes, which hydrolyze β-glucans to reduce viscosity and improve filtration efficiency. Alternatively, adding oat or rice hulls to the mash tun or lauter tun can enhance wort separation by improving the grain bed's permeability.[56]

PCA (Figure 5) illustrates the correlations between features and samples, providing a clear separation (95% confidence) between the quality control malt (Control) and BKM. The differences between the control and BKM samples are primarily explained by PC1, which accounts for 71.6% of the variation. PC1 associates the control malt with higher FAN, α-amylase, DP, and pH, while BKM are associated with higher β-glucan, filtration time, moisture, and color.

PC2, which explains 15.6% of the variation, primarily reflects differences among BKM samples, with the largest contribution from soluble protein and a secondary contribution from color. Extract was identified as the main contributor to PC3, which represents finer distinctions in malt characteristics.

Metabolomic analysis

Based on the MQA, we selected a fixed steeping time to further investigate the effects of SSF time on the metabolomic profile of BKM. BKM steeped for 6 h were chosen due to their favorable characteristics, including lower β-glucan content, faster filtration time (FT), and higher extract and α-amylase activity, making them more suitable for brewing applications.

In total, 2,657 metabolites were detected, 694 of which were annotated, resulting in 3,405 potential identifications. Among these, 39 compounds had identification scores >40 Sixteen were automatically accepted by the algorithm, and 23 were putatively identified using literature and databases.

The list of identified compounds, including their identification numbers, retention times and m/z values, description, chemical formula, and subclasses is presented in the appendix (Table A1.). A table with the putative identifications for each compound and full classification is presented in the Online Supplementary material (S1. Metabolites ID description and classification).

Evolution of metabolomic profile of koji-malts from 40 to 48 h of SSF

The Hierarchical Clustering Heatmap (Online Supplementary Figure S2.1) provides a clear visualization of how samples and features are clustered, displaying the concentration of compounds for each sample on a color-based scale. This analysis revealed distinct patterns in the formation and degradation of annotated compounds over fermentation time. BKM with 40 and 44 h of SSF (BK40 and BK44) shared more compounds with each other than with BK48, clustering together based on the Euclidean-Ward method.

Similarly, compounds were clustered into two main groups: those with higher concentrations in BK48 and those

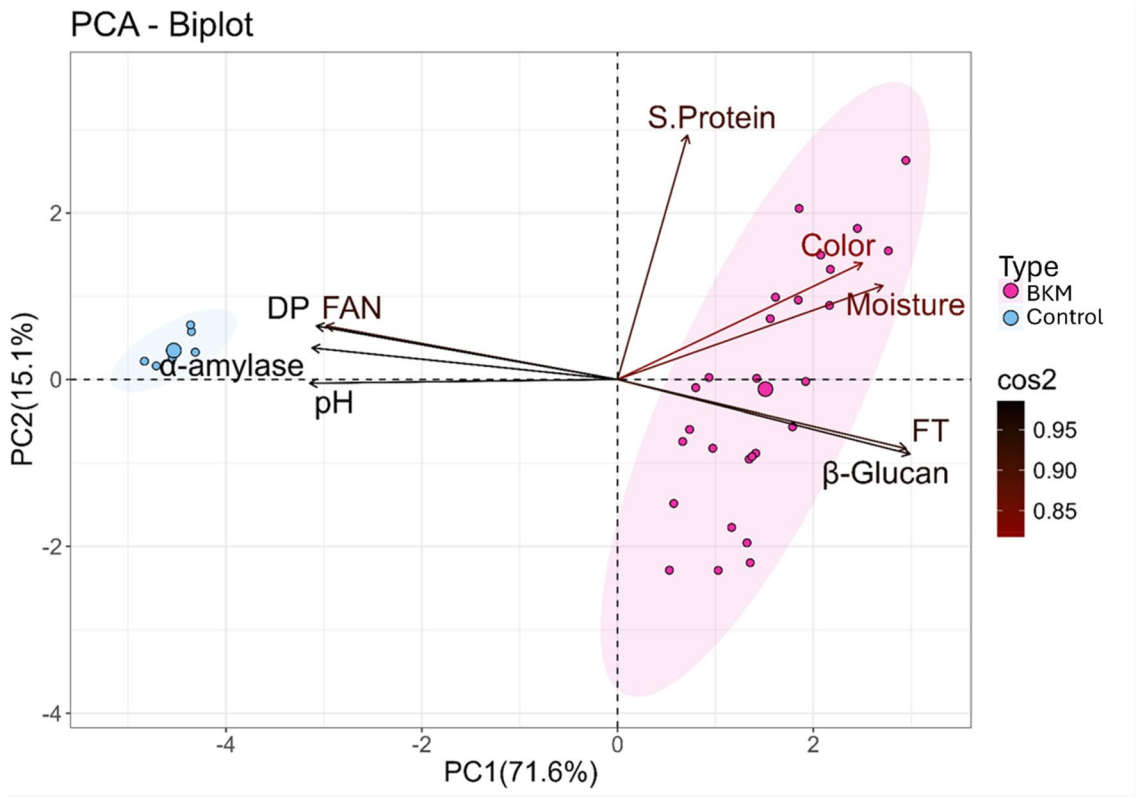

Figure 5. PCA biplot showing the correlations between the samples and features. Each categorial variable (control vs. BKM) is displayed with the correspondent confidence ellipses (95%). Variables are filtered by cos2 > 0.5. Abbreviations: PCA, principal component analysis; BKM, barley koji malt; cos2, cosine squared; Extract, extract fine grind dry basis; S. Protein, soluble protein; FAN, free amino nitrogen; DP, diastatic power; FT, filtration time.

predominant in BK40 and BK44. These clusters further branched into subgroups, indicating higher concentrations in either BK44 or BK40. Interestingly, some compounds peaked at 44 h of SSF before being mostly degraded by 48 h.

PCA (Online Supplementary Figure S2.2), an unsupervised multivariate method, reduced the dataset dimensions while preserving the variance between samples. PCA separated the BKM into three groups. PC1 (explaining 59.2% of variance) mainly distinguished BK48 from the other two groups, while PC2 (explaining 20.5% of variance) separated BK44 from the others. Permanova analysis confirmed a high R^2 for each group, indicating that fermentation time differences were well-explained by the PCA model. However, the p-value (0.1) suggests some overlap between groups.

Identified compounds and their trends

Of the 39 features with scores >40, most were lipids (26%), organic acids (20%), organooxygen compounds (18%), and nucleosides/nucleotides (13%) (Figure 6).

Statistical analysis showed patterns consistent with annotated compounds as shown in the Hierarchical Clustering Heatmap (Figure 7). In the PLS-DA scores graph (Figure 8), BK40 and BK44 were closer to each other, while BK48 formed a distinct group. PLS-DA, a supervised classification method, maximized covariance within groups and identified distinguishing variables through variable importance in projection (VIP) scores.[57]

PLS-DA generated three components with excellent performance ($R^2 > 0.95$, $Q^2 > 0.80$) and a low chance of results occurring by chance ($p = 0.04$). According to VIP results (Figure 8c), the most important compounds for the model were hydroxycinnamic acid (p-Coumaric acid or similar), adenosine, and guanine, which were higher in BK40 and decreased with fermentation time.

Hydroxycinnamic acid is a phenolic compound found in barley that imparts an astringent flavor and serves as a precursor for flavonoids, tannins, and curcuminoids (ASBC flavor database).[58] Guanine and adenosine are purine nucleotides involved in DNA and RNA synthesis, and essential in energy metabolism and other cellular functions.[59] However, excessive dietary intake of purines can lead to excessive accumulation of purine metabolites and, consequentially, uric acid in the body, which is associated with gout disease.[60] Some koji molds, including A. luchuensis[61] and certain sake strains,[62] have been reported to produce purine-degrading enzymes.

