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Comparative analysis of functional components in Sakekasu (Sake lees)

Yuto Nishidono[1,2], Shingen Misaka[3], Yuko Maejima[3], Kenju Shimomura[3], Ken Tanaka[1*]

[1]College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Noji-Higashi, Kusatsu, Shiga 525-8577, Japan; [2]Research Organization of Science and Technology, Ritsumeikan University, 1-1-1 Noji-Higashi, Kusatsu, Shiga 525-8577, Japan; [3]Department of Bioregulation and Pharmacological Medicine, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima, Fukushima 960-1295, Japan

***Corresponding author:** Ken Tanaka, Ph.D., College of Pharmaceutical Sciences, Ritsumeikan University1-1-1 Noji-Higashi, Kusatsu, Shiga 525-8577, Japan.

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ABSTRACT

Background: Sake lees (Sakekasu), a byproduct of sake production, has been recently attracting attention as a functional food. Sakekasu is rich in nutrients and contains glycerophosphocholine (GPC) and *S*-adenosylmethionine (SAM), which are well-known functional compounds. The content of these compounds in Sakekasu depends on a variety of factors, including fermentation conditions, especially the method and length of ripening. These differences are reflected prominently in the color of Sakekasu, which becomes darker due to the long ripening period and high drying temperature.

Objective: This study aimed to clarify the contents of functional components in Sakekasu with different color tones (i.e., ripening period).

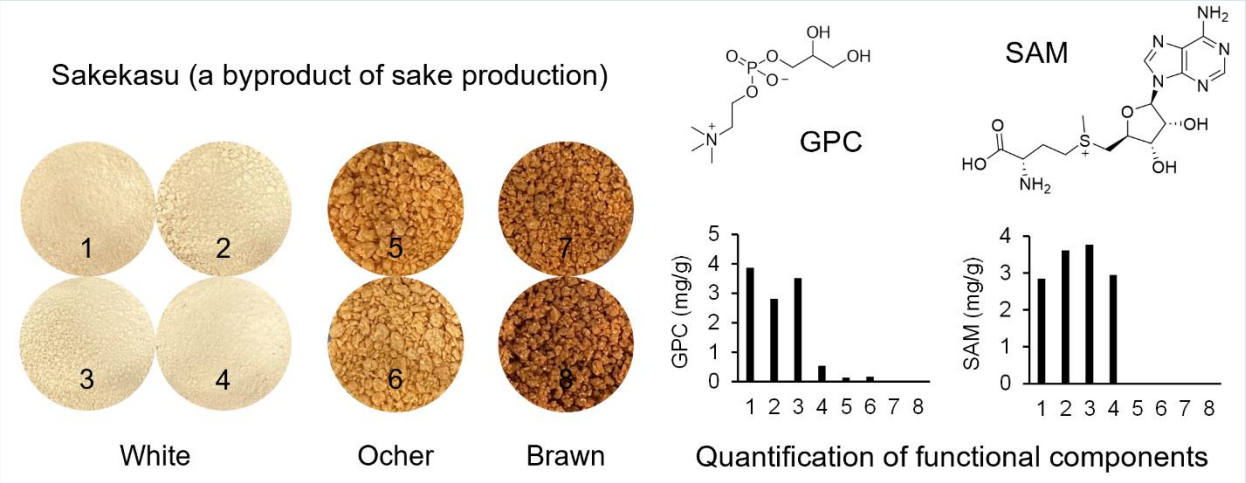
Methods: Three types of Sakekasu with different color tones (white, ocher, and brawn) were collected from several breweries. The contents of multiple functional components in their extracts were determined by liquid chromatography coupled to high resolution ion-trap/time-of-flight mass spectrometry.

Results: Sakekasu with white color had more abundant GPC, SAM, and fatty acids than those with darker color. However, ethyl glucoside and glyceryl glucosides did not differ significantly by color tone. Furthermore, the Maillard reaction products of sugar and dipeptide were mainly found in dark-colored Sakekasu, and their structures were annotated by

tandem mass spectrometry.

Conclusions: This study has clarified many functional compounds in Sakekasu in relation to color tone (i.e., ripening period) and highlighted the potential of Sakekasu with white color tone as a functional food.

Keywords: Sakekasu, functional components, glycerophosphocholine, *S*-adenosylmethionine, tandem mass spectrometer



INTRODUCTION

Consumers are growing increasingly aware of the significance of diet in human health leading to an increase in demand for functional foods [1, 2]. The debate over the definition of functional food has persisted for many years. In 2021, the Functional Food Center proposed the following definition of the term 'functional food': “Natural or processed foods that contain biologically-active compounds, which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms” [3]. To place foods into the functional food category, various approaches have been proposed, such as a 16-step course [4] and Functional Food Development Cycle [1]. One important step in these processes is the identification of functional components, which are the element of classifying functional foods [4].

