JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Relationship between Medium-Chain Fatty Acid Contents and Organoleptic Properties of Japanese Sake

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Supporting Information

ABSTRACT: Medium-chain fatty acids (MCFAs) and ethyl esters are considered to contribute to some organoleptic properties, such as fatty odor and bitterness in Japanese sake. However, the relationships between these compounds and the organoleptic properties of sake remain unclear. Here, we quantified MCFAs and ethyl hexanoate in *ginjo* sake using gas chromatography with a flame ionization detector (GC-FID). The hexanoic acid concentration strongly correlated with fatty odor ($p < 0.0001$). The octanoic acid/hexanoic acid ratio correlated with butanoic acid concentration, which is likely correlated with inharmonious bitter taste. Multiple comparison analysis revealed that the ethyl hexanoate level was negatively correlated with bitterness. We then identified other chemical compounds correlating with fatty odor and bitterness using comprehensive two-dimensional GC coupled with time-of-flight mass spectrometry. By performing correlation analysis between certain compounds and sensory values following statistical selection for chemical compounds, we identified several candidate compounds correlating with fatty odor and bitterness in sake.

KEYWORDS: *medium-chain fatty acid (MCFA), ethyl hexanoate, Japanese sake, alcoholic beverage, gas chromatography with a flame ionization detector (GC-FID), comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC × GC−TOFMS)*

INTRODUCTION

Medium-chain fatty acid (MCFA) ethyl esters in premium Japanese sake (*ginjo* sake) provide a pleasant, fruity apple-like flavor. This flavor is an essential trait for *ginjo* sake, and MCFA ethyl esters are considered to play an important role in flavor quality. On the other hand, MCFAs such as hexanoic acid and octanoic acid, precursors to ethyl esters, are recognized as a rancid or unpleasant fatty odor in reconstituted sake.[1] In sake fermentation, although the mechanism of MCFA ethyl ester formation during sake fermentation is poorly understood, it is considered that they are chemically generated (without enzymes) or by biosynthesis through enzymes in sake yeast, *Saccharomyces cerevisiae*.[2] Cerulenin-resistant sake yeast is generally used in modern *ginjo* sake brewing. This yeast harbors a point mutation at base 3748 of the fatty acid synthase 2 gene, encoding the alpha subunit of the fatty acid synthesis enzyme complex.[3,4] In this type of sake yeast, MCFAs, which are intermediates in the biosynthesis of long-chain fatty acids, accumulate to high concentrations. In turn, MCFAs combine during fermentation to form ethyl alcohol and generate MCFA ethyl esters.[3,5,6] Therefore, it is increasingly important to quantify MCFA concentrations during the brewing process and to develop techniques that control the production of these compounds during *ginjo* sake fermentation.

In our previous work, we developed a simple method for simultaneously quantifying MCFAs and ethyl hexanoate in sake using gas chromatography with a flame ionization detector (GC-FID). Based on these quantification data, we proposed a concept regarding the efficiency of ethyl ester production in sake.[7] In addition, this low-cost method is less labor intensive than other data analysis methods, and it is therefore suitable for processing large sample numbers. In contrast, this method is not suitable for analyzing target chemicals that are present in less than sub-ppm concentrations or for nontarget analyses.

In recent developments of analytical instruments and data processing techniques, the comprehensive two-dimensional gas chromatography (GC × GC) coupled with high-speed data acquisition using time-of-flight mass spectrometry (GC × GC−TOFMS) has emerged as a powerful analytical method that can be used to reveal large numbers of metabolites in complex samples. In combination with a statistical approach that combines multidimensional data, including first and second retention times, mass spectra, and variance of replicates, the compositional differences between samples can be effectively determined.[8,9] Many studies of relationships between brewing conditions, organoleptic properties, and metabolites have been published in the context of winemaking[10−15] and beer brewing.[16,17] However, whereas comprehensive metabolomics studies on sake were performed by Sakamoto et al.[18] using GC−MS, and by Sugimoto et al.[19,20] using capillary electro-

Received: May 2, 2014
Revised: July 31, 2014
Accepted: July 31, 2014
Published: July 31, 2014