Other distinguishing compounds included amino acids (e.g., dipeptides and L-pipecolic acid) and a compound derived from leukotriene lipid oxidation. Amino acids play multiple roles, including entering pathways for nitrogenous compounds, cofactors, nucleotides, and secondary metabolites like flavor compounds.[63] They can also participate in Maillard reactions, forming color and flavor compounds. Proline, asparagine, and glutamic acid, identified in the samples, contribute sweet or umami-like flavors, while

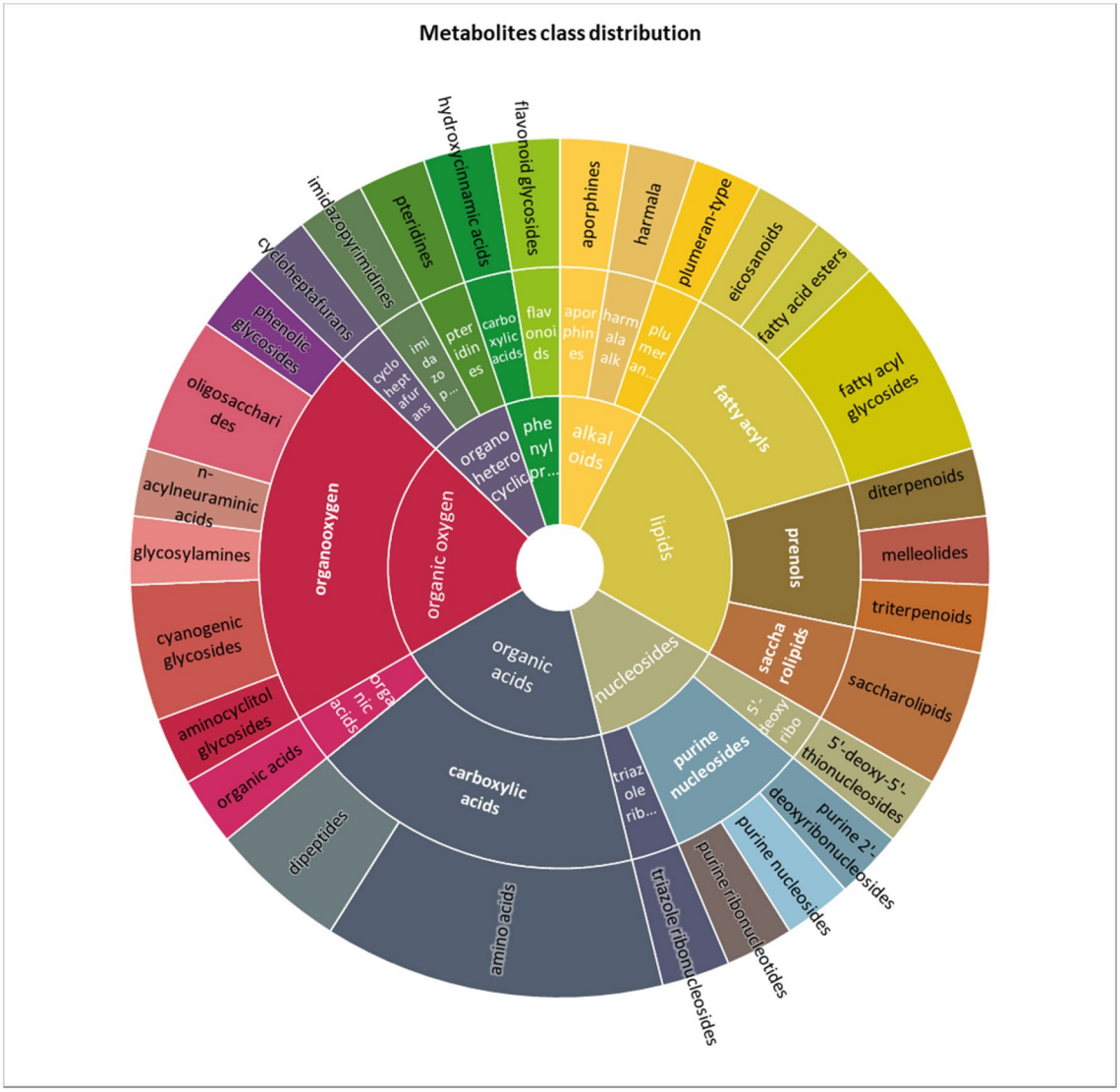

Figure 6. Class distribution of beer metabolites (based on frequency).

nucleotides such as adenosine monophosphate enhance umami perception (ASBC Beer Flavor Database[64–66]).

Compounds decreasing with SSF time included N-acetylneuraminic acid (Sialic acid, associated with fungal adhesion), and linamarin, a cyanogenic glycoside with a bitter taste.[67,68] The decrease in linamarin may reflect detoxification processes by *A. oryzae*, which is known to metabolize cyanogenic glycosides into safer compounds.[69]

Conversely, compounds increasing with SSF time included glycosylated metabolites, such as (37) oligosaccharides, (20) N2-Fructopyranosylarginine, and (23) 6-(alpha-D-Glucosaminyl)-1D-myo-inositol, which indicate fungal cell-wall modulation and stress responses. Stress-protective metabolites such as (S)-Homostachydrine also increased, reflecting fungal adaptation during fermentation.[70]

Pairwise comparison

To better understand metabolite evolution, pairwise comparisons of samples were performed using oPLS and volcano plots. Volcano plots (Figure 9a) identified significant features with high magnitude changes between BK40 and BK44, including guanine, adenosine, hydroxycinnamic acid, and sesquiterpenoids, all of which were higher in BK40.

The oPLS model (Figure 9b), a supervised multivariate approach based on PLS regression which maximizes class differences between two groups, identified additional key compounds. These included xanthoplanine, a benzylisoquinoline alkaloid, and tuberonic acid glucoside, a jasmonic acid derivative associated with stress signaling. Both compounds increased from 40 to 44 h of SSF, highlighting their role as secondary metabolites during fermentation. Tuberonic acid glucoside likely results from the enzymatic breakdown of jasmonic acid by *A. oryzae* β-glucosidases.[71,72]

Xanthoplanine belongs to the aporphine subclass of benzylisoquinoline alkaloids, which are known for diverse bioactive properties, including antimicrobial and cytotoxic activities, antioxidant effects, smooth muscle relaxation, and interactions with neurotransmitter receptors.[73] Its increasing concentration during SSF indicates a potential role in the microbial ecosystem or as a stress-response metabolite produced by *A. oryzae*.

