Lignin Is Linked to Ethyl-Carbamate Formation in Ume (Prunus mume) Liqueur

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Ethyl carbamate concentrations in oak barrel-aged ume (Prunus mume) liqueurs were measured, and possible explanations for elevated levels were examined. The average concentration was 0.30 mg/L, significantly higher than in ume liqueurs not aged in oak (0.08 mg/L). Oak powder extracts were prepared from both untoasted and toasted oak powder by extraction with aqueous ethanol, and these were used to make ume liqueurs. Relative to a no-oak control, the ethyl carbamate concentrations were 3.8 and 11 times higher in the untoasted and toasted oak powder extracts respectively. The extracts were loaded onto a C18 column, washed with water, and eluted with aqueous ethanol, and these were used to make ume liqueurs. Relative to a no-oak control, the ethyl carbamate concentrations in the 3-month old liqueurs were found to be 1.2 and 4.6 higher for the untoasted oak-powder and the toasted oak-powder respectively. Ethyl carbamate was formed when lignin was added to a 40% aqueous ethanol solution that contained potassium cyanide. The 13C-NMR spectra of the main constituents of the methanol elution fractions were consistent with those for lignin or fragments thereof. The methanol fractions were added to ume liqueur which was stored for 3 months. Relative to a control, the ethyl carbamate concentrations in the 3-month old liqueurs were found to be 1.2 and 4.6 higher for the untoasted oak-powder and the toasted oak-powder respectively. Ethyl carbamate was formed when lignin was added to a 40% aqueous ethanol solution that contained potassium cyanide. These observations suggest that lignin or fragments thereof promote the formation of ethyl carbamate.

Key words: ethyl carbamate; ume liqueur; lignin; oak barrel; contamination of ume liqueurs

Ethyl carbamate (urethane, C₂H₅OCNH₂, CAS no. 51-79-6) is known for its genotoxicity but is found in a wide range of food products, including fermented foods and alcoholic beverages (e.g., stone-fruit spirits, whiskey, sake). In 2007, the International Agency for Research on Cancer (IARC) upgraded ethyl carbamate to Group 2A (probably carcinogenic to humans). In Japan, there are currently no regulatory limits for ethyl carbamate in food or drink. The Joint FAO/WHO Expert Committee on Food Additives concluded at its 64th meeting that mitigation measures to reduce the concentrations of ethyl carbamate in some alcoholic beverages should be continued.

In previous work, we analyzed 38 ume liqueurs and found no significant differences in ethyl carbamate concentrations among ume liqueurs that used different sources of alcohol, e.g., sake and shochu. In recent years, a greater variety of ume liqueurs has become available, including barrel-aged products. The major ethyl carbamate precursor in whiskey and cachaças (a Brazilian distilled beverage made from fermented sugar cane) is cyanide, the same precursor reported in ume liqueur. Aylott et al. reported that when an ethanol oak extract was added to newly distilled grain spirits and stored, the ethyl carbamate concentration increased relative to unsupplemented grain spirits. Nóbrega et al. reported that when the ethyl carbamate levels of yellow (cask matured) and colorless single-distilled cachaças from the same distillery were compared, the yellow type contained much more. These findings indicate that barrel-aged ume liqueurs contain more ethyl carbamate than non-barrel aged ume liqueurs.

Baumann and Zimmerli have reported that in model solutions simulating stone-fruit distilled spirits, ethyl carbamate was not formed when the solutions were stored in the dark in the presence of diacetyl or 2,3-pentanedione, while it was formed when the solutions were stored in the dark in the presence of methylglyoxal. Nagao et al. have reported that bourbon whiskey had the highest concentration of methylglyoxal of alcoholic beverages tested. Since this whiskey is stored in a barrel with a charred surface, the methylglyoxal content of the whiskey may be related to the barreling process, stating that the methylglyoxal concentration in bourbon whiskey was 1.5 mg/L. Thus it is possible that methylglyoxal enhances the formation of ethyl carbamate in barrel-aged ume liqueur. To explore this possibility, the ethyl carbamate concentration of barrel-aged ume liqueurs was determined, and possible causes for the high levels were investigated.

Materials and Methods

Reagents. Unless otherwise indicated, high-quality reagents from Wako Pure Chemical Industries (Osaka, Japan) and Kanto Chemical (Tokyo) were used.