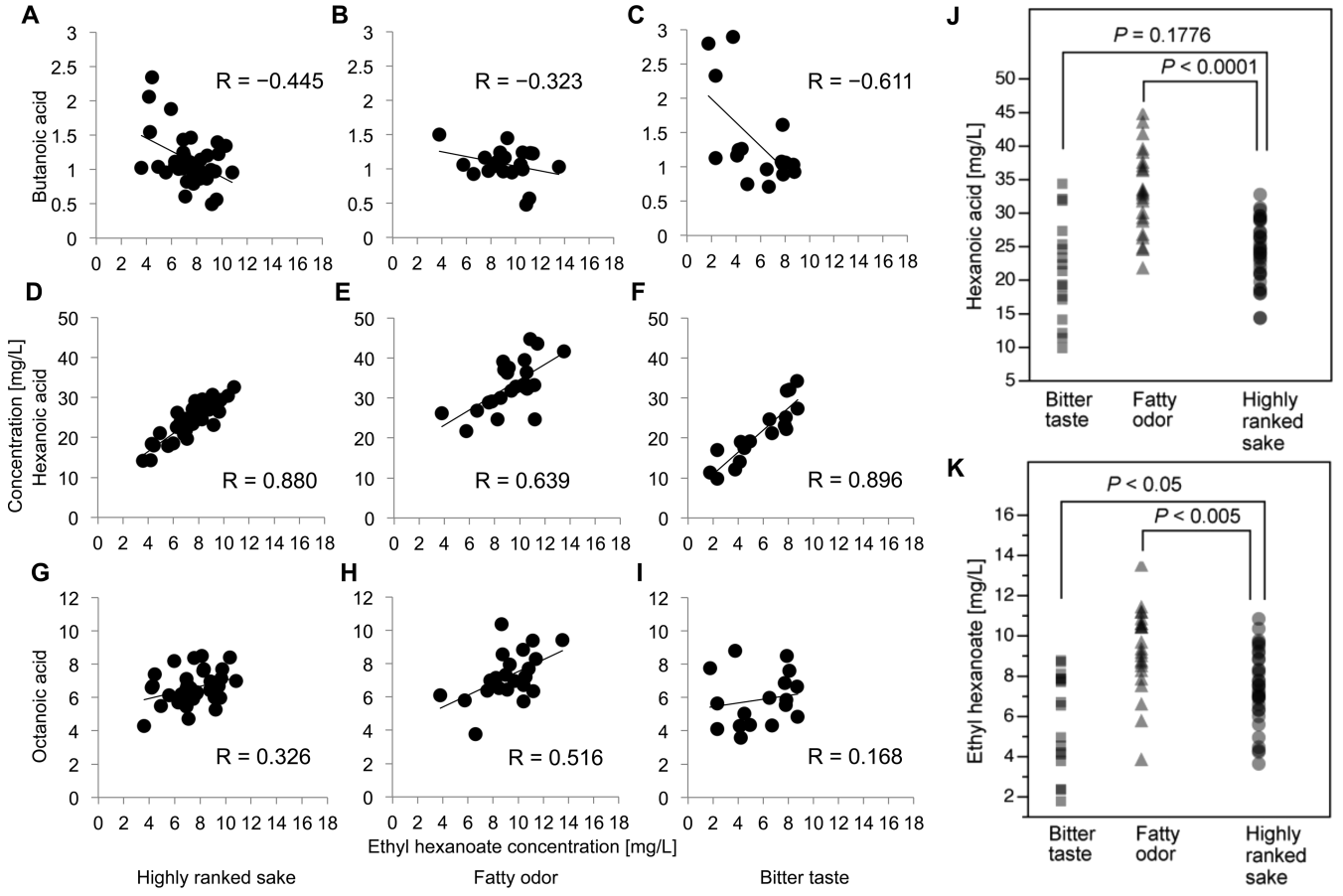

Figure 1. Correlation between ethyl hexanoate and MCFAs in Japanese sake. Indicated chemical compounds (ethyl hexanoate, butanoic acid, hexanoic acid, and octanoic acid) in sake were quantified using GC-FID. Graphs (A), (D), and (G) show results from the highly ranked sake (control). Graphs (B), (E), and (H) show results from fatty odor sake, and graphs (C), (F), and (I) show results from inharmonious bitter taste sake. Panels (J) and (K) show the statistically significant differences in hexanoic acid and ethyl hexanoate concentrations between highly ranked sake and fatty odor sake. Statistically significant differences were identified using the Dunnett's post hoc test. Typical data from three independent analytical procedures are shown.

phoresis TOFMS (CE−TOFMS), no metabolomics studies of sake report data from GC × GC−TOFMS analyses.

In this study, we determined MCFA and ethyl hexanoate concentrations in *ginjo* sake using GC-FID, and investigated correlations between MCFA and ethyl hexanoate with fatty odor and inharmonious bitter taste. Subsequent analysis of MCFAs and ethyl hexanoate in the sake mash *moromi* demonstrated that MCFA composition appeared to associate with ethyl hexanoate generation. We finally used GC × GC−TOFMS to analyze and identify other chemical compounds that correlate with fatty odor and inharmonious bitter taste in sake.

MATERIALS AND METHODS

Materials. Premium Japanese sake samples were selected from entries in the National New Sake Awards competition held by the National Research Institute of Brewing (NRIB) in Japan during 2009, 2010, and 2011. Other samples were obtained by brewing at the NRIB using 40 kg of polished rice (Japanese cultivar: *Yamadanishiki*) and a cerulenin-resistant sake yeast strain (Kyokai No. 1801). Sake mash (*moromi*) was sampled in the morning dairy on days 2−39. *Moromi* samples were paper-filtered, and liquid fractions were used for chemical analysis. Samples were dispensed and preserved at −30 °C until use.

All chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HP-FFAP capillary columns were purchased from Agilent Technologies (Palo, Alto, CA, USA), and DB-1 and BPX-50 capillary columns were purchased from Agilent Technologies and SGE Analytical Science Japan Inc. (Yokohama, Japan), respectively.

Sensory Evaluation. Sensory evaluations were performed by 15 well-trained panelists, as described in Japanese governmental reports.[21−23] To semiquantify sensory properties, the presence or absence of fatty odor and inharmonious bitter taste was determined on the basis of "check-all-that-apply (CATA)" assessments.[24,25] Sake samples in which fatty odor or inharmonious bitter taste were identified by more than four panelists were defined as fatty odor or inharmonious bitter taste sake, respectively.

Sample Pretreatment for GC-FID Analysis. Analysis of chemical components using GC-FID was performed according to a previously described Method[7] with some modifications. Briefly, 400-μL aliquots of sake samples were mixed with 2-propanol (1.2 mL). After centrifugation (17,000*g* for 5 min at room temperature), the supernatant was separated from the precipitate. An equal volume of 100% (v/v) 2-propanol was then added to the supernatant, diluting the sample. To estimate injection and detection efficiencies, an internal standard comprising 500 mg/L 1-pentanol (for ethyl hexanoate) and 200 mg/L heptanoic acid (for butanoic acid, hexanoic acid, and octanoic acid) in 17.0% ethanol was added to the diluted samples at a ratio of 9:1 (sample:internal standard).

Quantification of MCFAs and Ethyl Hexanoate Using GC-FID. A GC-FID (GC-17A-FID version 1; Shimadzu, Kyoto, Japan) coupled with an AOC-20i autosampler (Shimadzu, Kyoto, Japan) was used as previously described.[7] Splitless injections were performed for all samples with injection volumes of 1.2 μL. Two HP-FFAP columns

