

# Formation of Clusters in Whiskies During the Maturation Process

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**Abstract:** Maturation provides whisky with a mild and smooth texture by removing the irritating alcoholic flavor. However, the precise mechanism by which the whisky flavor is improved through the maturation process remains unknown. In this study, we performed mesoscopic structural measurements—dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS)—to elucidate the relationship between the liquid structure and flavor maturation of whiskies. Both techniques detected two scattering components corresponding to the clusters formed by the extractives from oak casks during maturation, which are not present in the new make (freshly distilled whisky). Analyzing the scattering profiles revealed that only the small clusters increase in concentration during maturation. It is concluded the small cluster component is crucial for obtaining flavorful whiskies, while the large cluster component, whose concentration is independent of the maturation time, is related to the alcoholic irritation of the whiskies, as demonstrated by the sonication test.

**Keywords:** clusters, dynamic light scattering, maturation, small angle X-ray scattering, whisky

## Introduction

Whisky is a distilled alcoholic beverage that undergoes prolonged storage maturation in wooden casks. It is believed that this aging technique was discovered by hiding untaxed moonshine in wooden casks in Scotland during the 18th century. The new make is distilled from the fermented product of malted barley and other cereal grains. Malt whisky is made using only malted barley as the fermentation feedstock and is typically subjected to two rounds of distillation in a pot still. Various cereals (that is, corn, rye and wheat) are used for producing whisky worldwide, and columns are still commonly used for distillation. A new make, which is clear and colorless, is unsuitable for drinking because of its high alcohol concentration (70% to 94%) and contamination with undesirable substances causing a strong bitter flavor. Thus, the new make is aged in charred wooden casks for a few years to a few decades. This maturation process is believed to be a critical procedure to imbue the whisky with its distinctive golden brown color and rich flavor. During the maturation process, various chemical reactions occur that not only produce valuable compounds but also alter or degrade undesirable substances (Conner, Paterson, & Piggott, 1993; Mosedale & Puech, 1998; Russell & Stewart, 2014).

Whisky contains a wide variety of compounds, such as alcohols, esters, acetates, terpenes, ketones, fatty acids, and phenols, derived from fermentation, distillation, and extraction from the wooden cask (Campo, Cacho, & Ferreira, 2007; Lehtonen, 1982; Poisson

& Schieberle, 2008; Wanikawa, Hosoi, Kato, & Nakagawa, 2002). The chemical reactions also contribute to the development of the characters of the whisky. The components and reactions in whisky have been thoroughly studied by chemical analyses, such as liquid and gas chromatography (LC and GC, respectively) and mass spectrometry (Wiśniewska, Dymerski, Wardencki, & Namieśnik, 2015). These analyses facilitate the discrimination of authentic whiskies from counterfeit products and allow the characterization of each brand of whisky at the molecular level (Câmara et al., 2007; Garcia et al., 2013). However, chemical analyses have not yet clarified the mechanism of flavor maturation of whisky.

According to previous studies in which calorimetry and nuclear magnetic resonance (NMR) were applied to aqueous ethanol solutions, the maturation process of whisky involves changes in the liquid structures of H<sub>2</sub>O and ethanol via hydrogen bonding. These structural changes help the whisky to become mellow and reduce the degree of alcoholic irritation (Nose & Hojo, 2006; Nose, Hojo, & Ueda, 2004; Nose, Hojo, Suzuki, & Ueda, 2004). Koga *et al.* suggested that these changes in the liquid structures are promoted by extractives from the casks; that is, amphiphilic molecules originating from the cask extractives likely develop unique structures and influence the hydrogen bonding between H<sub>2</sub>O and ethanol (Koga & Yoshizumi, 1977, 1979). However, the structures of these amphiphilic molecules arising from the cask extractives remain unknown.