Sake is a traditional Japanese alcoholic beverage. During its production process, steamed rice is first fermented with *Aspergillus oryzae* to make “*koji*” mold. Then, *koji* mold, steamed rice, and *Saccharomyces cerevisiae* (Sake yeast) are mixed, and ethanol fermentation occurs at the oar. Subsequently, the fermented product is filtered and separated into liquid and solid components. The solid portion is called “Sakekasu” (sake lees). Sakekasu is rich in protein, peptides, amino acids, carbohydrates, dietary fiber, fat, ash, and vitamins and has traditionally been used as an ingredient in food processing and as a moisturizer in cosmetics in Japan [5].

Sakekasu has been recently attracting attention as a functional food, with numerous reported effects, such

as antidiabetic [6], osteoporosis-preventive [7], and anti–colon cancer effects [8]. It also improves hepatic lipid accumulation [9, 10], inhibits acute alcohol-induced liver damage [11], alleviates pain sensitivity [12], and prevents allergic rhinitis–like symptoms [13]. Other than Sakekasu's nutritional components, its functional ingredients have been widely studied. For example, peptides obtained from the hydrolyzed products of Sakekasu inhibit angiotensin-converting enzyme and suppress hypertension [14]; they also inhibit prolyl endopeptidase, which is involved in amnesia development [15]. Furthermore, ethyl glucoside (**1**) (Figure 1), which is relatively abundant in Sakekasu, has hepatoprotective [16] and diuretic effects [17] and improves the functions of the stratum corneum [18]. Sakekasu also contains *S*-adenosylmethionine (SAM) (**3**) (Figure 1), which is reportedly effective in treating diseases such as alcoholic liver dysfunction and depression [19]. In recent years, glycerophosphocholine (GPC) (**6**) found in Sakekasu has attracted attention for its anti-aging and brain function improvement effects (Figure 1) [20]. The GPC- and SAM-associated metabolic pathways (choline pathway and methionine pathway, respectively) are related via betaine, and the relationship between their content remains insufficiently understood.

The composition of these functional components greatly varies depending on the fermentation conditions and especially length of aging of Sakekasu in different breweries. However, to date, only changes in Sakekasu composition caused by the drying method have been reported [21], and changes caused by aging remain uninvestigated. Differences in the degree of maturity of Sakekasu are reflected in the color. Therefore, in the present study, to clarify the relationship between the contents of the functional components in Sakakasu and color tones, we collected Sakekasu with white, ocher, and brawn color tones from several sake breweries and comprehensively analyzed their functional components.

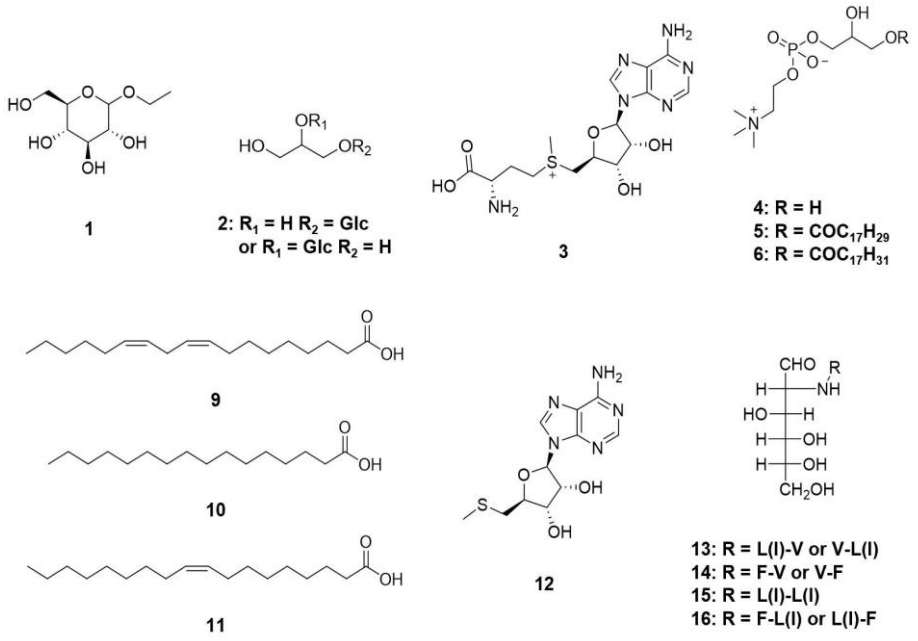


Figure 1. Structures of the compounds detected. **1**: ethyl glucoside, **2**: glyceryl glucosides, **3**: *S*-adenosylmethionine (SAM), **4**: glycerophosphocholine (GPC), **5**: linolenoyl-glycero-phosphocholine, **6**: linoleoyl-glycero-phosphocholine, **9**: linoleic acid, **10**: palmitic acid, **11**: oleic acid, **12**: 5'-deoxy-5'-methylthioadenosine, **13**: fructose-leucine (isoleucine)-valine or fructose-valine-leucine (isoleucine), **14**: fructose-phenylalanine-valine or fructose-valine-phenylalanine, **15**: fructose-leucine (isoleucine)-leucine (isoleucine), **16**: fructose-phenylalanine-leucine (isoleucine) or fructose-leucine (isoleucine)-phenylalanine. Glc, Glucose; L, leucine; I, isoleucine; V, valine, F, phenylalanine.