dx.doi.org/10.1021/jf502071d | J. Agric. Food Chem. 2014, 62, 8478−8485

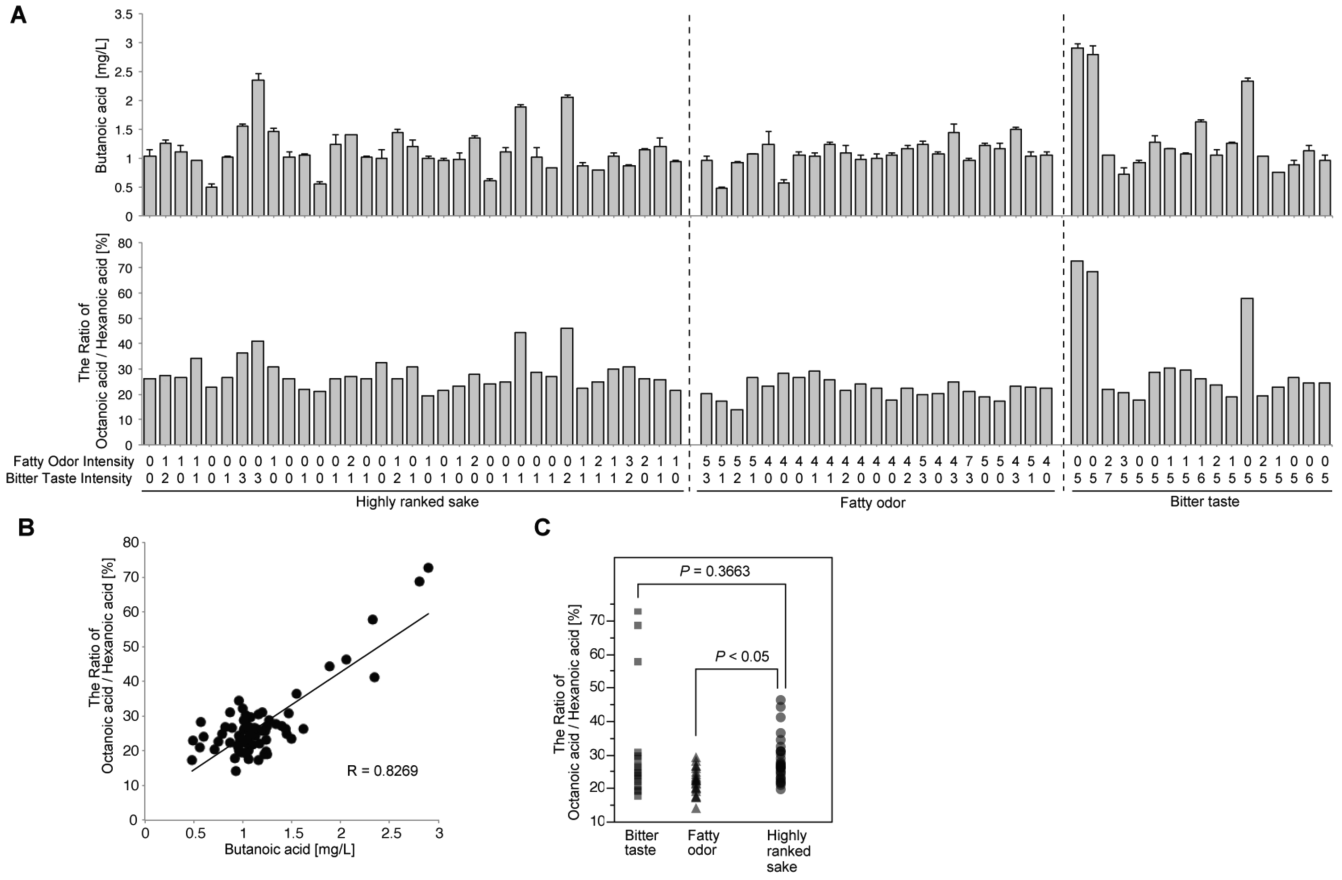

Figure 2. Positive correlation of butanoic acid concentrations and the octanoic acid/hexanoic acid (OA/HA) ratios in Japanese sake. Indicated chemical compounds (butanoic acid, hexanoic acid, and octanoic acid) were quantified using GC-FID. (A) *Upper panel*, The butanoic acid concentrations in sake are grouped according to the characteristics (highly ranked sake, fatty odor, and inharmonious bitter taste sake). Groupings are based on the numbers of sensory evaluation panelists who identified fatty odor or inharmonious bitter taste (shown below the graph). *Lower panel*, corresponding OA/HA ratios in sake. (B) Correlation between the OA/HA ratio and butanoic acid concentrations in all sake. (C) No statistically significant differences in OA/HA ratios between highly ranked and inharmonious bitter taste sake were identified using the Dunnett's post hoc test. Error bars indicate the variance between two technical replicates. Typical data from three independent analytical processes are shown.

(30 m × 0.32 mm i.d., 0.25-μm film thickness) were connected in tandem using a universal press-tight column connector (Restek, Bellefonte, PA, USA). The oven was initially set at 50 °C (for 3 min), and the temperature was increased from 50 to 235 °C at 7 °C/min, and was then maintained at 235 °C for 15 min. The temperature was subsequently increased from 235 to 240 °C at 30 °C/min and was maintained at 240 °C for 5 min. Peak areas on the chromatograms were quantified using GC-solution software version 2 (Shimadzu).

Analysis of General Components of *moromi* and Sake. The general components of the *moromi* during fermentation and those of sake were analyzed by the standard method established by the National Tax Administration Agency of Japan. In this analysis, ethanol content was determined using GC with an Rtx-624 column (Restek).

Analysis of Choline-Containing Phospholipids. The amount of choline-containing phospholipids in sake samples was determined using a colorimetric enzyme assay for detecting serum lipids with minor modification (Phospholipid C kit 433-36201, Wako Pure Chemicals, Osaka, Japan). Briefly, 10 μL of sake, filtered sake mash (*moromi*), or standards were mixed with 1.5 mL of assay reagent, incubated for 5 min at 37 °C, and the absorbance was measured at 600 nm.

Sample Pretreatment for GC × GC−TOFMS Analysis. The following manipulations were performed below 15 °C. Eleven sake samples (15 mL each), which were entries into the 2011 NRIB National New Sake Awards competition, were diluted with equal volumes of water and were passed through a 1-cc Oasis HLB cartridge containing 30 mg of sorbent (Waters, Milford, MA, USA) under slightly negative pressure (approximately from −0.002 to −0.005 MPa) using a GL-SPE vacuum manifold system (GL Science, Tokyo, Japan). After washing with 200 μL of water, the chemical components were eluted with 400 μL of organic solvent (2-propanol:methanol = 4:1), and 37.5-fold condensed elutes were tightly packed in glass vials and stored at 4 °C until further analysis.

GC × GC−TOFMS Analysis. The GC × GC system comprised an Agilent 7890N instrument equipped with an auto sampler 7683B series injector (Agilent). The nonpolar DB-1 (30 m × 0.25 mm i.d., 0.25-μm film thickness) was used as a primary separation column, and the BPX-50 (1.5 m × 0.1 mm i.d., 0.1-μm film thickness), which enables medium polarity separation, was used as a secondary column. A LECO Pegasus 4D TOFMS (LECO, St. Joseph, MI, USA) was connected in series with the GC × GC separation system. During modulation, cold pulses were generated using dry nitrogen gas cooled by liquid nitrogen, and heated dry air was used for hot pulses. Injector, transfer line, and ion source temperatures were set at 250 °C. The oven temperature program for the primary column began at 40 °C (for 5 min) and increased to 310 °C at 7 °C/min, and then the temperature was maintained at 310 °C for 1 min. The oven temperature program for the secondary column began at 55 °C (for 5 min) and increased to 325 °C at 7 °C/min, and then the temperature was maintained at 325 °C for 2 min. The modulator offset was +10 °C relative to the primary oven, and the modulation period was 5 s. Ultra-high-purity helium (>99.9999%) was used as a carrier gas with a constant flow rate of 1.5 mL/min. The injection volume was 1.0 μL.

Mass spectrometry parameters included electron ionization at 70 eV with a detector voltage of −1750 V, a mass range between 29 and 500

dx.doi.org/10.1021/jf502071d | J. Agric. Food Chem. 2014, 62, 8478−8485

Da, and an acquisition rate of 200 spectra/s. Data are presented as nominal mass.

Analyses were performed 4 times per sample, and the first data set obtained for each sample was discarded to avoid contamination by carryover compounds from the previous analysis. Data from the subsequent three analyses were then processed.