Determining the structure of the extractives and its role in the production of flavorful whisky is crucial to elucidating the mechanism of whisky maturation. In this study, we characterized the unique structure in whiskies using dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) measurements, which are typically used to determine the size and concentration of mesoscopic structures. The probes (visible light and X-ray) of these two scattering techniques cover a wide size-range of structures (DLS; ca. 10 to 1000 nm, SAXS; ca. sub nm – 100 nm). In this study, single malt whiskies were investigated after various maturation times.

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## Materials and Methods

### Materials

The whisky samples investigated in this study were “Hakushu” series, which were commercially produced by Suntory Spirits Ltd. (Tokyo, Japan). The samples are from several different casks matured for various ages. For example, “Hakushu 18 years old” is a mixture of several malt whiskies matured in different casks more than 18 years and then diluted with water to keep the alcohol content to be around 43%. It is noted that this work is not intended to commercial campaign but to exploration of the mechanism of maturation process of whisky. The non-matured spirit (new make) was also provided by Suntory. The alcohol concentrations of the samples were determined by using a vibration type densitometer (DA-520; Kyoto Electronics MFG Co. Ltd., Kyoto, Japan) and a conversion table between solution density and alcohol concentration provided by National tax agency JAPAN. The sonication of the whiskies was conducted using a BRANSON2510 (Yamato, Japan) instrument. The whisky samples were placed in glass vials and then sonicated. The temperature rise caused by the sonication process was controlled to 25 °C via the addition of ice.

### HPLC analysis

High-performance liquid chromatography (HPLC) measurements were performed to analyze the absolute concentrations of cask extractives in the whisky samples. The samples were pre-filtered using a 0.45- $\mu\text{m}$  syringe-type filter with a hydrophilic polytetrafluoroethylene (PTFE) membrane (Millex-LH, Merck, Darmstadt, Germany). HPLC was conducted using a Prominence system (Shimadzu Co. Ltd., Kyoto, Japan) equipped with dual pumps (LC-20AD), an HPLC degassing unit (DGU-20A5), a diode array detector (SPD-M20A; covering the absorbance in 600 to 250 nm), an auto sampler (SIL-20AC), a column oven (CTO-20AC), and a system controller (CBM-20A). The reversed-phase chromatography was performed using an Inertsil ODS-4 (250  $\times$  4.6 mm, 5  $\mu\text{m}$ , GL science, Tokyo, Japan) column with a solvent flow rate of 0.6 mL/min. The solvent system used was as follows: A 35-min linear gradient elution from 10% to 45% and a 10-min elution from 45% to 100% of acetonitrile containing 0.1% formic acid in H<sub>2</sub>O was followed by 5 min of isocratic elution with 90% of acetonitrile containing 0.1% formic acid in H<sub>2</sub>O. In this study, we determined the absolute concentrations of the 12 primary compounds derived from oak casks. The total concentration  $c_{\text{Total}}$  was defined by summing the concentrations of the 12 compounds.

### DLS measurements

DLS data were collected using an SLS/DLS-5000 light-scattering system (ALV, Germany). A He-Ne laser (wavelength,  $\lambda = 632.8$  nm) was used as the incident beam. The scattering intensity was measured at  $\theta = 150^\circ$ . The measurement temperature was 25 °C. The refractive index  $n$  and solvent viscosity  $\eta_0$  were determined using an Abbe refractometer (Atago, Japan) and an MCR 501 rheometer (Anton Paar, Austria), respectively.

The time-autocorrelation function of the scattering intensity  $g^{(2)}(\tau)$  measured by DLS was analyzed using the CONTIN method to obtain the relaxation spectrum  $G(\Gamma^{-1})$  of the relaxation time  $\Gamma^{-1}$ . The  $\Gamma^{-1}$  is associated with the hydrodynamic radius  $R_H$  of the particle via the Stokes–Einstein equation as

$$R_H = \frac{k_B T}{6\pi\eta_0\Gamma q^{-2}} \quad (1)$$

where  $k_B T$ ,  $q$  and  $\eta_0$  are the Boltzmann constant multiplied by the absolute temperature, the magnitude of the scattering vector  $q = (4\pi n/\lambda)\sin(\theta/2)$ , and the viscosity of the solvent, respectively.