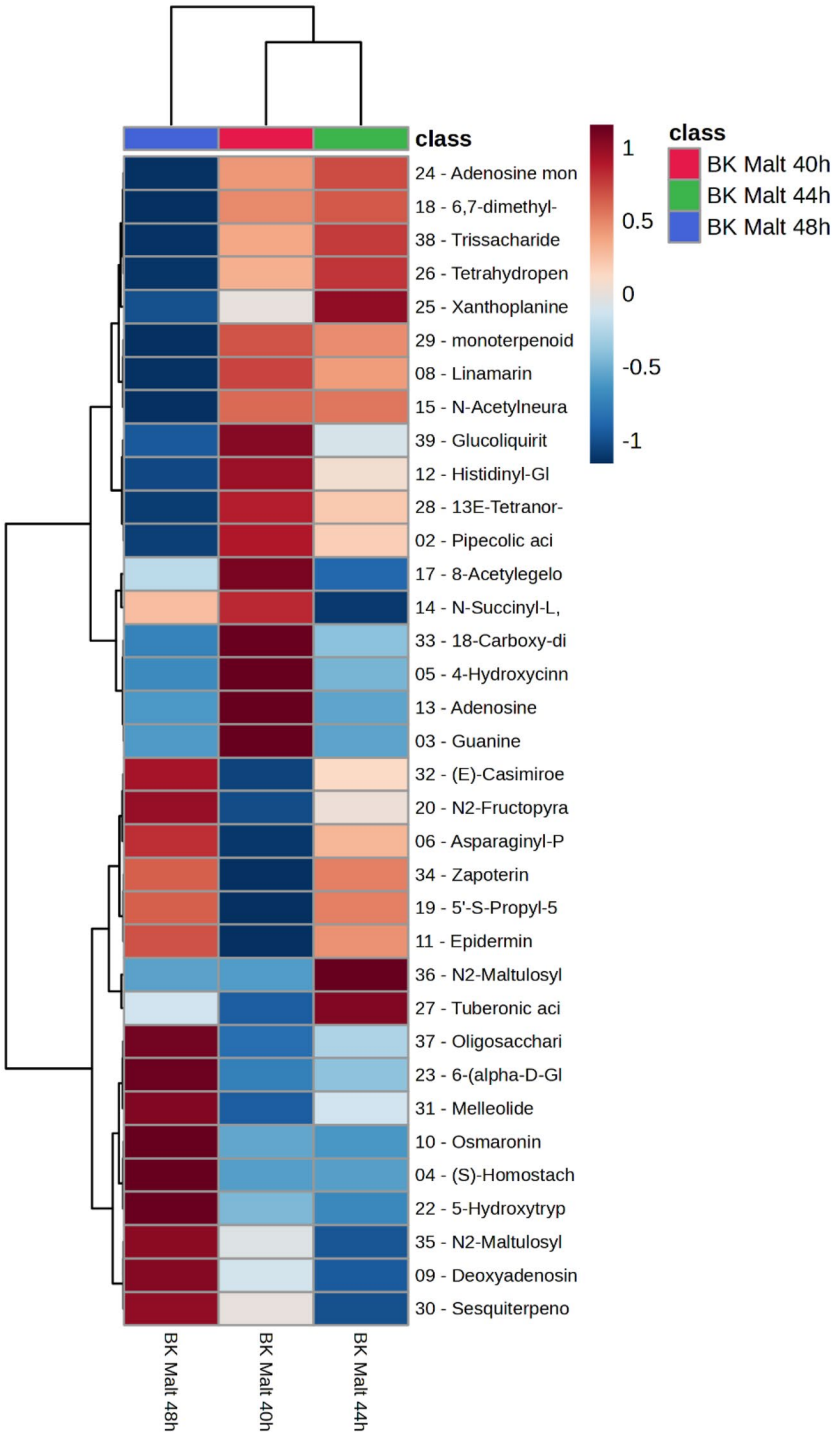

Figure 7. Hierarchical clustering heatmap of the identified compounds. Colored cells represent the concentration of the variables in the samples (red = high concentration, blue = low concentration).

Comparing BK44 and BK48, the most significant changes indicated by the volcano plot (Figure 10a) and the oPLS VIP plot (Figure 10b) included increases in sesquiterpenoids and (S)-Homostachydrine, alongside a decrease in trisaccharides. Additionally, an increase in (GPI)-anchors, such as 6-(alpha-D-Glucosaminyl)-1D-myo-inositol, and oligosaccharides potentially indicate mold growth and cell-wall modulation. These trends highlight the dynamic adaptation of *A. oryzae* during SSF, including cell-wall remodeling and metabolite production.

Comparison with control base malt

To investigate the main differences between the koji malts and the control malt and highlight the uniqueness of the koji malts, their metabolite profiles were evaluated. After data filtering, the initial 694 features (mz/rt data matrix) were reduced to 216 features that best represented the dataset. Samples were normalized by the sum, log-transformed, and pareto-scaled. Comparing the koji malts with the control (Online Supplementary Figure S3.1), it was noticeable that only one-third of the features were higher in the koji malts.

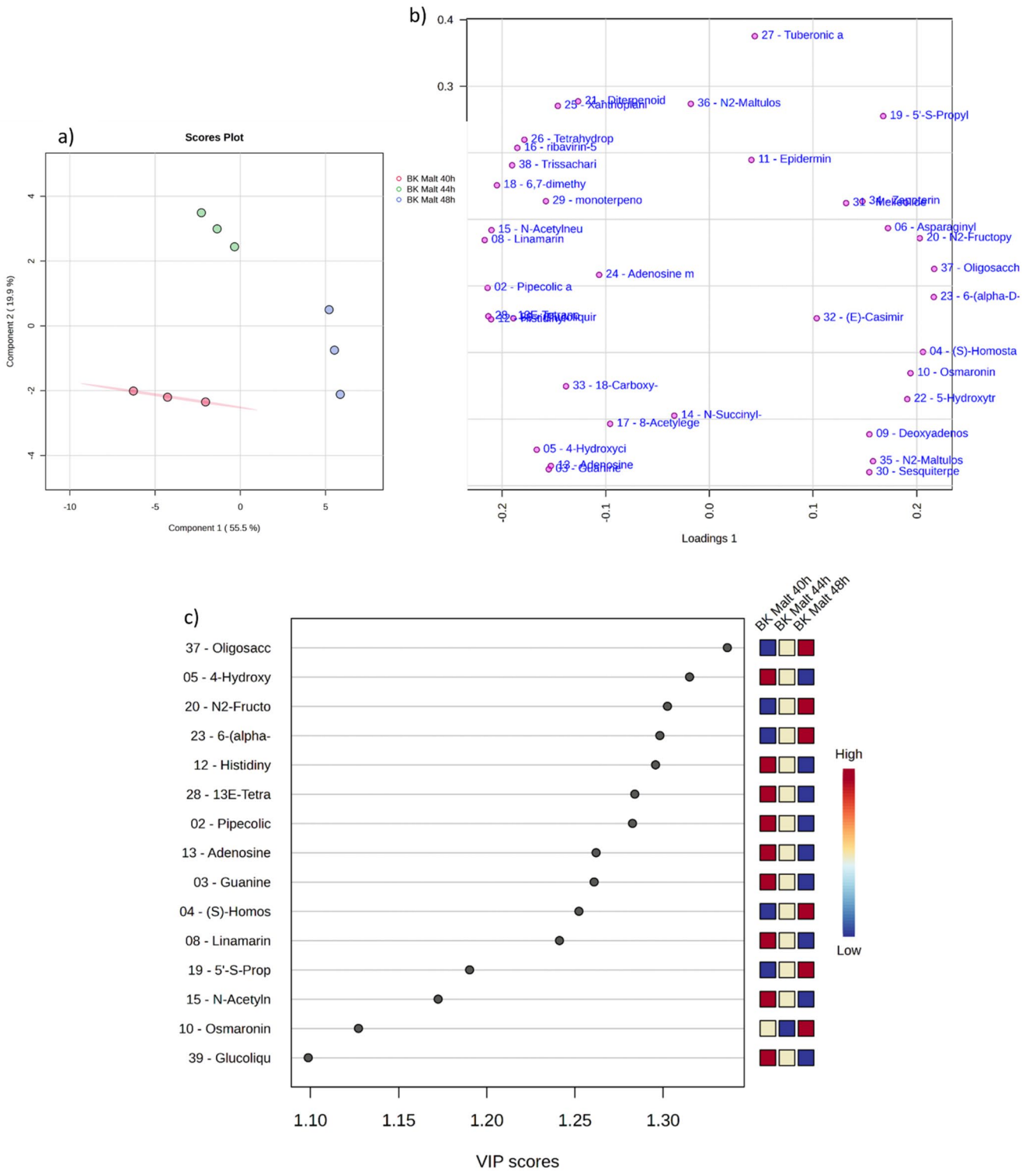

Figure 8. PLS-DA scores plot (a), loadings plot (b), and VIP compounds (c) of the identified compounds. Abbreviations: PLS-DA, partial least squares discriminative analysis; VIP, variable importance in projection.