Data Processing and Semiquantification. Deconvolution of GC × GC−TOFMS spectra was conducted using ChromaTOF software ver. 4.50, which was optimized for the LECO Pegasus 4D TOFMS instrument. Chromatograms were processed with a baseline offset of 0.5, automatic peak smoothing, peak find with a minimum signal-to-noise ratio (S/N) of 30, a first dimension peak width of 15 s, and a second dimension peak width of 0.1 s. Compound mass spectral data were compared against the Wiley09 database library to calculate "similarity" and "reverse" parameters. Analytical conditions and parameters are summarized in Supporting Information Table 1.

Statistical Analyses. Descriptive statistical, multiple variance, and principal component analyses were conducted using JMP software version 10.0.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Quantification of MCFAs and Ethyl Hexanoate in Sake Using GC-FID. Premium Japanese sake "*ginjo* sake" is known for its fruity flavor. However, little is known of the flavor-generating mechanisms in sake. In particular, sensory evaluations of sake sometimes identify problematic fatty odor and inharmonious bitter taste, although the relevant chemical compounds remain uncharacterized. To address this issue, we initially selected three types of sake, which were entries into the NRIB National New Sake Awards Competition from 2009 to 2011, for use as samples. Highly ranked sake samples were used as controls, and sake samples with fatty odor or inharmonious bitter taste were examined. The numbers of highly ranked sake were 9 (2009), 13 (2010), and 13 (2011). The numbers of sake with fatty odor were 5 (2009), 12 (2010), and 6 (2011), and the numbers of sake with inharmonious bitter taste were 4 (2009), 8 (2010), and 5 (2011).

After selection of sake samples, we quantified the concentrations of butanoic acid, hexanoic acid, octanoic acid, and ethyl hexanoate in sake using GC-FID. Figure 1 shows correlations between ethyl hexanoate and MCFAs. As expected, higher ethyl hexanoate and MCFAs concentrations were found in sake samples (Figure 1) than in commercial sake products that were previously determined.[7] Using multiple comparison analysis, we found that ethyl hexanoate and hexanoic acid were present in significantly higher quantities in fatty odor sake than in the highly ranked sake (Figure 1J and K). The average hexanoic acid concentration in highly ranked sake was 24.2 mg/L, whereas that in fatty odor sake was 9.9 mg/L higher (34.1 mg/L). In contrast, the average octanoic acid concentration differed by only 0.7 mg/L. Yamane et al. previously showed that the addition of 8 mg/L hexanoic acid to sake resulted in increased perceptions of unpleasant fatty-related odors,[1] which is consistent with our results. Furthermore, we performed verification tests by adding 20 mg/L hexanoic acid to three sake samples containing 14.2−19.6 mg/L hexanoic acid, resulting in increased perception of fatty odor ($p < 0.01$ by χ^2 test). As shown in Figure 1D−F, ethyl hexanoate was positively correlated with hexanoic acid, as previously described.[26] A stronger positive correlation between ethyl hexanoate and hexanoic acid ($R = 0.88$) was observed in highly ranked sake (Figure 1D). As expected, the addition of 6 mg/L ethyl hexanoate to sake did not increase any perception of fatty odor, but unexpectedly rather decreased perception of fatty odor ($p < 0.01$ by χ^2 test). These results suggested that increased hexanoic acid but not ethyl hexanoate concentration is a primary contributor to fatty odor in sake.

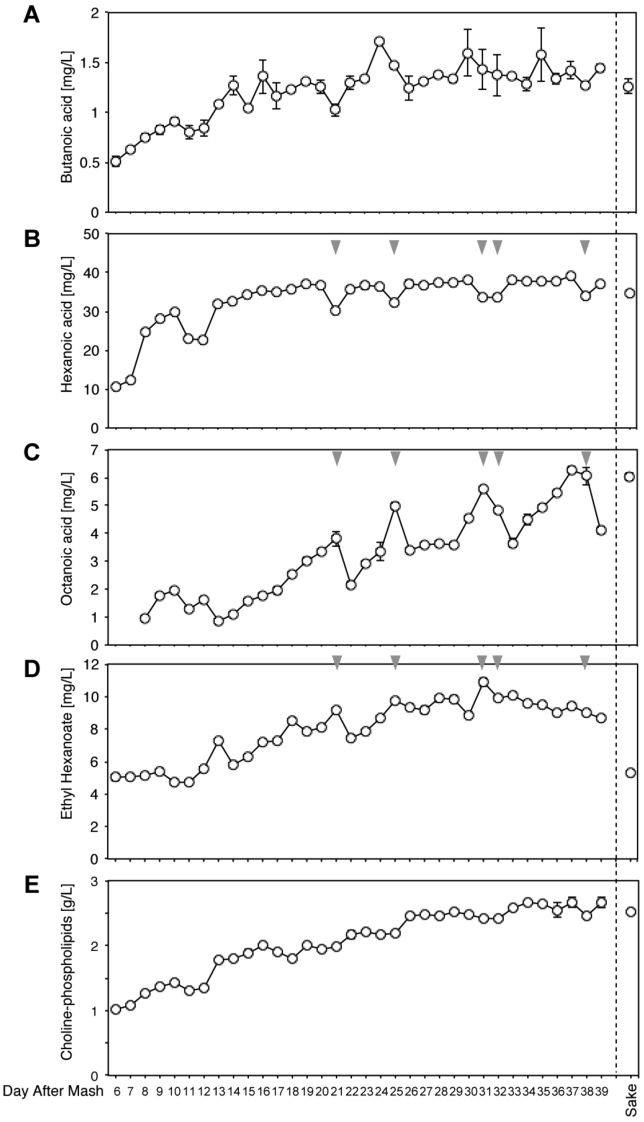

Figure 3. Variable patterns of MCFA and ethyl hexanoate concentrations in sake mash. Japanese sake was brewed on a semi-industrial scale, and chemical compounds concentrations were determined in samples taken during the fermentation period, and in samples of the final product (separated by dotted lines). (A) Butanoic acid, (B) hexanoic acid, (C) octanoic acid, (D) ethyl hexanoate, (E) choline-phospholipids concentrations in *moromi* samples and in final product; the *x*-axis shows the number of days after starting the mash. Gray arrowheads indicate days on which uncharacteristic patterns of hexanoic acid, octanoic acid, and ethyl hexanoate concentrations were observed. Errors bar indicate the variance between two technical replicates. Typical data from three independent analytical processes are shown.

No significant differences in hexanoic acid levels were found between highly ranked and inharmonious bitter taste sake. However, ethyl hexanoate levels were relatively lower in inharmonious bitter taste sake (Figure 1J and K), suggesting that ethyl hexanoate negatively correlated with inharmonious bitter taste.