### SAXS measurements

The SAXS measurements were conducted using the BL 40B2 (beam line status: [http://www.spring8.or.jp/wkg/BL40B2/instrument/lang-en/INS-0000001280/instrument\\_summary\\_view](http://www.spring8.or.jp/wkg/BL40B2/instrument/lang-en/INS-0000001280/instrument_summary_view)) at SPring-8, Hyogo, Japan. The wavelength, camera length and accumulation time were 0.1 nm, 1600 mm, and 300 s, respectively. The intensity of the scattered X-rays was measured using an imaging plate detector (R-AXIS, VII). To minimize the background and parasitic scattering, a flow cell, which was fixed on the optical system, was used for these measurements.

The excess scattering intensity  $I(q)$  was determined as

$$I(q) = I_{\text{whisky}}(q) - wI_{\text{ethanol}}(q) - (1-w)I_{\text{water}}(q) \quad (2)$$

where  $w$  is the alcohol content of each whisky:  $w = 0.40$  (10 years), 0.43 (12, 18, and 25 years), and 0.60 (new make). We analyzed  $I(q)$  using a scattering function, assuming two spheres with different sizes. The scattering function was defined using the form factor  $P_i(q)$  for spheres, as follows:

$$I(q) = n_{\text{Small}} V_{\text{Small}}^2 \Delta\rho_{\text{Small}}^2 P_{\text{Small}}(q) + n_{\text{Large}} V_{\text{Large}}^2 \Delta\rho_{\text{Large}}^2 P_{\text{Large}}(q) \quad (3)$$

$$P_i(q) = \left[ 3 \frac{\sin(qR_i) - (qR_i)\cos(qR_i)}{(qR_i)^3} \right]^2 \quad (4)$$

Here,  $n_i$  and  $V_i$  are the number and the volume of the small ( $i = \text{Small}$ ; sub nm) and large ( $i = \text{Large}$ ; hundreds nm) particles, respectively.  $\Delta\rho_i$  is the scattering length density difference between the particle and the medium. If the  $\Delta\rho$  and the mass density are identical for the small and large components, then the scattering function is represented in terms of the mass concentrations  $c_i$  and radii  $R_i$  as follows:

$$I(q) \propto c_{\text{Small}} R_{\text{Small}}^3 P_{\text{Small}}(q) + c_{\text{Large}} R_{\text{w, Large}}^3 P_{\text{z, Large}}(q) \approx c_{\text{Small}} R_{\text{Small}}^3 P_{\text{Small}}(q) + (c_{\text{Total}} - c_{\text{Small}}) R_{\text{H, Large}}^3 P_{\text{z, Large}}(q) \quad (5)$$

Here, the log-normal distribution of the molar mass is assumed for the large component;  $R_w$  and  $P_z$  denote the weight-average radius and z-average form factor, respectively. In this study, the polydispersity index is set as  $M_{w, \text{Large}}/M_{n, \text{Large}} = 4.5$  (When  $M_{w, \text{Large}}/M_{n, \text{Large}} \geq 4.5$ , the profile does not change), where  $M_w$  and  $M_n$  are the weight- and number-average molar masses, respectively. The total concentration  $c_{\text{Total}}$ , which was determined via HPLC, is assumed to be the summation of the concentrations of the small and large components:  $c_{\text{Total}} = c_{\text{Small}} + c_{\text{Large}}$ ; that is, we assume that the scattering particles (small and large clusters) are formed by association of cask extractives. Furthermore,  $R_{w, \text{Large}}$  is assumed to be identical to  $R_{H, \text{Large}}$ , which was determined via DLS. In Eq. (5), the free parameters for the curve fitting were  $c_{\text{Small}}$  and  $R_{\text{Small}}$ . Because these two parameters can be independently determined from curve fitting with Eq. (5), the two components are unambiguously characterized.