The Hierarchical Clustering Heatmap (Online Supplementary Figure S3.2), generated using the Euclidean-Ward method, shows the BKM clustered according to SSF time (BK_48, BK_44, BK_40), forming a distinct group separate from the control malt. Features were also grouped into two major clusters based on their concentrations: high in the control malt or high in the BKM. Overall, most features present in high concentrations in the control malts were at lower levels in the BKM, and vice versa.

PCA analysis (Online Supplementary Figure S3.3) further demonstrated the separation of BKM from the control malt. The first principal component (PC1) explained 94% of the variation, clearly separating the BKM from the control malt.

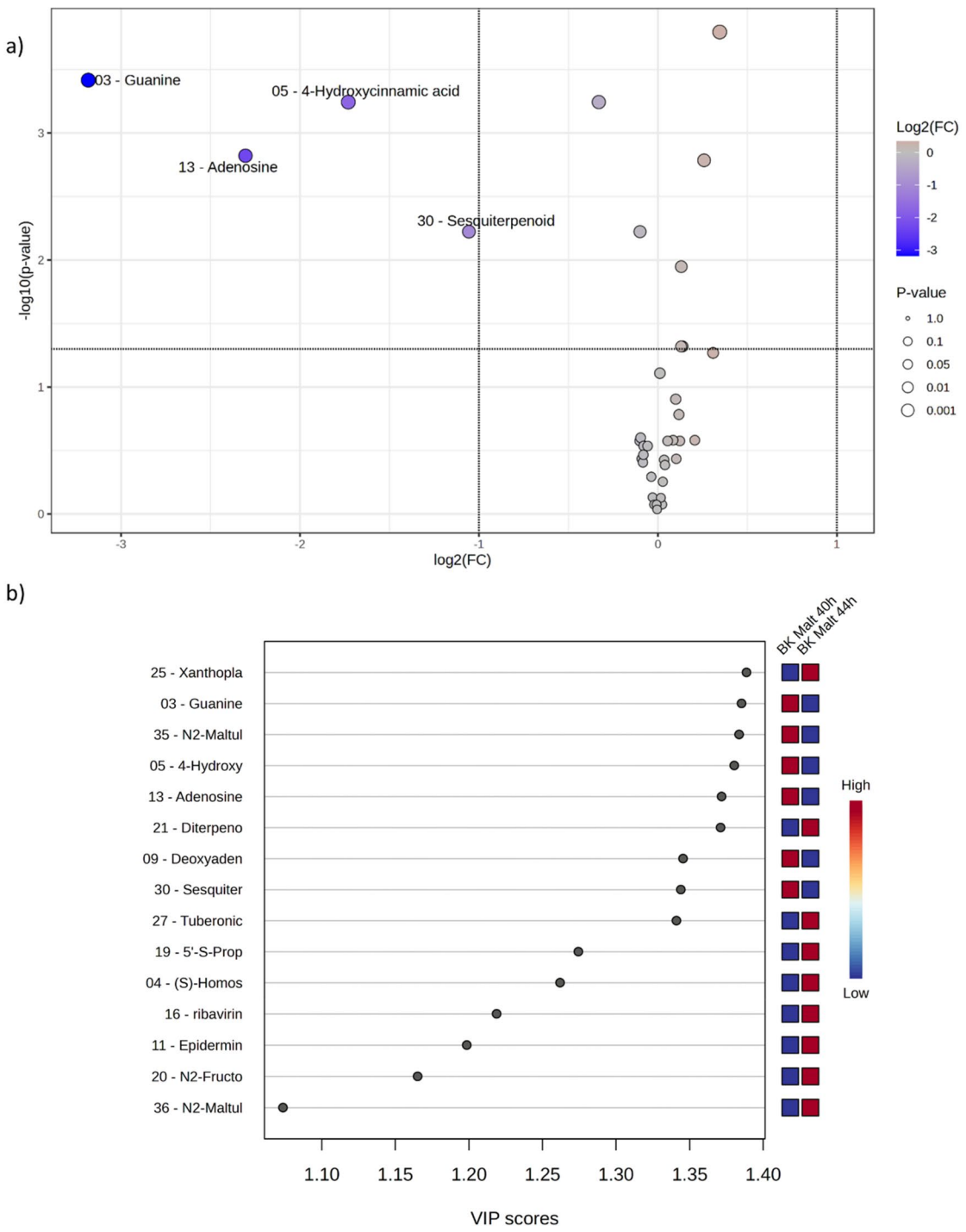

Figure 9. Volcano plot (a) and o-PLS (b) VIP plot for BK40 and BK44. The volcano plot considers BK44/BK40. The features in blue (left) are significant and down-regulated, and the red (right) significant and up-regulated, according to the threshold FDR < 0.1 and FC = 2.0. Abbreviations: o-PLS, orthogonal partial least squares; VIP, variable importance in projection; BK40, barley koji malt with 40 h of SSF; BK44, barley koji malt with 44 h of SSF; FDR, false discovery rate; FC, fold change.

The second principal component (PC2), accounting for 3.9% of the variation, separated the BK samples based on SSF time into three distinct groups.

Identified compounds and their trends

Focusing on the 39 identified compounds, statistical analysis showed similar clustering trends as observed with all annotated metabolites. These trends are visualized in the Hierarchical Clustering Heatmap (Figure 11) and PCA scores plot (Online Supplementary Figure S3.4). However, the identified features were less effective at distinguishing between BK40 and BK44.

PCA (permanova: F-value 52.763; R^2 = 0.84067; p-value = 0.004 based on 999 permutations) separated the control malt from the BK samples along PC1, which explained 82.3% of the variance, while PC2 (12.9%) captured variations among different SSF times.