Unexpectedly, ethyl hexanoate levels were found to negatively correlate with butanoic acid (Figure 1A−C). Butanoic acid concentration varied considerably even brewed

dx.doi.org/10.1021/jf502071d | J. Agric. Food Chem. 2014, 62, 8478−8485

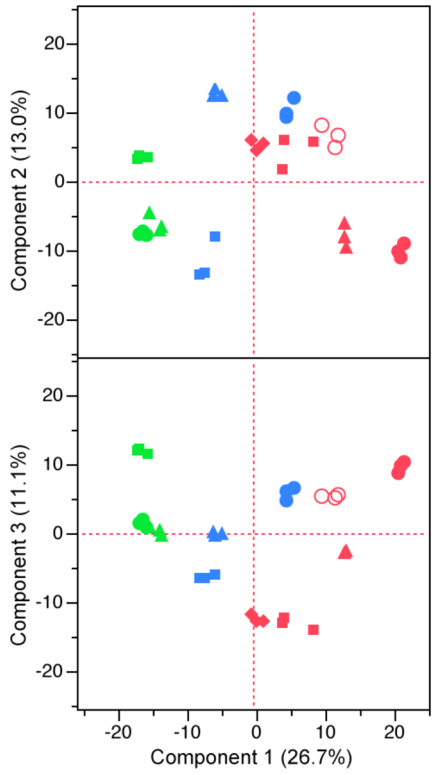

Figure 4. Principal component analysis (PCA) of Japanese sake. After selecting compounds using Fisher ratio criteria, PCA was performed for 11 sake samples in triplicate. Highly ranked sake (n = 5) is shown in red, fatty odor sake (n = 3) is shown in green, and inharmonious bitter taste sake (n = 3) is shown in blue.

using the same fermentation materials and under the same conditions, including rice species, yeast strain, and *shubo* (seed mash) type. However, butanoic acid concentration tended to be slightly higher in sake samples, brewed using rice species, *Omachi*.

Positive Correlation between Butanoic Acid and the Octanoic Acid/Hexanoic Acid Ratio. Because we were interested in our findings showing that MCFAs did not uniformly vary between sample groups (Figure 1), the molecular ratios of chemical compounds were analyzed. Figure 2A shows the butanoic acid concentrations and the octanoic acid/hexanoic acid (OA/HA) ratios. Some sake samples with high butanoic acid concentrations also had high OA/HA ratios (Figure 2A), which were likely to be correlated with inharmonious bitter taste. As shown in Figure 2B, butanoic acid concentration correlated with the OA/HA ratios. Although inharmonious bitter taste was not significantly correlated with butanoic acid or the OA/HA ratios (Figure 2C), some inharmonious bitter taste sake samples contained higher butanoic acid concentrations, or higher OA/HA ratios (Figure 2A and B).

Variable Patterns of MCFA and Ethyl Hexanoate Concentrations in Sake Mash. To determine when MCFAs and ethyl hexanoate were generated during the sake brewing process, we brewed *ginjo* sake. In the sake brewing process, *moromi* stirring was minimized to promote the discharge of CO_2 gas during fermentation. The final ethanol concentration in the sake product was 16.2%. Parts A–C of Figure 3 show changes in butanoic acid, hexanoic acid, and octanoic acid concentrations, respectively, over time in *moromi*. Hexanoic acid concentrations reached a maximum level during the early-to-middle stage of the fermentation period (Figure

Table 1. Positive Correlation of Chemical Compounds with Fatty Odor Sake[a]

no.	1st RT	2nd RT	unique mass	name	corr coeff	similarity	reverse	type
1	815	1.405	74	octanoic acid, methyl ester (CAS)	0.792	884	888	MCFA
2	590	1.335	96	2-furancarboxaldehyde (CAS)	0.790	666	903	furfural
3	2225	1.535	57	eicosane, 2-methyl- (CAS)	0.788	770	834	long-chain alkane
4	560	1.325	74	hexanoic acid, methyl ester (CAS)	0.781	868	955	ethyl hexanoate
5	1570	1.825	104	butanoic acid, 3-methyl-, 2-phenylethyl ester (CAS)	0.773	844	852	ester
6	2300	1.735	71	nonadecane, 2-methyl- (CAS)	0.773	882	884	long-chain alkane
7	1540	3.19	135	adenine	0.768	871	879	adenine
8	2040	2.98	143	pentanamide, *N*-[2-(indol-3-yl)]ethyl-	0.761	776	778	ester
9	1735	1.855	104	butanoic acid, 3-methyl-, 2-phenylethyl ester (CAS)	0.758	751	815	ester
10	2245	1.645	71	nonadecane, 2-methyl- (CAS)	0.737	908	911	long-chain alkane
11	750	2.46	42	2H-pyran-2-one, tetrahydro-6-methyl- (CAS)	0.730	928	938	cyclohexanoate
12	830	1.245	54	1,2-nonadiene (CAS)	0.717	768	850	alkene
13	1280	1.66	159	1S,*cis*-calamenene	0.711	812	875	wood-like
14	2190	1.585	71	nonadecane, 2-methyl- (CAS)	0.693	848	882	long-chain alkane
15	1110	1.535	60	decanoic acid	0.693	909	909	MCFA
16	1060	1.425	74	decanoic acid, methyl ester (CAS)	0.690	819	868	MCFA
17	2215	1.58	71	eicosane, 2-methyl- (CAS)	0.685	770	834	long-chain alkane
18	2065	1.505	71	eicosane (CAS)	0.678	860	888	long-chain alkane
19	285	1.395	85	1-methoxy-3-pentene	0.669	694	884	alkene ether
20	1805	2.28	120	tyrosol acetate	0.669	686	793	ester
21	1900	2.875	143	pentanamide, *N*-[2-(indol-3-yl)]ethyl-	0.664	768	782	indole-related
22	1530	3.525	144	1H-indole-3-carboxaldehyde (CAS)	0.660	889	904	indole-related
23	1120	2.37	130	1H-indole, 3-methyl- (CAS)	0.656	931	931	indole-related
24	1300	2.65	143	quinoline, 3-methyl- (CAS)	0.645	857	880	quinoline
25	2130	1.54	71	nonadecane, 2-methyl- (CAS)	0.625	903	905	long-chain alkane

[a]Retention time is provided as seconds.

dx.doi.org/10.1021/jf502071d | J. Agric. Food Chem. 2014, 62, 8478–8485

Table 2. Positive Correlation of Chemical Compounds with Inharmonious Bitter Taste Sake[a]

no.	1st RT	2nd RT	unique mass	name	corr coeff	similarity	reverse	type
1	1430	1.725	81	2-octylfuran	0.69	694	741	furan
2	315	1.15	86	2-pentanone (CAS)	0.636	907	912	ketone
3	975	1.7	95	ethanone, 1-(2-furanyl)- (CAS)	0.622	659	752	furan
4	955	1.725	135	(+)-(1*R*,5*R*)-2(10)-PINEN-4-ONE	0.605	834	843	ketone
5	990	1.93	82	2-cyclohexen-1-one, 3,5-dimethyl-	0.579	682	762	ketone
6	620	1.26	101	ethyl 4-hydroxypent-2-enoate	0.555	698	856	ester

[a]Retention time is provided as seconds.