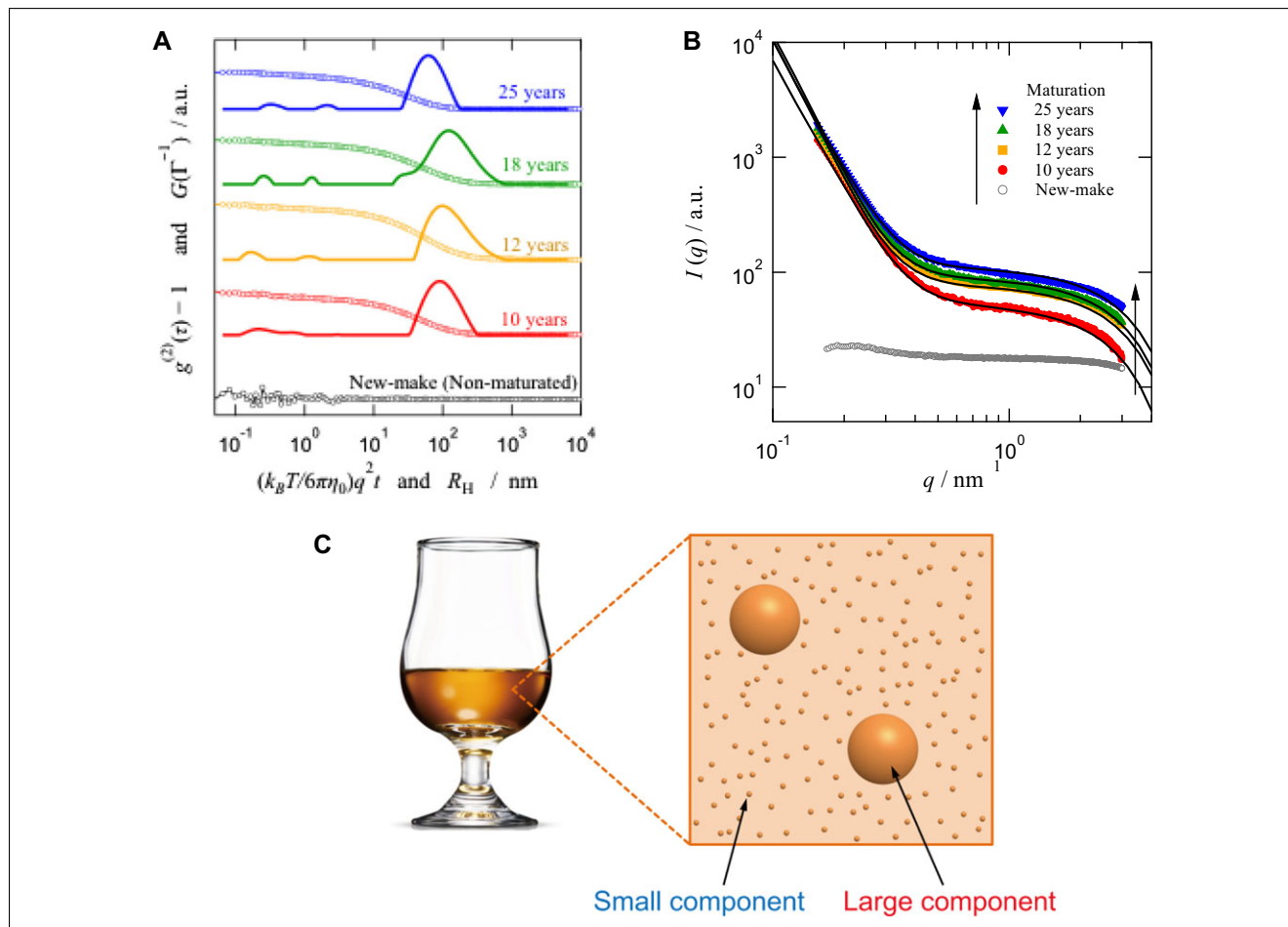


Figure 1—(A) DLS measurement results obtained at scattering angle of  $\theta = 150^\circ$ . The symbols show the time-autocorrelation function of the scattering intensity  $g^{(2)}(\tau) - 1$ , and the solid lines represent the distribution  $G(\Gamma^{-1})$  of the hydrodynamic radius  $R_H$  for whiskies with different maturation times. (B) SAXS profiles (the excess scattering intensity  $I(q)$ ) for whiskies with different maturation times. The solid lines represent the scattering functions calculated using Eq. (5). (C) Schematic illustration of the large and small scattering components in whisky.

## Results and Discussion

To measure the absolute concentrations of the main compounds in whiskies, high-performance liquid chromatography (HPLC) analysis was conducted. Although whiskies consist of a wide variety of compounds, 12 well-known major compounds (Nose et al., 2004; Owens, Zimmerman, Gardner, & Lowe, 2015) (Figure S1) were quantified in this work. The total concentration  $c_{\text{Total}}$  of the 12 compounds increases fairly proportionally as the maturation time  $t_{\text{mat}}$  increases, as shown in Figure S2. Thus, these compounds play an important role in the development of flavorful and mellow whiskies during the maturation process. This increase in the concentration has two origins: increased extractives from oak casks and the evaporation of alcohol and water (that is, the angel's share). Here, we note that the main cask extractives have amphiphilic chemical structures and, hence, they may form clusters by self-assembly in an aqueous alcohol solution.