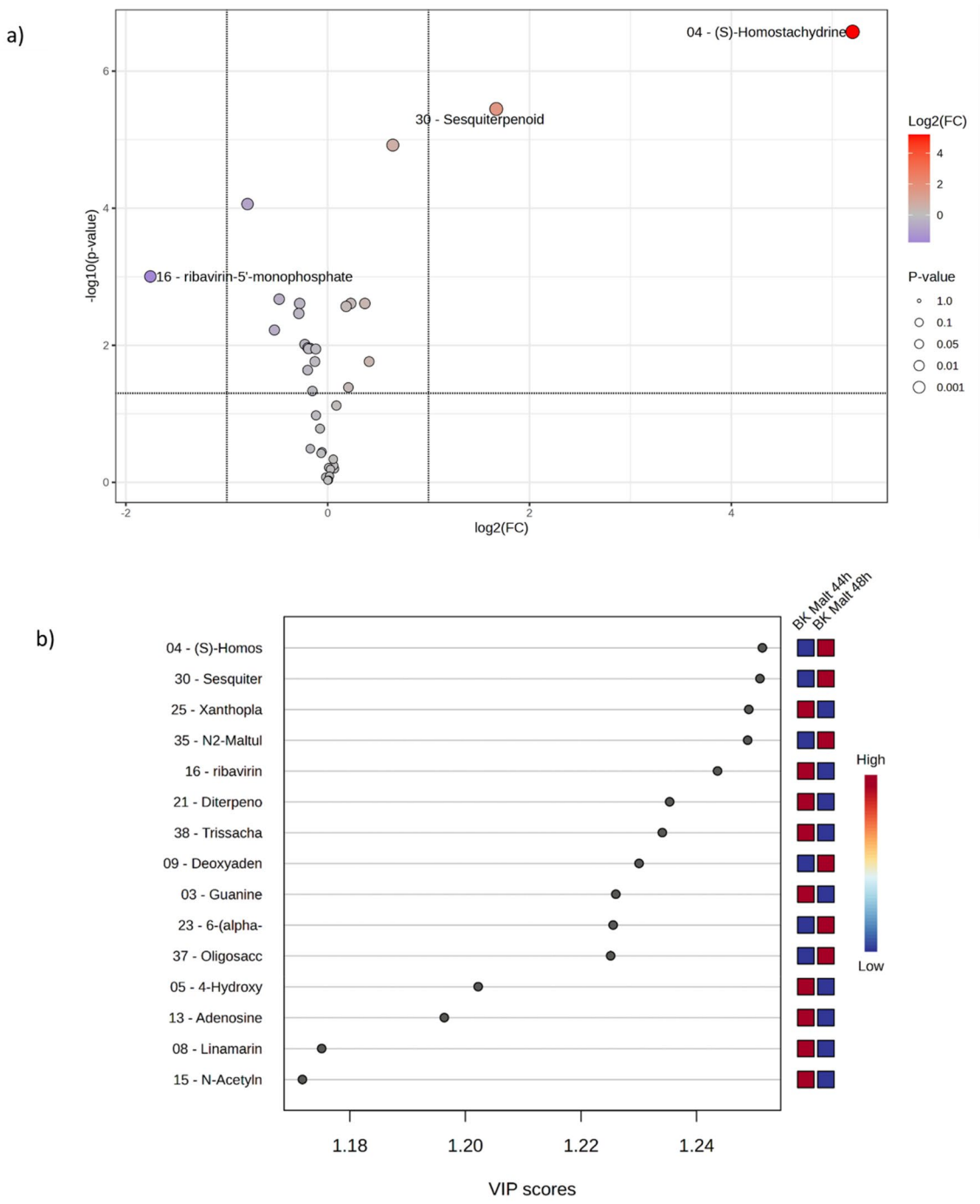

Figure 10. Volcano plot (a) and o-PLS (b) VIP plot for BK44 and BK48. The volcano plot considers BK48/BK44. The features in blue (left) are significant and down-regulated, and the red (right) significant and up-regulated, according to the threshold FDR < 0.1 and FC = 2.0. Abbreviations: o-PLS, orthogonal partial least squares; VIP, variable importance in projection; BK44, barley koji malt with 44 h of SSF; SSF, solid-state fermentation; BK48, barley koji malt with 48 h of SSF; FDR, false discovery rate; FC, fold change.

Pairwise comparison

The volcano plot and oPLS model (Figure 12) were used to identify the most important features for differentiating the control from the BKM. The p1 component of the oPLS model, which separates the two groups, explained 75.5% of the variation, with R2Y = 99.5%, R2X = 99.3%, and a p-value of 0.002.

According to the VIP and Volcano plot (Figure 12), key compounds with significantly higher concentrations in the koji malts included sesquiterpenoids (melleolides), monoterpenoid indole alkaloids, phenolic glycosides (5-Hydroxytryptophol glucuronide), and dipeptides (Asparaginyl-Proline). Terpenoids, such as melleolides, are biologically active compounds known for their antimicrobial properties.[74] Monoterpenoid indole alkaloids, including tabersonine and vindoline, have been associated with anticancer activity.[75] These findings underscore the metabolic diversity of koji malts and their potential bioactivity.

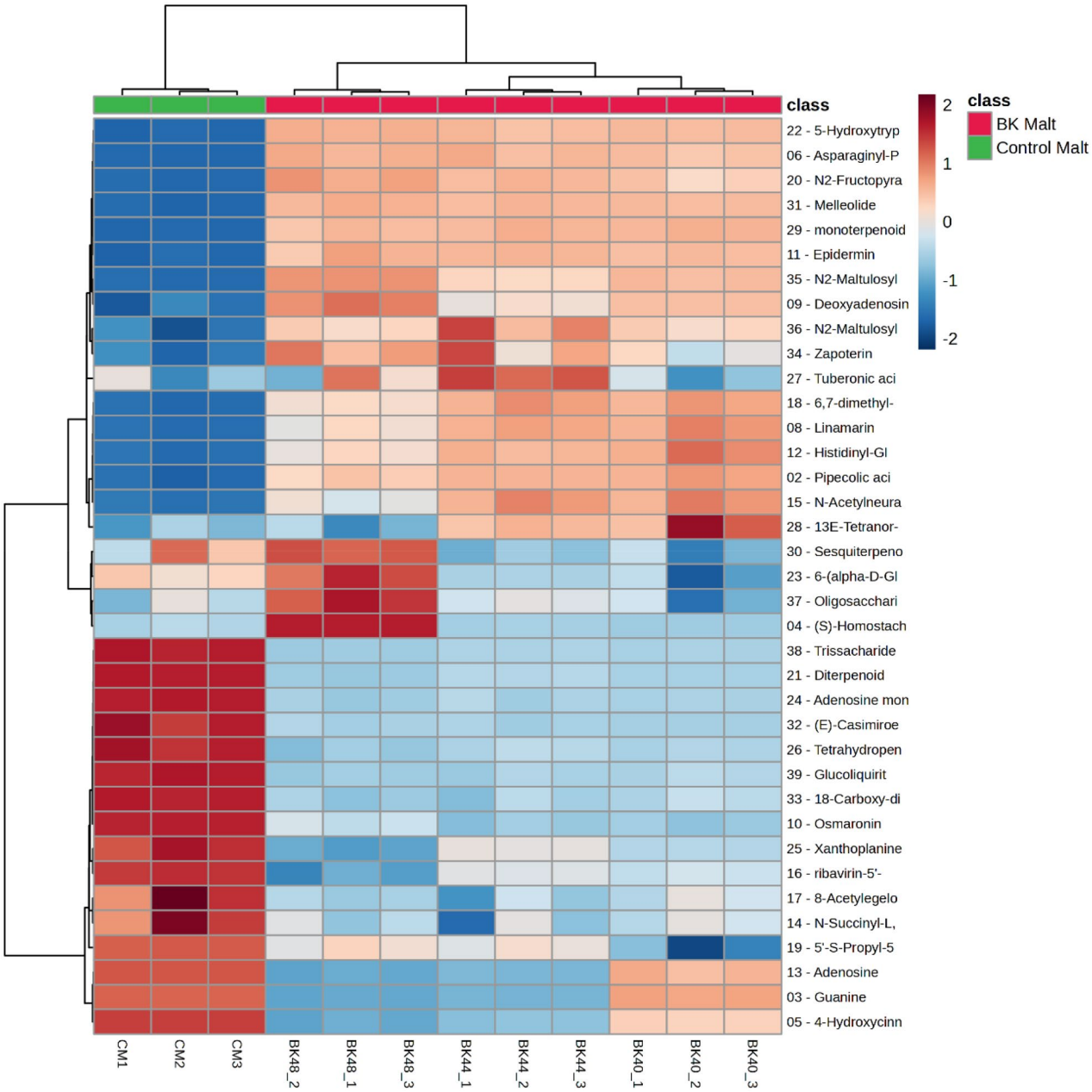

Figure 11. Hierarchical clustering heatmap comparing the base malt (M) and the BK-malts (BK40, BK44, BK48), based on the 39 metabolites identified. Samples and features were clustered using the Euclidean-Ward method. Colored cells represent the concentration of the variables in the samples (red = high concentration, blue = low concentration). Abbreviations: BK40, barley koji malt with 40 h of SSF; SSF, solid-state fermentation; BK44, barley koji malt with 44 h of SSF; BK48, barley koji malt with 48 h of SSF.