Analysis of commercial ume liqueurs. The ume liqueurs used in this study were purchased from retailers in February 2010. Ethyl carbamate was quantified by gas chromatography-mass spectrometer using butyl carbamate as internal standard. Free hydrogen cyanide was quantified using the 4-pyridinecarboxylic acid pyrazolone method. Total acidity was measured in by the wine analysis method of the Official Method of Analysis of the National Tax Agency of Japan. The analytical values for ethyl carbamate, free HCN, and total acidity were corrected for ethanol content assuming an alcohol concentration of 20% v/v based on the labeled alcohol content of the commercial products.

Preparation of oak-powder extracts. Five hundred g of untoasted and toasted commercial oak powder were placed separately in
Erlenmeyer flasks with 3 L of 40% aqueous ethanol and stored in a dark place at 30 °C for 12 d. The solutions were filtered through filter paper to remove insoluble oak powder solids, the alcohol content was measured by the method for whiskey in the Official Method of Analysis of the National Tax Agency of Japan, and Milli-Q water was added to adjust the alcohol content to 35%. Both extracts were assayed, and were found not to contain ethyl carbamate.

Preparation of ume liqueur using oak-powder extract. Frozen Nankobai ume fruit grown and harvested in 2009 in Wakayama Prefecture, Japan, was thawed and used. The crystal sugar used was from a commercial source (Nissin Sugar Manufacturing, Tokyo). Ume fruit (300 g) and crystal sugar (150 g) were steeped in the untoasted or toasted oak-powder extract or a 35% aqueous ethanol solution (540 mL), and stored in the dark at 30 °C. Ethyl carbamate was measured in triplicate after 3 months of storage.

Quantification of glyoxal and methylglyoxal in the oak-powder extracts. The glyoxal and methylglyoxal in the oak-powder extracts were measured by gas chromatography-mass spectrometry by the method developed by De Revel et al. Water (15 mL) and the internal standards, 1.2 g/L of 2,3-hexanedione in 50% aqueous ethanol (25 mL) and 6.5 g/L of o-phenylenediamine (2.5 mL), were added to the oak-powder extract (10 mL), and the pH was adjusted to pH8 using a sodium hydroxide aqueous solution. The solution was heated for 3 h at 70 °C and cooled to room temperature, after which a 1-mL sample was loaded onto a diatonic column and eluted using 5 mL of dichloromethane. Sodium sulfate was added to the eluate, which was subsequently dried and subjected to gas chromatography-mass spectrometry (Focus DSQ, Thermo Fisher Scientific, Waltham, MA) using a DB-WAX capillary column (60 m x 0.25 mm, 0.25 μm) (Agilent Technologies, Santa Clara, CA). The mass selective detector was operated in electron impact mode with an ionization energy of 70 eV. Helium (1.0 mL/min) was used as the carrier gas, with splitless 1-μL injections. The column was subjected to the following temperature program: 70 °C for 1.5 min, heating to 200 °C at a rate of 25 °C/min, heating from 200 to 250 °C at a rate of 10 °C/min, and holding at 250 °C for 20 min. The temperatures of the injector, the transfer line, and the ion source were 240, 240, and 280 °C respectively. Quantification was done in single ion monitoring mode at m/z 130 for quinoxaline (glyoxal), 144 for 2-methylquinoxaline (methylglyoxal), and 158 for 2-methyl-3-propylquinoxaline (2,3-hexanedione, internal standard).

Preparation of ume liqueur supplemented with glyoxal or methylglyoxal. The same ingredients and recipe as above were used. A 35% aqueous ethanol solution, containing glyoxal or methylglyoxal (10 mg/L), ume fruits, and crystal sugar were steeped in the dark at 30 °C. Ethyl carbamate was measured monthly in triplicate.

Analysis of freeze-dried oak powder extract. The solvent was removed from the extract using a rotary evaporator, and the extract was then freeze-dried overnight. Solid-state cross polarization and magic angle spinning 13C-NMR were measured using an ECA 500 spectrometer (JEOL, Tokyo), under the following conditions: frequency 125.76 MHz, sweep width 50.35 kHz, relaxation delay 5 s, acquisition time 40 ms, magic angle spinning 15 kHz, and contact time 2 ms. Chemical shifts were referenced to the methyl carbon signal (17.3 ppm) of hexamethylbenzene.