The DLS results indicate the existence of such clusters. Figure 1(A) shows the relaxation time distribution  $G(\Gamma^{-1})$  obtained by the time-autocorrelation function  $g^{(2)}(\tau) - 1$  of the scattering intensity. The hydrodynamic radius  $R_H$  is calculated from the relaxation time  $\Gamma^{-1}$  using the Stokes–Einstein equation (Eq. (1)). The distributions  $G(\Gamma^{-1})$  for the whiskies are bimodal or trimodal; that is, the scattering particles in the whiskies have multiple sizes (Shibayama, Karino, & Okabe, 2006). A clear peak

at  $R_H \sim 100$  nm is observed for all whiskies, regardless of the maturation time. Additionally, small peaks at  $R_H \sim 0.2$  and  $\sim 2$  nm are occasionally observed. We call the former (the peak at  $R_H \sim 100$  nm) the “large component” and the latter (small peaks at  $R_H \sim 0.2$  and  $\sim 2$  nm) the “small component”. Although the small component appears to split at  $R_H \sim 0.2$  and  $2$  nm, we regard the small peaks as unimodal because of the uncertainty of the CONTIN method for the weak relaxation signal (Shibayama et al., 2006). Note that  $g^{(2)}(\tau) - 1$  for the new make, which does not show any relaxation (i.e., no peaks in  $G(\Gamma^{-1})$ ). Thus, the scattering components observed for the whiskies are formed during the maturation process.

The SAXS profiles also confirm the existence of the large and small components in whiskies. Figure 1(B) shows the excess scattering intensities  $I(q)$  for the whiskies and the new make. The profiles of the whiskies show a unique  $q$  (scattering vector)-dependence: an upturn in the low  $q$ -region ( $q \leq 0.3$  nm $^{-1}$ ) and a shoulder at  $q \sim 2$  nm $^{-1}$ . Each  $q$ -dependence reflects the existence of both the large ( $>$  tens of nm) and small (sub-nm) components in the whiskies, which is consistent with the DLS results. The SAXS profiles of the new make exhibited different behaviors, which is independent of  $q$  and is similar to those obtained for general solvents such as water. Therefore, the two scattering components appear during the maturation process. Based on the results of both DLS