3B). In contrast, octanoic acid concentrations increased with ethyl alcohol production (data not shown) and reached a maximum at a later stage of the fermentation period (Figure 3C). These differences may reflect differences in hydrophobicity. Figure 3D shows the ethyl hexanoate concentration in *moromi*, which reached a maximum during the middle stage, and was markedly decreased in the final product. This phenomenon has long been observed in the sake industry and was expected because ethyl hexanoate is thought to exist in the yeast cells, although details of its localization are unknown. Interestingly and unexpectedly, we observed several points: the lower hexanoic acid concentrations, but higher concentrations of octanoic acid and ethyl hexanoate (Figure 3B−D). These changes in concentrations were probably attributable to the minimal *moromi* stirring before sampling. In previous reports, yeast ethyl ester biosynthesis 1 (Eeb1) and ethanol hexanoyl transferase 1 (Eht1) catalyzed the esterification of hexanoic acid (hexanoyl-coenzyme A) and ethanol, producing ethyl hexanoate.[2] Saerens et al. also showed that the *EEB1* gene was particularly considered to be robustly upregulated in the presence of octanoic acid.[27] Thus, the variable concentration of hexanoic acid, octanoic acid, and ethyl hexanoate in *moromi* may reflect the expression of genes such as *EEB1 and EHT1*. The butanoic acid concentrations in *moromi* did not change significantly over time (Figure 3A) and were not associated with the OA/HA ratio, indicating that butanoic acid concentration does not always correlate with OA/HA ratio.

Choline-containing phospholipids in *moromi* were positively correlated with hexanoic acid (Figure 3E). Although we did not identify predominating choline-containing phospholipid species and fatty acids in cerulenin-resistant yeast, the concentration of choline-containing phospholipids in *moromi* was positively correlated with hexanoic acid, but not with octanoic acid.

In conclusion, MCFA and ethyl hexanoate concentrations did not uniformly increase in *moromi*. This pattern has not been previously observed in the sake fermentation process. In yeast cells, MCFAs are primarily generated in the cytosol by the traditional fatty acid synthase complex. However, MCFAs such as octanoic acid, which is involved in the formation of α-lipoic acid, are also generated in the mitochondria.[28,29] Hence, cytosolic MCFA composition may differ from that generated in the mitochondria. In the present study, sake fermentation was performed under completely anaerobic conditions, indicating that sake yeast does not require the tricarboxylic acid cycle to produce energy in mitochondria. However, the mechanisms behind MCFA generation and accumulation in sake yeast during the brewing process remain unknown, and further targeted cell biology studies are required.

Comprehensive Analysis of Compounds Using GC × GC−TOFMS. We showed that fatty odor and inharmonious bitter taste in sake correlate with MCFA contents, indicating that yeast metabolism may change during sake brewing. Thus, the present comparisons of sake metabolites other than MCFA in these three types of Japanese sake (highly ranked sake, fatty odor sake, and inharmonious bitter taste sake) are critical for understanding the factors that contribute to certain organoleptic properties. Hence, a comprehensive analysis of volatile compounds in these three types of sake was performed using a GC × GC−TOFMS system.

In these experiments, 11 sake samples, including 5 highly ranked sake samples, 3 fatty odor sake samples, and 3 inharmonious bitter taste sake samples, were selected from entries into the NRIB National New Sake Awards competition held in 2011. Sake samples were analyzed in triplicate using GC × GC−TOFMS equipped with a LECO Pegasus 4D system, giving a total of 33 sets of two-dimensional total ion chromatogram (TIC) data. Analytical conditions are summarized in Supporting Information Table 1. Typical TIC data for each type of sake are shown in Supporting Information Figure 1A−C, and corresponding intensity data are shown in Supporting Information Figure 1D−F. Supporting Information Figure 1 shows similar patterns of most peaks from the three different types of sake, although some differences are indicated. Subsequently, after performing peak deconvolution using ChromaTOF software, an automatic data mining program was applied, and detected peaks were aligned using the statistical-compare function of ChromaTOF software.

Correlations of Chemical Compounds with Fatty Odor and Inharmonious Bitter Tastes. GC × GC−TOFMS analyses showed >3,000 peaks. For convenience, peaks with poor reproducibility were denoted as "unknown", and peaks with good reproducibility (with similarities of >600) were denoted as "analytes". Specific compound names were assigned to peaks with similarities >650. A total of 1,182 analytes were thus identified, and specific chemical compounds were inferred for 1,110 of these. Using the area values of these compounds, we performed a Fisher ratio calculation using ANOVA to identify significant differences between samples. Consequently, 568 peaks remained after Fisher ratio selection with F values of >5.0. Principal component analysis (PCA) was performed using semiquantification data from the 568 peaks as parameters. The results of PCA show clear separation of fatty odor sake from highly ranked sake and inharmonious bitter taste sake (Figure 4). Highly ranked sake may not have been clearly separated from inharmonious bitter taste sake because nonvolatile compounds contributed more to the bitter taste than volatile compounds. Although the replicates were repeatable, the five highly ranked sake samples were not tightly clustered (Figure 4). Nonetheless, fatty odor sake was clearly distinguished by component 1, indicating that numerous chemical compounds in sake contribute to its characteristics. Hence, loading plot analysis after PCA was not used to estimate correlations of chemical compounds with class separation. Instead, we correlated certain compounds with semiquantitative sensory

data (numbers of notations for fatty odor and inharmonious bitter taste) using a relational analysis method.

Compounds that correlated with fatty odor and inharmonious bitter taste are listed in Tables 1 and 2, respectively. Several compounds, such as long-chain alkanes, some MCFA-related compounds, and some indole-related compounds, were positively correlated with fatty odor (Table 1). Alkanes in yeast can be generated intracellularly by the decarboxylation of fatty acids.[30] MCFAs and fatty acids with longer carbon chains may be converted to more volatile alkanes in decarboxylation reactions and may enhance fatty odor. MCFAs are toxic to microorganisms.[31−33] Hence, for protection against exposure to MCFAs, yeast may express proteins that esterify or export MCFAs.[34,35] Fungi similarly express enzymes that decarboxylate MCFAs such as *E,E*-2,4-dihexenoic acid (common name, sorbic acid).[36] Thus, the presence of alkanes in sake may reflect microbe-assisted decarboxylation reactions. Accordingly, MCFA-related compounds were correlated with fatty odor (Table 1), and this finding corresponded with the results shown in Figure 1. Although hexanoic acid was not identified in fatty odor sake, the derivative lactone (2H-pyran-2-one, tetrahydro-6-methyl-) was present (Table 1), despite the strong correlation between hexanoic acid concentrations and fatty odor. Some failures in peak picking were caused by peak saturation of some high concentration compounds such as hexanoic acid, and they may have lowered the Fisher ratio value to <5.0 in this experiment. Semiquantitative analysis of decanoic acid using GC-FID showed that decanoic acid positively correlated with both octanoic acid and hexanoic acid with correlation coefficients of $R = 0.59$ and $R = 0.33$, respectively (data not shown). For further information on MCFA-related compounds and alkanes, targeted analysis for these compounds is required.