METHODS

Specimens and reagents: We obtained eight samples of Sakekasu from Sasanokawa Shuzo Co. Ltd. (Fukushima, Japan) and two other brewery companies. (Aichi, Japan and Kyoto, Japan). Samples 1–4 were white (R: 255, G: 242, B: 207), 5–6 were ocher (R: 223, G: 170, B: 105), and 7–8 were brawn (R: 169, G: 98, B: 53). These color differences are caused by the ripening period and temperature of the Sakekasu. The color of samples becomes darker due to the long ripening period and high drying temperature [22]. All samples were lyophilized and stored at 4°C until analysis. We purchased analytically grade chemicals and chromatographic solvents (liquid chromatography–mass spectrometry [LC–MS] grade) from Fujifilm Wako Pure Chemical (Osaka, Japan), and SAM and GPC from Nacalai Tesque (Kyoto, Japan).

Instrumentation and analysis: For LC–MS analyses, we used the mass spectrometer Shimadzu LC–IT–TOF (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface. The ESI parameters were as follows: source voltage, +4.5 kV in positive ion mode and −3.5 kV in negative ion mode; capillary temperature, 200°C; and nebulizer gas flow rate, 1.5 L/min. We used the mass spectrometer in positive and negative ion modes and recorded the scans from *m/z* 150 to 1500. GPC and its relating compounds were separated using the HILIC column Waters XBridge BEH amide (2.1 × 150 mm, 5 µm) at 40°C consistently. The binary mobile phase consisted of (A) 5 mM CH_3COONH_4 in water and (B) CH_3CN. These compounds were eluted using the following gradient conditions: 0–30 min, linear gradient from 95% to 45% B, and 30–40 min of isocratic solution at 45% B. Furthermore, SAM was separated using the column Thermo Fisher Scientific Hypercarb (2.1 × 100 mm, 3 µm, 40°C), and the binary mobile phase consisted of (A) 0.1% HCOOH in water and (B) CH_3CN. The compound was eluted using the following gradient conditions: 0–10 min, linear gradient from 0% to 60% B, 10–12 min linear gradient from 60% to 80% B, and 12–15 min of isocratic solution at 80% B. Other compounds were separated using the ODS column Waters Atlantis T3 (2.1 × 150 mm, 5 µm, 40°C), and the binary mobile phase consisted of (A) 5 mM CH_3COONH_4 in water and (B) CH_3CN. The compounds were eluted using the following gradient conditions: 0–30 min, linear gradient from 10% to 100% B, and 30–40 min of isocratic solution at 100% B.

Extraction of constituents from Sakekasu: To extract GPC and SAM, we first pulverized freeze-dried Sakekasu specimens and mixed each 500 mg of the sample with 5 mL of 50% methanol. These extraction mixtures were ultrasonicated for 10 min and then left overnight at room temperature. On the following day, we filtered the extracts through 0.45 µm Millipore filter units (Advantec, Tokyo, Japan) and injected 1 µL of the sample into the LC–MS. Other compounds were extracted using other conditions. For example, 10 g of the fine powder of freeze-dried Sakekasu specimens was accurately weighted, and the constituents were extracted with methanol using the Extraction System B-811 LSV (BUCHI, Flawil, Switzerland) under reflux conditions for 100 min. The organic solvent was evaporated in vacuo to collect the methanol extract. Additionally, 2 mg of the extract was dissolved in 1 ml of methanol–water mixture (1:1 by vol.). All extracts were filtered through 0.45 µm Millipore filter units (Advantec), and 1 µL of the sample was injected into the LC–MS.

RESULTS AND DISCUSSIONS

Quantitation of GPC and SAM in Sakekasu: The total ion

chromatograms and the mass chromatograms of the extracts analyzed using the HILIC column are shown in Figure 2. Especially, Figure 2 (f) illustrates the mass chromatograms monitored by the M^+ ion of GPC (**4**, *m/z* 258.1102), while Table 1 lists the quantitation results of GPC in Sakekasu samples. The white Sakekasu in samples 1–4, which are thought to have been aged for a shorter period, had higher GPC concentrations than aged samples 5–8 (Table 1). Notably, it was not detected in the brawn colored Sakekasu (samples 7 and 8), which appeared to have been aged for a long time (Table 1). It is well known that GPC is abundant in dried salmon and rainbow trout [23], and the GPC content of white Sakekasu was comparable to these products. In addition, samples with high GPC concentrations contained linolenoyl-glycero-phosphocholines (**5**) and linoleoyl-glycero-phosphocholines (**6**) [Figure 2 (g) and (h)]. These compounds are active ingredients that inhibit inflammatory cytokine production [24] and contribute to Sakekasu functionality. In experiments using aged mice, Matsubara *et al.* reported that the deposition of transthyretin, an amyloidogenic protein, in the brain was reduced by additional 17% GPC intake [20]. In the present study, the GPC concentration in Sakekasu is very high and is considered to be a concentration at which efficacy can be expected with daily ingestion.

Table 1. Concentrations of glycerophosphocholine and *S*-adenosylmethionine in Sakekasu samples.