Fractionation of oak-powder extract. Bondesil C18 (Agilent Technologies, Santa Clara, CA) (25 g) was packed in a glass column (inner diameter, 2 cm) and conditioned with methanol (300 mL) and water (300 mL). The oak-powder extract (50 mL) was concentrated to 20 mL using a rotary evaporator to remove ethanol, loaded onto the Bondesil C18 column, and washed with water (500 mL), and eluted with methanol (500 mL). The solvent was removed from the elution fractions with a rotary evaporator, and the fractions were freeze-dried overnight to yield 240 mg of untoasted oak-powder extract and 230 mg of toasted oak-powder extract.

Ethyl carbamate formation in ume liqueur. Ume liqueur was prepared by steeping ume fruit as described in the aforementioned recipe, storing it in the dark at 30 °C, and removing the fruit after 3 months. A sample (5 mL) of the ume liqueur was transferred to a vial, oak-powder extract (25 mg) purified using a C18 column was added, and the vial was sealed and kept in the dark at 30 °C. Ethyl carbamate was assayed in triplicate after 3 months.

Ethyl carbamate formation in model solution. Referring to experiments by Baumann and Zimmerli and Taki et al., potassium cyanide (10 mM) was added to a 40% aqueous ethanol solution to which lignin (dealkalized, Tokyo Chemical Industry, Tokyo) was also added (1 or 5 g/L). These model solutions were kept in the dark at 30 °C and assayed monthly in triplicate for ethyl carbamate.

Statistical analysis. Statistically significant differences were assessed by Student’s t-test. A p-value of <0.05 was considered significant.

Results

The analyses of the ume liqueurs are shown in Table 1. The average ethyl carbamate concentration in the non-barrel aged products was 0.08 mg/L, while that in the barrel-aged products was significantly higher, at 0.30 mg/L. No significant differences were detected in free hydrogen cyanide or total acidity.

Preparation of ume liqueures using oak-powder extracts

Because the ethyl carbamate concentrations were found to be higher in the barrel-aged ume liqueurs than in the liqueurs not aged in barrels, we speculated that the wooden barrels were the source of a substance(s) that facilitated ethyl carbamate formation. Because barrels used for alcoholic beverages (e.g., whiskey and brandy) are generally made of oak, we prepared ume liqueurs using the oak-powder extracts and measured ethyl carbamate concentrations following 3 months of storage. As shown in Fig. 1, relative to a no-oak extract control, the ethyl carbamate concentrations were 3.8 and 11 times higher with the untoasted oak-powder and toasted oak-powder extracts respectively.

Preparation of ume liqueur containing glyoxal or methylglyoxal

Baumann and Zimmerli have reported that ethyl carbamate formed in a model solution in the presence of methylglyoxal even when the solution was stored in the dark. Hence we measured glyoxal and methylglyoxal in the oak-powder extracts (Table 2). To determine whether glyoxal or methylglyoxal would promote ethyl carbamate formation, ume liqueurs were made containing these substances at a concentr-

Table 1. Compositional Data for Barrel-Aged and Non-Barrel Aged Ume Liqueurs

<table>
<thead>
<tr>
<th>Type of Ume Liqueur</th>
<th>Ethyl Carbamate</th>
<th>Free HCN</th>
<th>Total Acidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barrel-aged products</td>
<td>0.30 (0.12–0.69)</td>
<td>4.9</td>
<td>17.6</td>
</tr>
<tr>
<td>Non-barrel aged products</td>
<td>0.08 (0.06–0.13)</td>
<td>4.5</td>
<td>17.6</td>
</tr>
</tbody>
</table>

*Converted assuming an alcohol content of 20% v/v; **Range; *Significantly different from non-barrel aged products (p ≤ 0.05, Student’s t-test).
red-oak lignin data reported by Haw were reduced. The chemical shifts were identified using solid state cross-polarization and magic angle spinning (14%), but significantly relative to control (Fig. 2). When methylglyoxal was used, the ethyl carbamate concentration did not increase significantly relative to control. When glyoxal was used, the ethyl carbamate concentration was higher. We suggest that both toasted and untoasted oak-powder methanol fractions were applied to a C18 column and removed the hemicellulose, toasted and untoasted oak-powder elution fraction respectively (Fig. 4).