In contrast, compounds with significantly higher concentrations in the control malt included flavonoid glycosides (Glucoliquiritin apioside), trisaccharides, adenosine monophosphate, and diterpenoids. Diterpenoids, which are involved in plant lipid metabolism (e.g., cell signaling and membrane stabilization), were notably more abundant in the control malt.[59] It is also important to highlight that the control malt retained barley's husk and bran, whereas most of the lipids, terpenoids, flavonoids and other micronutrients are located in the outer layers of the barley, which are removed during the pearling process.[76]

The higher concentration of these compounds in the control malt may also suggest that the enzymatic activity of koji fermentation facilitated more extensive breakdown and transformation of certain metabolites. Conversely, the malting process appeared to preserve barley-derived compounds such as flavonoids and diterpenoids, which may contribute to the control malt's distinct profile and technological properties.

Association between metabolomics and MQA

To explore the relationship between metabolites and malt quality parameters (MQA), we conducted O-PLS regressions using metabolites as predictors (X variables) and MQA as responses (Y variables).

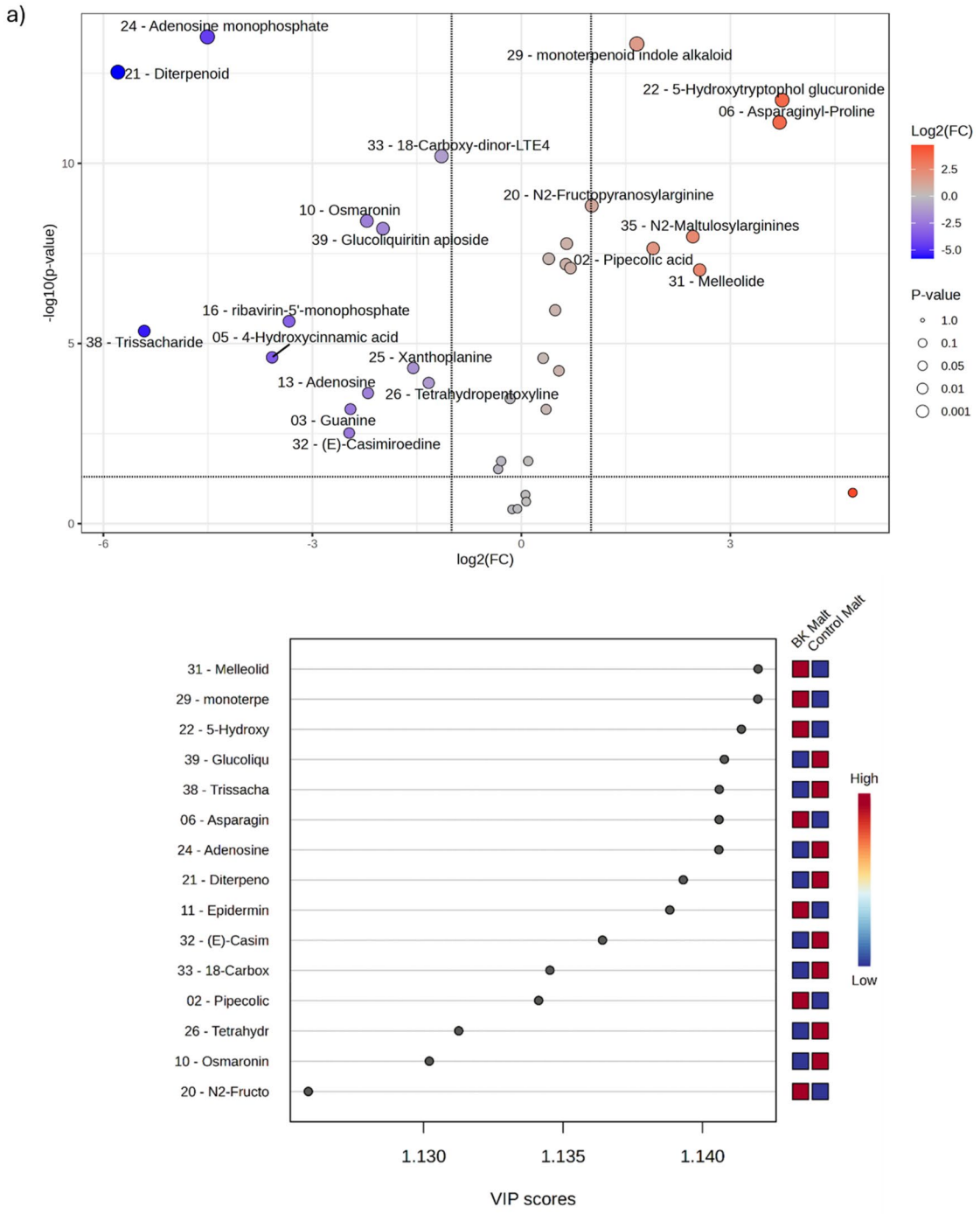

Figure 12. Volcano plot (a) and o-PLS (b) VIP plot for BKM and Control malts. The volcano plot considers BK/control. The features in blue (left) are significant and down-regulated in BKM and the red (right) significant and up-regulated in BKM, according to the threshold FDR < 0.1 and FC = 2.0. Abbreviations: o-PLS, orthogonal partial least squares; VIP, variable importance in projection; BKM, barley koji malt; FDR, false discovery rate; FC, fold change.

Trends in koji malts

When evaluating the three koji samples at different SSF times (Figure 13), the model generated four components with cumulative $R^2X = 0.98$, $R^2Y = 0.547$, and $Q^2 = 0.38$. Although the model captured most of the variability in X (predictors), it only moderately explained Y (responses), resulting in low classification power. It struggled to accurately classify some of the BK40 and BK44 samples, likely due to their similar technological characteristics.

Features near the border of the correlation plot were better represented in the model and primarily drove group separation. Models for color and β-glucan had strong predictive power ($R^2 > 0.7$, $Q^2 > 0.6$), while models for α-amylase and pH showed moderate predictive ability. Only the color model was statistically significant ($p < 0.05$).