Compounds	**Samples (mg/g)**							
	1	2	3	4	5	6	7	8
Glycerophosphocholine	3.87	2.82	3.52	0.55	0.14	0.17	0.00	0.00
***S*-Adenosylmethionine**	2.84	3.62	3.77	2.95	0.00	0.00	0.00	0.00

Figure 2 also shows a mass chromatogram monitored by the ion M^+ of SAM (**3**) [Figure 2 (e)]. The peaks show a broad shape, making quantitation by this method difficult. Krijt *et al.* reported SAM quantitation using a Hypercarb column [25]. Hypercarb columns have unique properties that distinguish them from conventional columns, and they excel in retaining and separating highly polar compounds. Figure 3 shows the total ion chromatograms analyzed using a Hypercarb column and mass chromatograms monitored by the M^+ ion of SAM. The amount of SAM in Sakekasu varied greatly from sample to sample, with a very high content in white Sakekasu (samples 1–4) [Figure 3 (c)]. Conversely, SAM was not detected at all in ocher and brawn-colored samples (samples 5–8) (Table 1). SAM is known for its mood-improving, anti-hepatotoxic, and anti-arthritic effects [26]. Sakekasu with a high SAM content is considered useful as a functional material.

The metabolic pathways associated with GPC (choline pathway) and SAM (methionine pathway) are related via betaine. In our study, samples with high GPC also had high SAM; when Sakekasu containing high GPC and SAM contents is ingested, a synergistic effect of both functionalities can be expected.

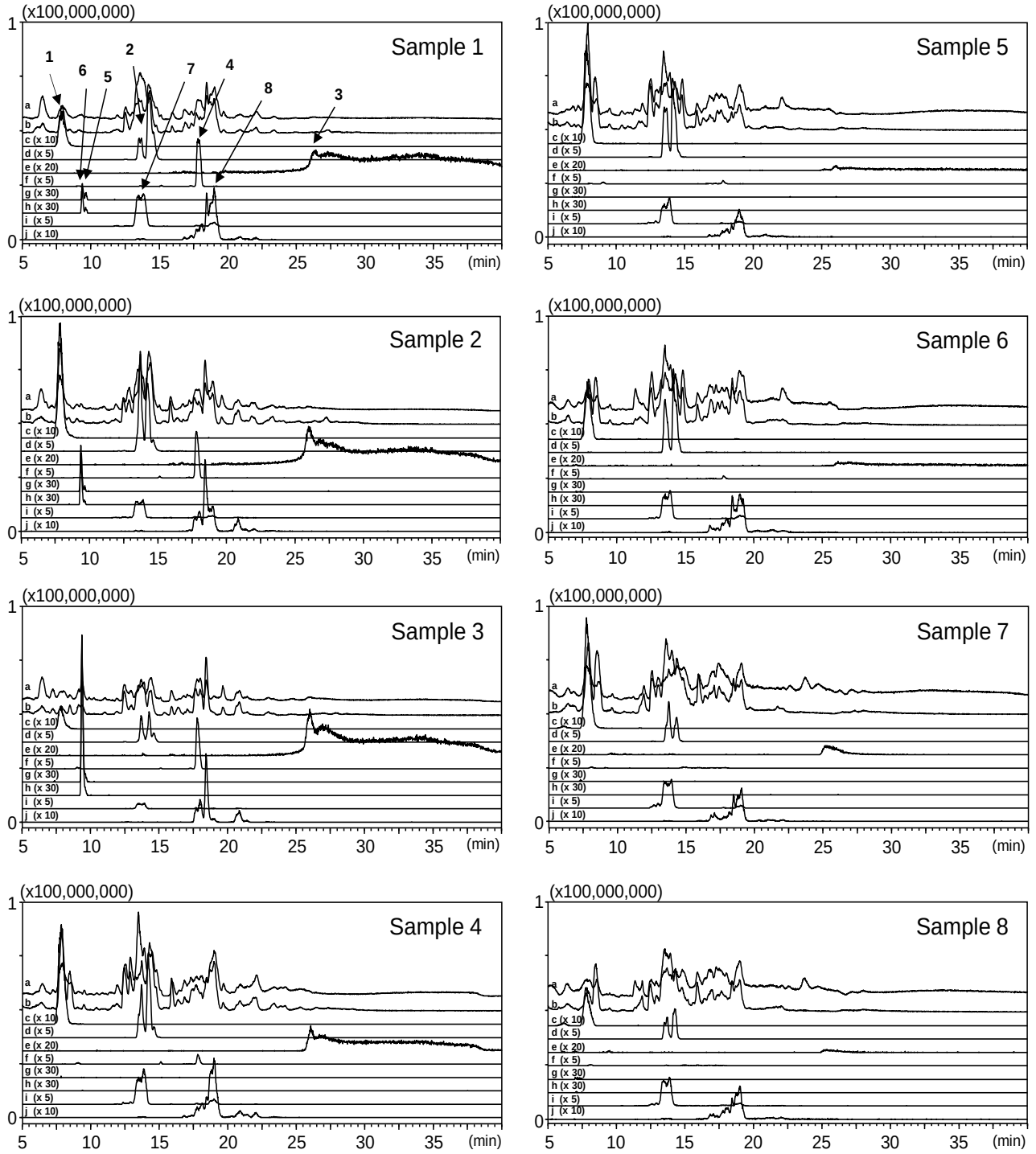


Figure 2. Total ion chromatograms and mass chromatograms of the extracts analyzed using the HILIC column.