**Table 2. Glyoxal and Methylglyoxal Contents of Oak-Powder Extracts**

<table>
<thead>
<tr>
<th></th>
<th>Glyoxal (mg/L)</th>
<th>Methylglyoxal (mg/L)</th>
</tr>
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<tbody>
<tr>
<td>Untoasted</td>
<td>1.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Toasted</td>
<td>1.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Oak powders were added to 40% v/v aqueous ethanol solutions. The solutions were stored in the dark at 30°C for 12 d and filtered, and the final ethanol concentrations was adjusted to 35% v/v with Milli-Q water.

**Analysis of freeze-dried oak powder extract**

The freeze-dried oak powder extract was analyzed by solid state cross-polarization and magic angle spinning 

The spectra obtained were consistent with the spectrum for red oak lignin reported by Haw et al., except that the 65–85 ppm peaks we obtained were higher. We suggest that both toasted and untoasted oak-powder freeze-dried extracts contain lignin or fragments thereof. Because broad-leaved trees consist of about 30% hemicellulose, we assume that the peaks between 65 and 85 ppm were caused by carbons bound to the oxygens of the hemicellulose. To remove the hemicellulose, toasted and untoasted oak-powder extracts were applied to a C18 column and eluted with methanol. The methanol fractions were freeze-dried following solvent removal and subjected to solid state cross-polarization and magic angle spinning

As a result, the 65–85 ppm peaks were reduced. The chemical shifts were identified using red-oak lignin data reported by Haw et al., as a guide. In the methanol elution fraction for the untoasted oak powder, a 15–50-ppm peak was attributed to aliphatic carbon not bonded to oxygen, a 56-ppm peak to the carboxyl group and/or the carbon of its ester. As for the methanol elution fraction for the toasted oak powder, a 15–50-ppm peak was attributed to aliphatic carbon not bonded to oxygen, a 56-ppm peak to the carboxyl group and/or the carbon of its ester. Hence we suggest that the main constituent in both the toasted and the untoasted oak-powder methanol fractions was lignin or fragments thereof.

**Ethyl carbamate formation in the ume liqueurs**

The aforementioned methanol elution fractions were added (5 g/L) to ume liquor which was then stored in the dark at 30°C for 3 months. Relative to control, the ethyl carbamate concentrations were found to be 1.2 and 4.6 higher for the untoasted oak-powder elution fraction and the toasted oak-powder elution fraction respectively (Fig. 4).

**Ethyl carbamate formation in the model solutions**

Figure 5 shows the results of an experiment in which lignin was added to the model distilled spirits solution. The concentration of ethyl carbamate increased with storage time as well as with the amount of lignin added. When lignin was not added (control), only a small amount of ethyl carbamate formed.

**Discussion**

Unlike free hydrogen cyanide and total acidity, for which no significant correlations with ethyl carbamate were found, significantly more ethyl carbamate was detected in the barrel-aged ume liqueurs than in the non-barrel aged ones (Table 1). Because this implied barrel-specific substances, we analyzed oak extracts, since oak is the main wood used in making barrels.

To determine whether constituents of oak promote the formation of ethyl carbamate, ume liquor was made...
using oak-powder extracts. As shown in Fig. 1, the ethyl carbamate levels were found to be 3.8 and 11 times higher with the untoasted and toasted oak-powder extracts respectively, relative to the no-oak control. These findings are consistent with the possibility that the oak powder extracts contained substances that did indeed promote ethyl carbamate formation.

According to Baumann and Zimmerli, methylglyoxal is involved in the formation of ethyl carbamate. Independently, Nagao et al. reported a methylglyoxal concentration of 1.5 mg/L in bourbon whiskey. Hence we assayed the oak-powder extracts for methylglyoxal and glyoxal (a compound of similar structure) and detected both, at concentrations from 1.5 to 4.6 mg/L (Table 2).

To determine whether the glyoxal or methylglyoxal in ume liqueur promotes ethyl carbamate formation, these compounds were added to an aqueous ethanol solution to a final concentration of 10 mg/L. While glyoxal was not found to increase ethyl carbamate levels, the addition of methylglyoxal was found to increase the ethyl carbamate concentration, but only by 14% (Fig. 2). This suggests that neither glyoxal nor methylglyoxal is a significant factor in causing increased levels of ethyl carbamate in barrel-aged ume liqueur.