BK48 appeared distinct from the other two samples and strongly associated with higher color and soluble protein, as well as lower FAN, pH, β-glucan, and α-amylase. It was

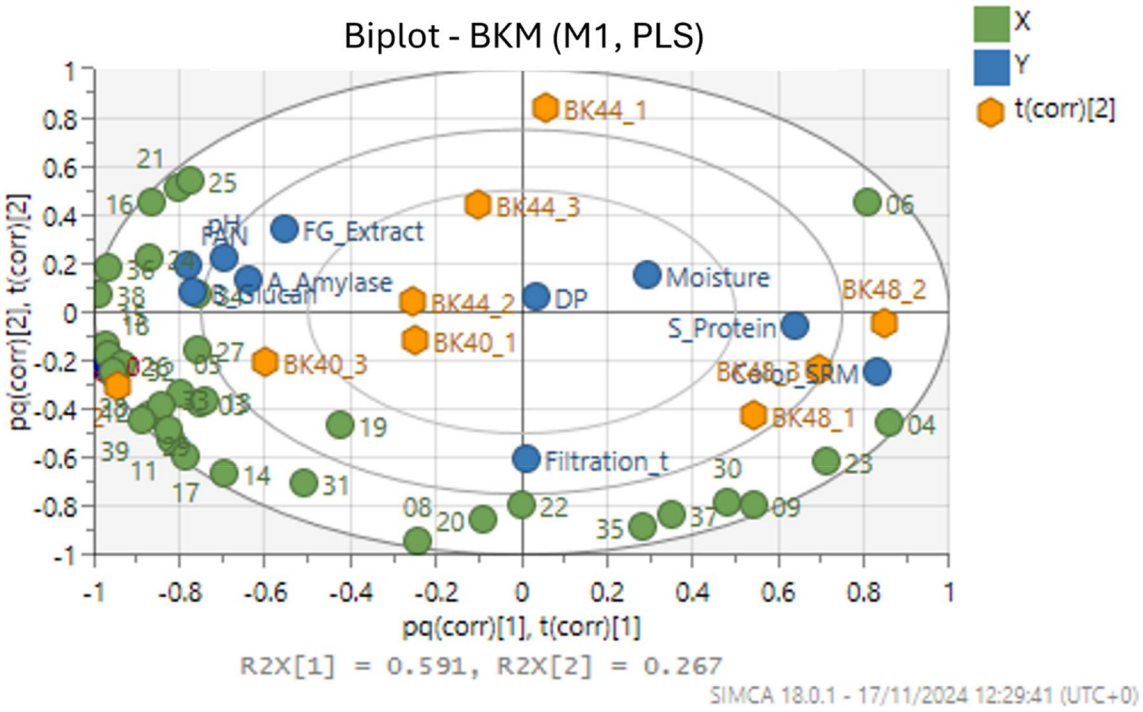

Figure 13. OPLS of koji malts identified metabolites (x) and MQA (y). Correlation scaled OPLS scores are plotted as orange hexagons, while loadings are represented as blue circles for MQA and green circles for the metabolites (numbers). Abbreviations: OPLS, orthogonal projection to latent structures; MQA, malt quality analysis; FG_Extract, extract fine grind dry basis; S_Protein, soluble protein; FAN, free amino nitrogen; DP, diastatic power; Filtration_t, filtration time. The numbers correspond to the metabolites.

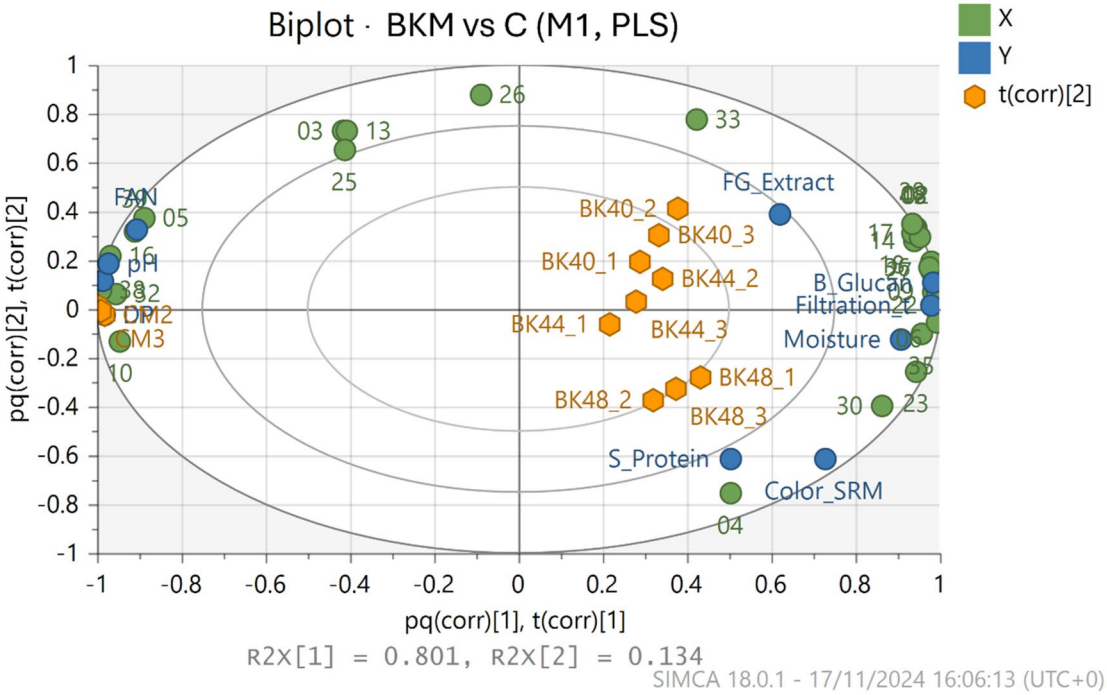

Figure 14. OPLS of koji and control malts identified metabolites (x) and MQA (y). Correlation scaled OPLS scores are plotted as orange hexagons, while loadings are represented as blue circles for MQA and green circles for the metabolites (numbers). Abbreviations: OPLS, orthogonal projection to latent structures; MQA, malt quality analysis; FG_Extract, extract fine grind dry basis; S_Protein, soluble protein; FAN, free amino nitrogen; DP, diastatic power; Filtration_t, filtration time. The numbers correspond to the metabolites.

linked to higher concentrations of metabolites such as Asparaginyl-Proline, (S)-Homostachydrine, and 6-(alpha-D-Glucosaminyl)-1D-myo-inositol. In contrast, it exhibited lower concentrations of most other compounds, like trisaccharides, acyl carnitine, pipecolic acid, the cyanogenic glycoside epidermin, and adenosine monophosphate.

The VIP compounds (Online Supplementary Figure S4.1) highlighted carbohydrate-derived compounds (e.g., 38, 15, 36), amino acid derivatives (e.g., 02, 12, 06), and products of lipid metabolism (e.g., 07). These metabolites are potential contributors to flavor and color formation (via Maillard reactions) and reflect enzymatic activity during fermentation. Alkaloids peaked at 44 h, suggesting they are secondary metabolites linked to mid-stage fermentation. Some compounds, such as riboflavin precursors (compound 18), terpenoids, flavonoids, and peptides also have potential health benefits.

Comparison with control malt

Comparing BKM with the control malt (M), the model generated two components ($R^2X = 0.935$, $R^2Y = 0.87$, $Q^2 = 0.736$), clearly separating the two groups in the OPLS biplot (Figure 14) due to large variations in metabolite concentrations and distinct technological properties.

The control malt (M) was positively associated with FAN, pH, DP, and metabolites like 4-Hydroxycinnamic acid, trisaccharides, and flavonoid glycosides (Glucoliquiritin apioside), reflecting typical malt characteristics and minimal enzymatic breakdown compared to koji malts.

Conversely, BKM were associated with β-glucan, filtration time, and moisture, highlighting the presence of undegraded polymers from barley or mold cell walls. Metabolites associated with BKM, such as tuberonic acid glucoside and the riboflavin precursor 6,7-dimethyl-8-(1-D-ribityl)lumazine, highlight the unique enzymatic and fungal metabolism during koji fermentation.

A clear separation among koji samples by SSF time was also visible in the biplot, strongly influenced by extract and compound 33 (18-Carboxy-dinor-LTE4), which were highly associated with BK40. In contrast, BK48 was associated with soluble protein, color, and metabolites such as (S)-Homostachydrine, Sesquiterpenoids, and 6-(alpha-D-Glucosaminyl)-1D-myo-inositol.