Samples 1–4 were white, 5–6 were ocher, and 7–8 were brown. The following ions were monitored (numbers in brackets indicate magnification). a: positive ion, b: negative ion, c: *m/z* 231.0829 ([M+Na]$^+$ ion of ethyl glucoside [**1**]), d: *m/z* 277.0897 ([M+Na]$^+$ ion of glyceryl glucosides [**2**]), e: *m/z* 399.1460 (M$^+$ ion of *S*-adenosylmethionine [**3**]), f: *m/z* 258.1102 (M$^+$ ion of glycerophosphocholine [**4**]), g: *m/z* 520.3428 (M$^+$ ion of linolenoyl-glycero-phosphocholines [**5**]), h: *m/z* 522.3232 (M$^+$ ion of linoleoyl-glycero-phosphocholines [**6**]), i: *m/z* 179.0575 ([M–H]$^-$ ion of monosaccharides [**7**]), j: *m/z* 341.1105 ([M–H]$^-$ ion of disaccharides [**8**]).

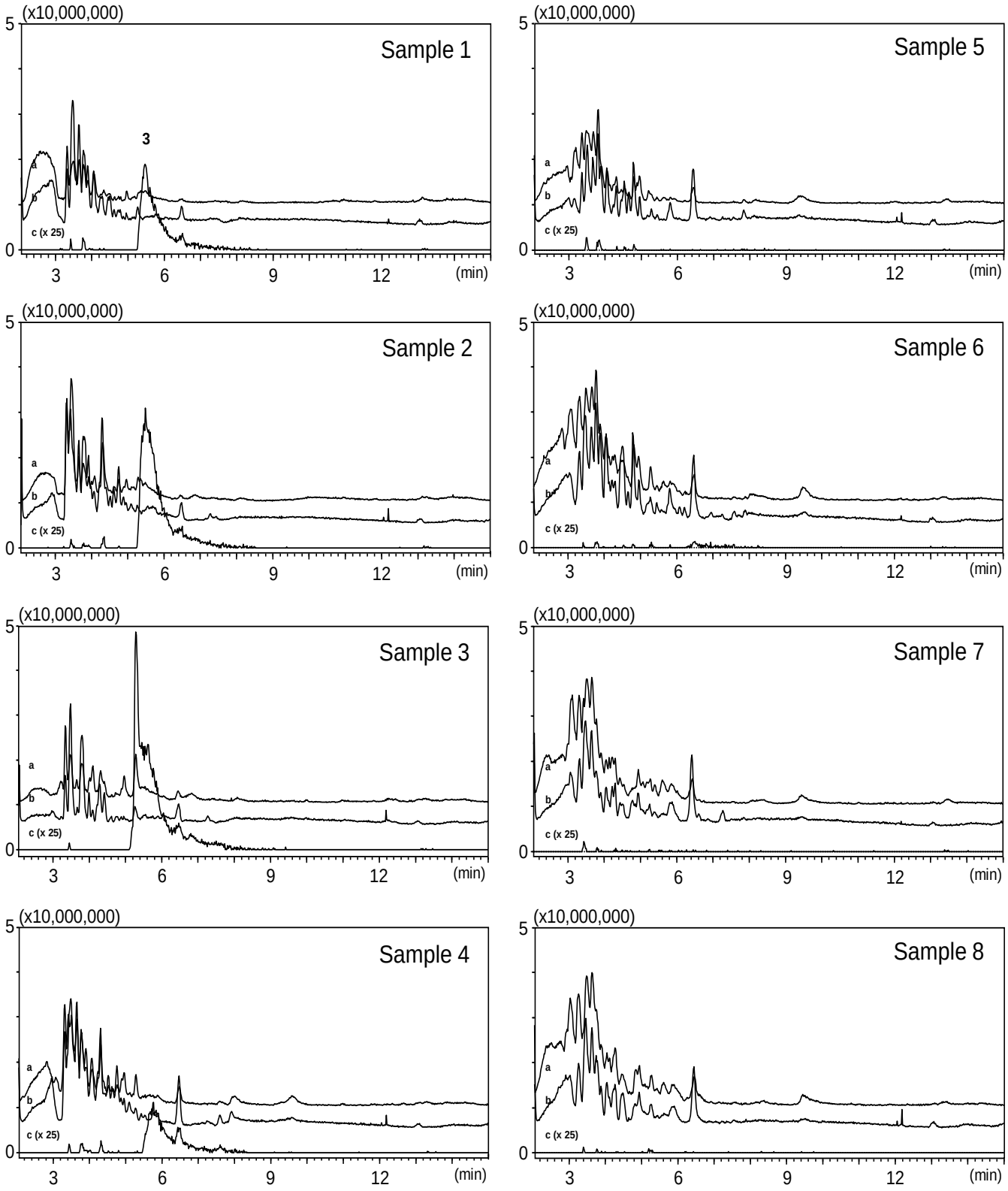


Figure 3. Total ion chromatograms and mass chromatograms of the extracts analyzed using the Hypercarb column. Samples 1–4 were white, 5–6 were ocher, and 7–8 were brawn. The following ions were monitored (numbers in brackets indicate magnification). a: positive ion, b: negative ion, c: *m/z* 399.1460 (M^+ ion of *S*-adenosylmethionine [**3**]).