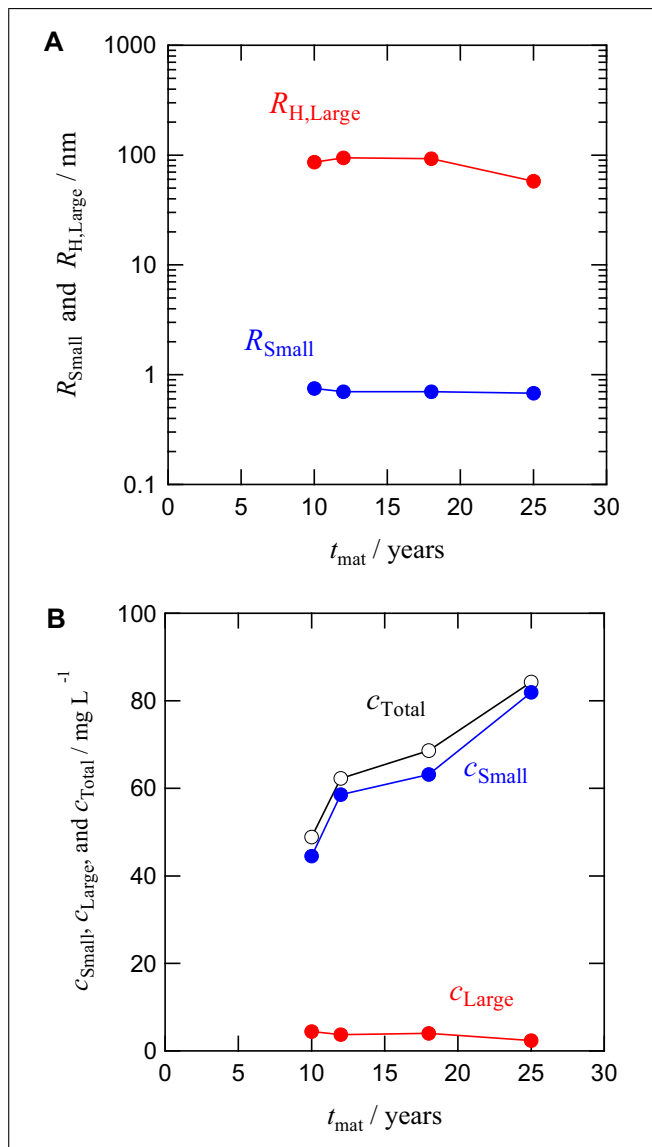


Figure 2—(A) Maturation time  $t_{\text{mat}}$  dependences of the sizes of the large and small components in whiskies.  $R_{\text{H,Large}}$  and  $R_{\text{Small}}$  were determined via DLS and SAXS, respectively. (B) Maturation time  $t_{\text{mat}}$  dependences of the concentrations of the large and small components. The total concentration  $c_{\text{Total}}$  was obtained by HPLC.

and SAXS, we conclude that the clusters in the whiskies comprise the cask extractives which are the only substances providing contrast for scattering in the alcohol/water solution. The mellow flavor of mature whisky must derive from the two scattering components; this finding is crucial for elucidating the maturation mechanism.

The size and concentrations of each scattering component in whisky were characterized using the DLS and SAXS results. The hydrodynamic radius of the large component  $R_{\text{H,Large}}$  was determined using the value of  $R_{\text{H}}$  at the clear  $G(\Gamma^{-1})$  peak. In contrast, the radius of the small component  $R_{\text{Small}}$  and the concentrations  $c_{\text{Large}}$  and  $c_{\text{Small}}$  were determined via model fitting of the SAXS profiles using a scattering function assuming the form factor  $P(q)$  for large and small spheres. The theoretical curve of Eq. (5) reproduced the experimental results well, as shown in Figure 1(B) and Figure S3.