Conclusion

BKM produced with *Aspergillus oryzae* during SSF present a unique and innovative alternative to traditional specialty malts, with great potential for use in craft brewing. This study demonstrates their distinctive properties, including a high extract comparable to conventional base malts and rapid production time, and darker color profile even when using gentle kilning conditions. Their flavor profile and contributions to beer properties will be addressed in a forthcoming manuscript.

Steeping and SSF times were found to strongly influence malt characteristics. Extended steeping increased moisture content and decreased β-glucan degradation, whereas shorter steeping enhanced enzymatic activity. Therefore, we recommend a maximum of 6h of steeping for improved brewing applications.

Prolonged SSF enhanced soluble protein and malt color, attributed to substrate breakdown and Maillard reactions, while lowered the pH values, possibly due to the production of organic acids by *A. oryzae*. The decrease in β-glucan over 48 h was not enough to impact filtration time. Different malt color and potentially flavor profiles could be explored by varying SSF times up to 48 h. However, extended times increase the probability of sporulation, which could result in the development of off-flavors.

Future work should focus on exploring species and strain selection to uncover additional functional properties and improved β-glucan breakdown. Additionally, testing varied substrates, such as drought resistant and naked grains, would further expand the versatility, sustainability, and applications of BKM in brewing.

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CRediT: **Aline Duarte Correa De Brito**: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft; **Paulo Tulio De Souza Silveira**: Writing – review & editing; **Hannah Ulhmann**: Formal Analysis, Data Curation, Methodology; **Flavio Luis Schmidt**: Supervision, Writing – review & editing; **Jamie Sherman**: Supervision.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix

Table A1. List of identified compounds, including their retention times and m/z values.

Selected ID	Compound (retention time_m/z)	Description	Mass (g)	Retention time (s)	Chemical Formula	Score	Subclass
1	2.96_118.0863m/z	Valine	118.0863395	2.960	C5H11NO2	39.5	Amino acids, peptides, and analogues
2	6.96_130.0869m/z	Pipecolic acid	130.0869276	6.959	C6H11NO2	40.4	Amino acids, peptides, and analogues
3	4.80_152.0577m/z	Guanine	152.0577039	4.797	C5H5N5O	52.6	Purines and purine derivatives
4	0.86_158.1177m/z	(S)-Homostachydrine	158.117651	0.855	C8H15NO2	39.6	Amino acids, peptides, and analogues
5	6.83_165.0553m/z	4-Hydroxycinnamic acid	165.0553346	6.831	C9H8O3	42.9	Hydroxycinnamic acids and derivatives
6	9.91_212.1049m/z	Asparaginyl-Proline	212.1048554	9.913	C9H15N3O4	40.4	Amino acids, peptides, and analogues
7	6.49_216.1234m/z	Acyl Carnitine	216.1234326	6.492	C18H32O16	50.2	Fatty acid esters
8	6.65_247.1062n	Linamarin	248.1134313	6.649	C10H17NO6	41.4	Carbohydrates and carbohydrate conjugates
9	7.90_252.1083m/z	Deoxyadenosine	252.1082851	7.897	C10H13N5O3	49.7	Purine 2′-deoxyribonucleosides
10	7.94_259.1058n	Osmaronin	260.1130361	7.944	C11H17NO6	46.8	Fatty acyl glycosides
11	7.27_261.1214n	Epidermin	262.1286434	7.269	C11H19NO6	53.6	Carbohydrates and carbohydrate conjugates
12	6.65_266.1240m/z	Dipeptide	266.124026	6.649	C11H17N5O4	52	Amino acids, peptides, and analogues
13	4.57_268.1054m/z	Adenosine	268.1053508	4.565	C10H13N5O4	48.1	
14	8.20_273.1089m/z	N-Succinyl-L,L-2,6-diaminopimelate	273.1089247	8.200	C11H18N2O7	39.5	Amino acids, peptides, and analogues
15	7.45_292.1043m/z	N-Acetylneuraminic acid	292.1042963	7.448	C11H19NO9	40.4	Carbohydrates and carbohydrate conjugates
16	0.99_305.0290m/z	ribavirin-5′-monophosphate	305.0290303	0.994	C8H11N4O8P-2	39.6	
17	8.18_308.1232n	8-Acetylegelolide	309.1304358	8.179	C16H20O6	40.3	
18	7.25_326.1238m/z	6,7-dimethyl-8-(1-D-ribityl) lumazine	326.1237776	7.251	C13H17N4O6-	42.9	
19	6.96_326.1292m/z	5′-S-Propyl-5′-thioadenosine	326.1292205	6.959	C13H19N5O3S	43.2	5′-deoxy-5′-thionucleosides
20	8.40_337.1723m/z	N2-Fructopyranosylarginine	337.1722996	8.404	C12H24N4O7	46.7	Organic acids and derivatives
21	0.84_337.1799m/z	Diterpenoid	337.1798803	0.844	C20H26O3	43.5	Diterpenoids
22	4.18_340.1399m/z	5-Hydroxytryptophol glucuronide	340.1398662	4.180	C16H21NO7	44.9	Carbohydrates and carbohydrate conjugates
23	6.93_342.1406m/z	6-(alpha-D-Glucosaminyl)-1D-myo-inositol	342.1405897	6.934	C12H23NO10	46.8	Carbohydrates and carbohydrate conjugates
24	9.68_348.0713m/z	Adenosine monophosphate	348.0713253	9.677	C10H14N5O7P	39.6	Purine ribonucleotides
25	0.90_356.1880n	Xanthoplanine	357.1952339	0.901	C21H26NO4+	40	
26	7.09_366.1462n	Tetrahydropentoxyline	367.1535258	7.088	C17H22N2O7	42	
27	7.24_387.1666n	Tuberonic acid glucoside	388.1738682	7.244	C18H27O9-	43.9	Fatty acyl glycosides
28	8.46_396.1513m/z	13E-Tetranor-16-carboxy-LTE4	396.151328	8.465	C19H27NO7S	43.2	Amino acids, peptides, and analogues
29	7.28_406.1825m/z	(3R)-3-hydroxy-16-methoxy-1,2-didehydro-2,3-dihydrotabersonine	406.1825251	7.284	C22H27N2O4+	41.9	
30	8.03_413.2134m/z	Sesquiterpenoids	413.2134335	8.026	C19H34O8	42.9	Fatty acyl glycosides
31	6.57_425.1934m/z	Melleolide	425.1934126	6.574	C23H30O6	44.5	Sesquiterpenoids
32	8.59_440.1775m/z	(E)-Casimiroedine	440.177535	8.589	C21H27N3O6	41.7	Carbohydrates and carbohydrate conjugates
33	7.98_442.1931m/z	18-Carboxy-dinor-LTE4	442.1930719	7.983	C21H31NO7S	39.9	Eicosanoids
34	8.85_470.1977n	Zapoterin	471.2050154	8.846	C26H30O8	43.1	Triterpenoids
35	8.51_481.2135m/z	N2-Maltulosylarginine	481.2135392	8.511	C18H34N4O12	48.7	
36	8.70_499.2253m/z	N2-Maltulosylarginine	499.225347	8.511	C18H34N4O12	48.7	
37	7.62_504.1960m/z	beta-D-Galactopyranosyl-(1->4)-2-amino-2-deoxy-beta-D-glucopyranosyl-(1->6)-D-mannose	504.1960197	7.616	C18H33NO15	43	Carbohydrates and carbohydrate conjugates
38	6.36_527.1595m/z	Oligosaccharide	527.1595457	0.000	C18H32O16	50.2	Carbohydrates and carbohydrate conjugates
39	7.61_735.2081m/z	Glucoliquiritin apioside	735.2081181	7.609	C32H40O18	44.3	Flavonoid glycosides