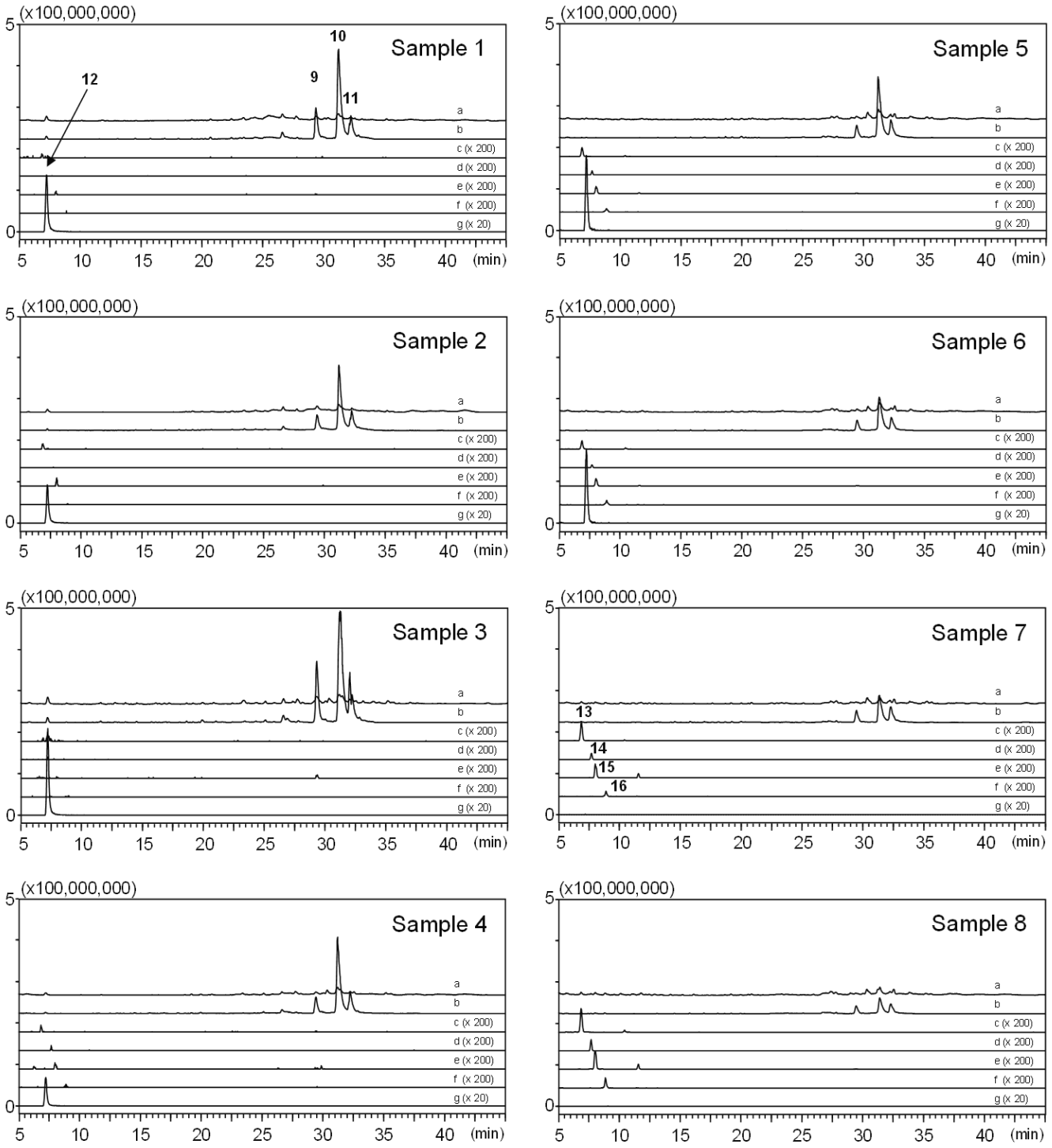


Figure 4. Total ion chromatograms and mass chromatograms of the extracts analyzed using the ODS column.

Samples 1–4 were white, 5–6 were ocher, and 7–8 were brawn. The following ions were monitored (numbers in brackets indicate magnification). a: positive ion, b: negative ion, c: *m/z* 393.2227 ($[M+H]^+$ ion of fructose-leucine (isoleucine)-valine and fructose-valine-leucine (isoleucine) [**13**]), d: *m/z* 427.2089 ($[M+H]^+$ ion of fructose-phenylalanine-valine and fructose-valine-phenylalanine [**14**]), e: *m/z* 407.2393 ($[M+H]^+$ ion of fructose-leucine (isoleucine)-leucine (isoleucine) [**15**]), f: *m/z* 441.2226 ($[M+H]^+$ ion of fructose-phenylalanine-leucine (isoleucine) and fructose-leucine (isoleucine)-phenylalanine [**16**]), g: *m/z* 298.0950 ($[M+H]^+$ ion of 5'-deoxy-5'-methylthioadenosine [**12**]).