To identify the substances that promoted ethyl carbamate formation, the freeze-dried oak-powder extracts were analyzed using solid-state cross polarization and magic angle spinning 13C-NMR (Fig. 3). Lignin or fragments thereof and hemicellulose were identified as candidate factors in both the toasted and the untoasted oak-powder extracts. Oaks are broad-leaved trees that consist of 50% cellulose, 30% hemicellulose, and 20% lignin. Because cellulose is insoluble in most solvents, we reasoned that the oak-powder extracts did not contain cellulose, but rather comprised a mixture of lignin or fragments thereof and hemicellulose. Otoguro et al. have reported that the hemicellulose and lignin contents were 687 mg/100 g and 24 mg/100 g in ripe

![Fig. 3. Solid-State Cross Polarization and Magic Angle Spinning 13C-NMR Spectra.](image)
a. Freeze-dried untoasted oak-powder extract; b. freeze-dried toasted oak-powder extract; c. freeze-dried methanol elution fraction obtained by loading untoasted oak-powder extract onto the C18 column; d. freeze-dried methanol elution fraction obtained by loading toasted oak-powder extract onto the C18 column.

![Fig. 4. Promotion of Ethyl Carbamate Formation in Ume Liqueur by the Methanol Elution Fraction.](image)

The oak-powder extract was loaded onto a C18 column, and the methanol elution fraction obtained was freeze-dried. The dried material was added to ume liqueur at a concentration of 5 g/L, and the liqueur was stored at 30 °C in the dark for 3 months. Values are means ± SD (n = 3). Values with different letters are significantly different at p ≤ 0.05 by Student’s t-test.

![Fig. 5. Promotion of Ethyl Carbamate Formation by Lignin in a Model Distilled Beverage.](image)

A 40% v/v aqueous ethanol solution containing KCN at 10 mM and added lignin was stored at 30 °C in the dark. Values are means ± SD (n = 3). *Significantly different from control (p ≤ 0.05, Student’s t-test).
Koshu koume ume (Prunus mume) fruit, respectively. We suggest that it is unlikely that hemicellulose was responsible for promoting the formation of ethyl carbamate in the ume liqueur that contained the oak-powder extracts. Therefore, lignin or fragments thereof appear to be the most likely candidate. To confirm this possibility, the oak-powder extracts were loaded onto a C18 column and eluted with methanol. Lignin or fragments thereof were determined to be a major constituent of the freeze-dried powders. The freeze-dried material was added to ume liqueur to determine whether it would raise the ethyl carbamate levels. Relative to a control to which no methanol elution fraction had been added, ethyl carbamate levels were found to be 1.2 and 4.6 times higher for the untoasted and toasted oak-powder methanol elution fractions respectively (Fig. 4). These results are consistent with the possibility that lignin or fragments thereof promote ethyl carbamate formation in oak barrel-aged ume liqueurs.

According to Nagata et al., the average ethyl carbamate concentration in whiskey was 25.5 ppb (13 samples of domestic whiskey), 40.8 ppb (13 samples of Scotch whiskey), 14.3 ppb (3 samples of Canadian whiskey), and 204.3 ppb (38 samples of bourbon whiskey, which is aged in charred barrels). The elevated levels reported in the bourbon whiskey are consistent with our finding of more ethyl carbamate in ume liqueur when toasted rather than untoasted oak powder or extracts thereof were used. The solid-state cross polarization and magic angle spinning 13C-NMR spectra of lignin and of fragments thereof (Fig. 3c, d) exhibited different peak shapes at 120–160 ppm as between the untoasted and toasted oak-powder methanol elution fractions, indicating that toasting changed the structure of lignin or fragments thereof.

An experiment using a model solution containing alcohol and potassium cyanide confirmed the ethyl carbamate formation promotion effect of lignin (Fig. 5). Hence we suggest that in both whiskey and cachaca, lignin or fragments thereof promote the formation of ethyl carbamate.

To our knowledge, this is the first study to evaluate the effects of lignin and fragments thereof on ethyl carbamate formation in a fruit liqueur. Because the genuine ethyl carbamate precursor in this system is believed to be cyanate (oxidized cyanide), we suggest that lignin or fragments thereof are involved in some way in the oxidation of cyanide. This possibility requires further investigation.

On a practical level, we suggest that ethyl carbamate levels be monitored in barrel-aged ume liqueurs, and that consideration be given to blending barrel-aged and non-barrel aged products as a means of reducing the levels of ethyl carbamate in the barrel-aged beverages.

References