Furan and ketone compounds were positively correlated with inharmonious bitter taste (Table 2). Furan is a well-known thermally generated compound that imparts food a bitter taste. Moreover, among ketones, 2-en ketone was particularly correlated with inharmonious bitter taste. However, neither direct nor indirect mechanisms have been described for the relationship between inharmonious bitter taste and these compounds.

Because GC analysis detects only volatile compounds, nonvolatile compounds were not identified. Thus, the GC analysis of derivatized compounds, and analysis using alternative instruments such as liquid chromatography and CE, may be required to investigate the roles of nonvolatile compounds in sensory qualities.

In conclusions, we quantified MCFAs and ethyl hexanoate in premium Japanese *ginjo* sake using GC-FID. These experiments showed a strong correlation between hexanoic acid and fatty odor, and a possible correlation between butanoic acid and the OA/HA ratio, and inharmonious bitter taste. The variable MCFA content in *moromi* apparently correlates with ethyl hexanoate concentrations, and can be linked to the expression of yeast genes. Furthermore, the chemical compounds found in fatty odor and inharmonious bitter taste sake were investigated using comprehensive GC × GC−TOFMS analysis. These experiments revealed direct correlations of MCFA derivatives and long-chain alkanes, and fatty odor. Taken together, MCFAs, MCFA derivatives, and long-chain alkanes could contribute to unpleasant fatty odor in sake. The present data indicate relationships between volatile compounds and the organoleptic properties of sake and provide fundamental knowledge for future research into the production of alcoholic beverages.

APPENDIX

Names for sake brewing procedures are written in *italics* in this manuscript, in accordance with the English edition of the sake handbook, published by the NRIB of Japan (http://www.nrib.go.jp/English/sake/pdf/sl_e.pdf; http://www.nrib.go.jp/English/sake/pdf/guidesse01.pdf; http://www.nrib.go.jp/English/sake/pdf/SakeNo01_en.pdf: most recent access date, 20 July 2014).

ASSOCIATED CONTENT

Supporting Information

Table S1, Analytical conditions of comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC × GC−TOFMS); Figure S1, Representative contour plots of Japanese sake of total ion chromatographs from comprehensive two-dimensional gas chromatography coupled with TOFMS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work involved the study of *ginjo* sake samples, which were supplied by entrants into the NRIB National New Sake Awards competition. We greatly appreciate all the participating breweries, particularly those that entered in the competition in 2011. We wish to thank Ms. Fumie Kabashima (LECO Corporation Japan) and Ms. Hiromi Kohno for scientific help.

REFERENCES

(1) Yamane, Y.; Takemiya, S.; Kawase, N.; Saiki, H. Sensory Properties of Some Fatty Acids in Sake. *J. Brew. Soc. Jpn.* **1997**, *92* (3), 224−227.

(2) Saerens, S. M.; Verstrepen, K. J.; Van Laere, S. D.; Voet, A. R.; Van Dijck, P.; Delvaux, F. R.; Thevelein, J. M. The Saccharomyces cerevisiae EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *J. Biol. Chem.* **2006**, *281* (7), 4446−56.

(3) Aritomi, K.; Hirosawa, I.; Hoshida, H.; Shiigi, M.; Nishizawa, Y.; Kashiwagi, S.; Akada, R. Self-cloning yeast strains containing novel FAS2 mutations produce a higher amount of ethyl caproate in Japanese sake. *Biosci. Biotechnol. Biochem.* **2004**, *68* (1), 206−14.

(4) Inokoshi, J.; Tomoda, H.; Hashimoto, H.; Watanabe, A.; Takeshima, H.; Omura, S. Cerulenin-resistant mutants of Saccharomyces cerevisiae with an altered fatty acid synthase gene. *Mol. Gen. Genet.* **1994**, *244* (1), 90−6.

(5) Ichikawa, E.; Hosokawa, N.; Hata, Y.; Abe, Y.; Suginami, K.; Imayasu, S. Breeding of a Sake Yeast with Improved Ethyl Caproate Productivity. *Agric. Biol. Chem.* **1991**, *55* (8), 2153−2154.

(6) Verstrepen, K. J.; Derdelinckx, G.; Dufour, J. P.; Winderickx, J.; Thevelein, J. M.; Pretorius, I. S.; Delvaux, F. R. Flavor-active esters: adding fruitiness to beer. *J. Biosci. Bioeng.* **2003**, *96* (2), 110−8.