Identification of other metabolites in Sakekasu: Figure 2 (c), (d), (i), and (j) shows the mass chromatograms monitored by the $[M+Na]^+$ ion of ethyl glucoside (**1**, *m/z* 231.0829), $[M+Na]^+$ ion of glyceryl glucosides (**2**, *m/z* 277.0897), $[M–H]^–$ ion of monosaccharides (**7**, *m/z* 179.0575), and $[M–H]^–$ ion of disaccharides (**8**, *m/z* 341.1105). These sugar derivatives were detected in all samples, although their concentrations varied. Ethyl glucoside (**1**) is particularly abundant in sake; it originates from the unique fermentation method of sake wherein starch saccharification and alcohol fermentation occur simultaneously [27]. Ethyl glucoside not only affects the taste of sake but also possesses several functional properties, such as improving skin texture [27, 28]. Given that the amount of ethyl glucoside does not depend on the color-tone, it is expected to have a constant functionality. Furthermore, glyceryl glucoside (**2**), which has moisturizing properties, was also detected in sake [29]. Considering the differences in the position and conformation of sugar bonds in glycerol, several compounds have been detected, but the analytical conditions used in the present study did not allow them all to be separated. However, they were largely separated as two peaks [Figure 2 (d)], possibly owing to the difference in the position of sugar binding in glycerol (**2**). In addition, their content was not dependent on the color tone of Sakekasu.

The methanol extracts of each sample were analyzed using an ODS column and the results are shown in Figure 4. Samples 1–4 contained higher amounts of fatty acids [linoleic acid (**9**), palmitic acid (**10**), and oleic acid (**11**)] than brown-colored samples 7 and 8. In Figure 4, the mass chromatograms shown in c–f are Amadori or Heyns products derived from the Maillard reaction of sugar and dipeptide monitored by the $[M+H]^+$ ion of the respective compounds, whereas that shown in Figure 4 (g) is 5′-deoxy-5′-methylthioadenosine (MTA, **12**) monitored by the $[M+H]^+$ ion. As shown clearly in Figure 4, white Sakekasu samples 1–4 contained fewer compounds derived from the Maillard reaction of sugar and dipeptides (**13**–**16**) than the brown-colored samples 7 and 8. In addition, MTA (**12**) was reduced in samples 7 and 8 [Figure 4 (g)], which were ripened. Tadenuma *et al.* reported that MTA and its precursor SAM are unique to sake and not found in other brews, and that MTA is formed from SAM during sake storage [30, 31]. They also observed that the elution of SAM from yeast increased after the addition of brewing ethanol, indicating that the MTA and SAM amounts are proportional to the ethanol concentration [30, 31]. Considering that the detailed production methods for samples 1–4 analyzed in our study are unknown, we cannot confirm whether such result is a characteristic of the yeast used or of the production process; however, the high MTA and SAM contents in samples 1–4 may be a characteristic of these samples. Moreover, compounds derived from the Maillard reaction of sugar and dipeptide tended to be extremely low in samples 1–4 than in other Sakekasu samples.

The compounds derived from the Maillard reaction were annotated by tandem mass spectrometry (MS/MS) analysis. Figure 5 shows the MS/MS analysis of the peak of compound **13** observed in the mass chromatogram depicted in Figure 4 (c). Compound **13** possessed a $[M+H]^+$ ion at *m/z* 393.2227 with a composition of $[C_{17}H_{32}N_2O_8+H]^+$. According to the composition of the ions, the Amadori or Heyns product of a dipeptide was assumed to be composed of hexose, leucine (isoleucine), and valine. Andruszkiewicz *et al.* reported that the reaction of fructose with peptides produces mainly the Heyns product and that the reaction of glucose with peptides produces mainly the Amadori product [32]. Furthermore, Yuan *et al.* investigated in detail the fragmentation attribution in the MS analysis of Heyns products [33, 34]. Based on these reports, the structure

of compound **13** was estimated by MS/MS analysis. The MS/MS analysis of compound **13** from the $[M+H]^+$ ion at *m/z* 393.2227 detected a $[M+H–3H_2O–H_2CO]^+$ ion at *m/z* 309.1806 as the characteristic ion, and the MS^3 analysis from the $[M+H–3H_2O–H_2CO]^+$ ion provides fragment ions at *m/z* 150.0916 $[Fructose\text{-}L(I)\text{-}V+H–3H_2O–H_2CO–x2]^+$ and *m/z* 164.1070 $[Fructose\text{-}V\text{-}L(I)+H–3H_2O–H_2CO–x2]^+$, where *x2* indicates a type of dipeptide cleavage (Figure 5). Figure 5 also shows the results of the estimation based on Yuan *et al.*'s report [33, 34]. According to these results, compound **13** is a Heyns product composed of fructose (Fru) and leucine (isoleucine)-valine or valine-leucine (isoleucine). Similarly, the MS analysis results considered Fru-F-V and Fru-V-F as compound **14**, Fru-L(I)-L(I) as compound **15**, and Fru-F-L(I) and Fru-L(I)-F as compound **16**.

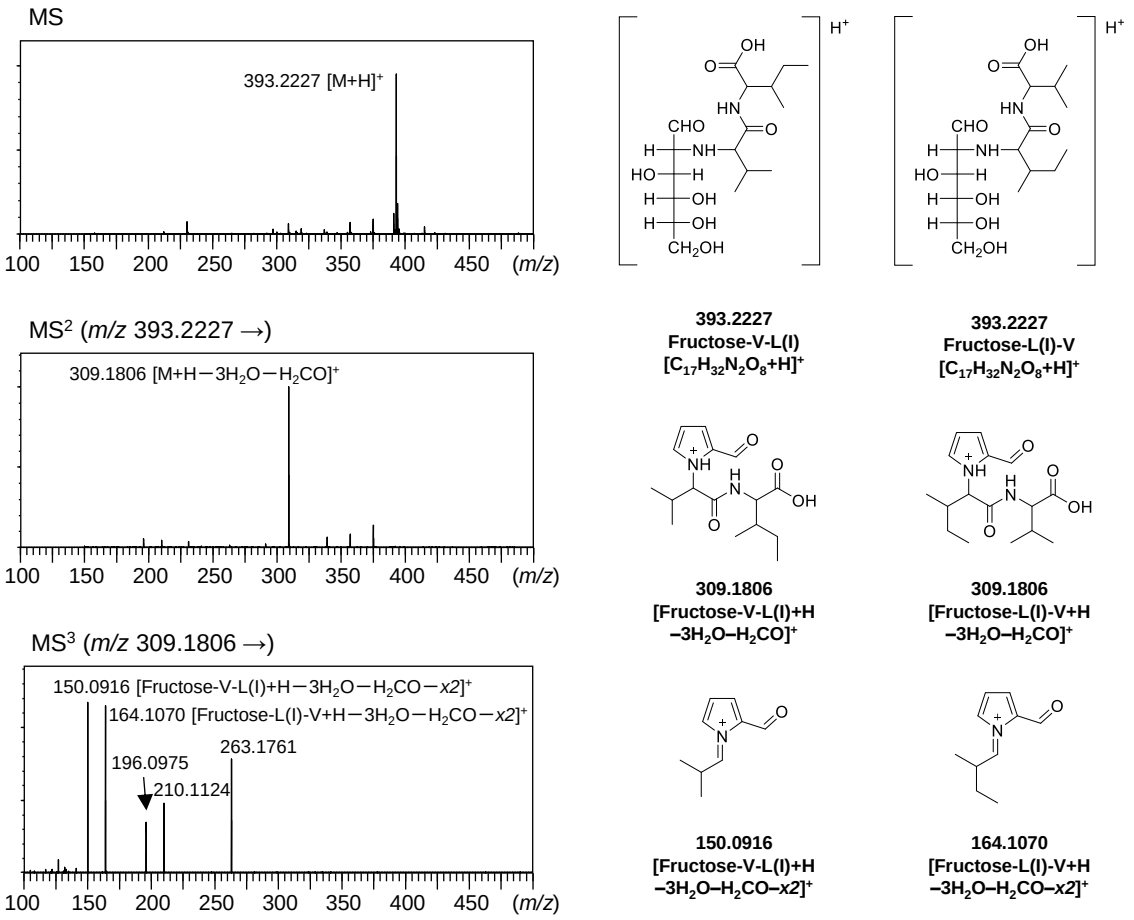


Figure 5. MS^n spectra from *m/z* 393.2227 ($[M+H]^+$ ion of fructose-leucine [isoleucine]-valine or fructose-valine-leucine [isoleucine] [**13**]) observed in Figure 4, and estimated structure of their ions. L, leucine; I, isoleucine; V, valine.

CONCLUSION

Several types of Sakekasu with different color tones were collected and analyzed for major functional ingredients such as GPC and SAM. Sakekasu with white color had large amounts of GPC and SAM. The concentration of GPC in Sakekasu was the highest ever found in a food product, and the daily consumption of this product may be sufficient for its efficacy. Moreover, compounds with suggested functionality, such as ethyl glucoside and glyceryl glucosides, were detected, and they were not

greatly influenced by color tone (i.e., aging period). Furthermore, the dark colored Sakekasu samples contained more Maillard reaction products of sugars and dipeptides, whereas the white ones showed more fatty acids. Through MS/MS based analysis, the Maillard reaction products of sugars and dipeptides were assessed. Thus, this study has clarified many functional compounds in Sakekasu in relation to color tone (i.e., ripening period) and emphasized the potential of Sakekasu with white color tone, which are thought to have been aged for a shorter period, as a functional food. Further biological studies on Sakekasu containing functional components such as GPC and SAM are expected.

Competing interest: There are no conflicts of interest to declare.

Author contribution: YN analyzed data and editing and revised the manuscript. SM, YM, and KS designed the study. KT obtained fundings, designed the study, performed the experiments, analyzed data, wrote the manuscript, and provided overall supervision. All authors have read and approved the final manuscript.

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