(7) Takahashi, K.; Goto-Yamamoto, N. Simple method for the simultaneous quantification of medium-chain fatty acids and ethyl hexanoate in alcoholic beverages by gas chromatography-flame ionization detector: Development of a direct injection method. *J. Chromatogr., A* **2011**, *1218* (43), 7850−6.
(8) Groger, T.; Schaffer, M.; Putz, M.; Ahrens, B.; Drew, K.; Eschner, M.; Zimmermann, R. Application of two-dimensional gas chromatography combined with pixel-based chemometric processing for the chemical profiling of illicit drug samples. *J. Chromatogr., A* **2008**, *1200* (1), 8−16.
(9) Pierce, K. M.; Hoggard, J. C.; Hope, J. L.; Rainey, P. M.; Hoofnagle, A. N.; Jack, R. M.; Wright, B. W.; Synovec, R. E. Fisher ratio method applied to third-order separation data to identify significant chemical components of metabolite extracts. *Anal. Chem.* **2006**, *78* (14), 5068−75.
(10) Perestrelo, R.; Barros, A. S.; Camara, J. S.; Rocha, S. M. In-depth search focused on furans, lactones, volatile phenols, and acetals as potential age markers of Madeira wines by comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry combined with solid phase microextraction. *J. Agric. Food Chem.* **2011**, *59* (7), 3186−204.
(11) Robinson, A. L.; Boss, P. K.; Heymann, H.; Solomon, P. S.; Trengove, R. D. Development of a sensitive non-targeted method for characterizing the wine volatile profile using headspace solid-phase microextraction comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. *J. Chromatogr., A* **2011**, *1218* (3), 504−17.
(12) Robinson, A. L.; Boss, P. K.; Heymann, H.; Solomon, P. S.; Trengove, R. D. Influence of yeast strain, canopy management, and site on the volatile composition and sensory attributes of cabernet sauvignon wines from Western Australia. *J. Agric. Food Chem.* **2011**, *59* (7), 3273−84.
(13) Welke, J. E.; Manfroi, V.; Zanus, M.; Lazarotto, M.; Alcaraz Zini, C. Characterization of the volatile profile of Brazilian Merlot wines through comprehensive two dimensional gas chromatography time-of-flight mass spectrometric detection. *J. Chromatogr., A* **2012**, *1226*, 124−39.
(14) Welke, J. E.; Manfroi, V.; Zanus, M.; Lazarotto, M.; Alcaraz Zini, C. Differentiation of wines according to grape variety using multivariate analysis of comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection data. *Food Chem.* **2013**, *141* (4), 3897−3905.
(15) Bordiga, M.; Rinaldi, M.; Locatelli, M.; Piana, G.; Travaglia, F.; Coisson, J. D.; Arlorio, M. Characterization of Muscat wines aroma evolution using comprehensive gas chromatography followed by a post-analytic approach to 2D contour plots comparison. *Food Chem.* **2013**, *140* (1−2), 57−67.
(16) Charry-Parra, G.; Dejesus-Echevarria, M.; Perez, F. J. Beer volatile analysis: optimization of HS/SPME coupled to GC/MS/FID. *J. Food Sci.* **2011**, *76* (2), C205−11.
(17) Van Opstaele, F.; De Causmaecker, B.; Aerts, G.; De Cooman, L. Characterization of novel varietal floral hop aromas by headspace solid phase microextraction and gas chromatography-mass spectrometry/olfactometry. *J. Agric. Food Chem.* **2012**, *60* (50), 12270−81.
(18) Sakamoto, K.; Mitsuya Shimoda; Osajima, Y. Concentration in Porapak Q Column of Volatile Compounds in Sake for Analysis. *Nippon Nogeikagaku Kaishi* **1993**, *67* (4).
(19) Sugimoto, M.; Koseki, T.; Hirayama, A.; Abe, S.; Sano, T.; Tomita, M.; Soga, T. Correlation between sensory evaluation scores of Japanese sake and metabolome profiles. *J. Agric. Food Chem.* **2010**, *58* (1), 374−83.
(20) Sugimoto, M.; Kaneko, M.; Onuma, H.; Sakaguchi, Y.; Mori, M.; Abe, S.; Soga, T.; Tomita, M. Changes in the charged metabolite and sugar profiles of pasteurized and unpasteurized Japanese sake with storage. *J. Agric. Food Chem.* **2012**, *60* (10), 2586−93.
(21) Sudo, S.; Isogai, A.; Fujita, A.; Iwata, H.; Hiramatsu, J. *Analysis of Sake Components Presented to the Sake Contest in 2009*; NATIONAL RESEARCH INSTITUTE OF BREWING: Higashi-hiroshima city, Hiroshima, Japan, 2010; pp 1−15.
(22) Sudo, S.; Isogai, A.; Fujita, A.; Hiramatsu, J. *Analysis of Sake Components Presented to the Sake Contest in 2010*; NATIONAL RESEARCH INSTITUTE OF BREWING: Higashi-hiroshima city, Hiroshima, Japan, 2011; pp 1−15.
(23) Matsumaru, K.; Isogai, A.; Fujita, A.; Sudo, S.; Kizaki, Y. *Analysis of Sake Component Presented to Sake Contests in 2011*; NATIONAL RESEARCH INSTITUTE OF BREWING: Higashi-hiroshima city, Hiroshima, Japan, 2012; pp 1−15.
(24) Dooley, L.; Lee, Y.-s.; Meullenet, J.-F. The application of check-all-that-apply (CATA) consumer profiling to preference mapping of vanilla ice cream and its comparison to classical external preference mapping. *Food Qual. Prefer.* **2010**, *21* (4), 394−401.
(25) Reinbach, H. C.; Giacalone, D.; Ribeiro, L. M.; Bredie, W. L. P.; Frøst, M. B. Comparison of three sensory profiling methods based on consumer perception: CATA, CATA with intensity and Napping. *Food Qual. Prefer.* **2014**, *32* (Part B), 160−166.
(26) Utsunomiya, H.; Yamada, O.; Hashiguchi, T. Analysis of Free Fatty Acids, Higher Alcohols and Esters in Ginjyo-shu Produced in the Northern Part of Kyushu. *J. Brew. Soc. Jpn.* **2000**, *95* (3), 214−218.
(27) Saerens, S. M.; Delvaux, F.; Verstrepen, K. J.; Van Dijck, P.; Thevelein, J. M.; Delvaux, F. R. Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. *Appl. Environ. Microbiol.* **2008**, *74* (2), 454−61.
(28) Hiltunen, J. K.; Schonauer, M. S.; Autio, K. J.; Mittelmeier, T. M.; Kastaniotis, A. J.; Dieckmann, C. L. Mitochondrial fatty acid synthesis type II: more than just fatty acids. *J. Biol. Chem.* **2009**, *284* (14), 9011−5.
(29) Tehlivets, O.; Scheuringer, K.; Kohlwein, S. D. Fatty acid synthesis and elongation in yeast. *Biochim. Biophys. Acta* **2007**, *1771* (3), 255−70.
(30) Radakovits, R.; Jinkerson, R. E.; Darzins, A.; Posewitz, M. C. Genetic engineering of algae for enhanced biofuel production. *Eukaryot. Cell* **2010**, *9* (4), 486−501.
(31) Liu, P.; Chernyshov, A.; Najdi, T.; Fu, Y.; Dickerson, J.; Sandmeyer, S.; Jarboe, L. Membrane stress caused by octanoic acid in Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* **2013**, *97* (7), 3239−51.
(32) Viegas, C. A.; Rosa, M. F.; Sa-Correia, I.; Novais, J. M. Inhibition of Yeast Growth by Octanoic and Decanoic Acids Produced during Ethanolic Fermentation. *Appl. Environ. Microbiol.* **1989**, *55* (1), 21−8.
(33) Ullah, A.; Orij, R.; Brul, S.; Smits, G. J. Quantitative analysis of the modes of growth inhibition by weak organic acids in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **2012**, *78* (23), 8377−87.
(34) Legras, J. L.; Erny, C.; Le Jeune, C.; Lollier, M.; Adolphe, Y.; Demuyter, C.; Delobel, P.; Blondin, B.; Karst, F. Activation of two different resistance mechanisms in Saccharomyces cerevisiae upon exposure to octanoic and decanoic acids. *Appl. Environ. Microbiol.* **2010**, *76* (22), 7526−35.
(35) Piper, P.; Mahe, Y.; Thompson, S.; Pandjaitan, R.; Holyoak, C.; Egner, R.; Muhlbauer, M.; Coote, P.; Kuchler, K. The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J.* **1998**, *17* (15), 4257−65.
(36) Stratford, M.; Plumridge, A.; Pleasants, M. W.; Novodvorska, M.; Baker-Glenn, C. A.; Pattenden, G.; Archer, D. B. Mapping the structural requirements of inducers and substrates for decarboxylation of weak acid preservatives by the food spoilage mould Aspergillus niger. *Int. J. Food Microbiol.* **2012**, *157* (3), 375−83.