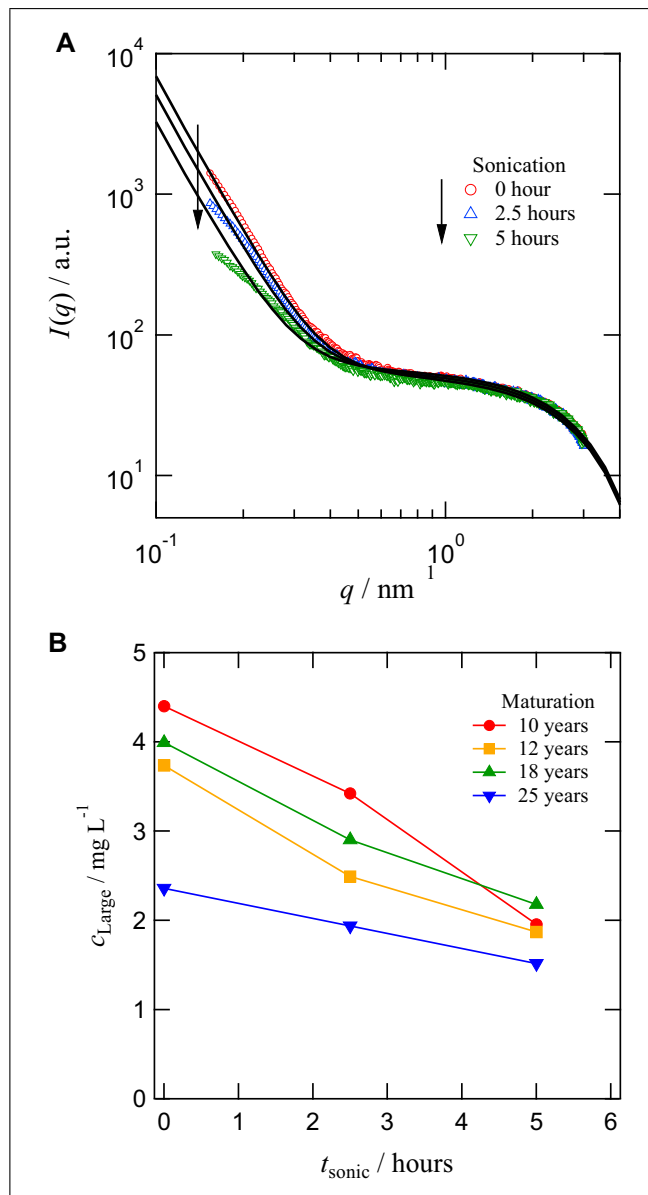


Figure 3—(A) SAXS profiles of sonicated whiskies ( $t_{\text{mat}} = 10$  years) after different sonication times. The solid lines represent the scattering function calculated using Eq. (5). (B) Sonication time  $t_{\text{sonic}}$  dependences of  $c_{\text{Large}}$  determined by fitting the data of whiskies after different maturation times.

The characterized sizes are shown in Figure 2(A). The value of the small component ( $R_{\text{Small}} \sim 0.75$  nm) is only a few times larger than that of aromatic cask extractive molecules. Therefore, the small component comprises clusters of several cask extractive molecules. On the other hand, the large component is a cluster formed by association of amphiphilic cask extractives, because other substances (such as coloring additives) are not added to the whisky. Note that  $R_{\text{H,Large}}$  (DLS) and  $R_{\text{Small}}$  (SAXS) are approximately independent of the maturation time,  $t_{\text{mat}}$ . Thus, each cluster has an optimum size.

The concentrations  $c_{\text{Large}}$  and  $c_{\text{Small}}$  determined from fitting analysis using Eq. (5) and the fitting curves are shown in Figure 2(B).  $c_{\text{Small}}$  depends on  $t_{\text{mat}}$ , whereas  $c_{\text{Large}}$  is independent. Based on these results, it was confirmed that the small component is the major cluster in whiskies and depends on the maturation time. To acquire additional information about the solution states of whiskies, we

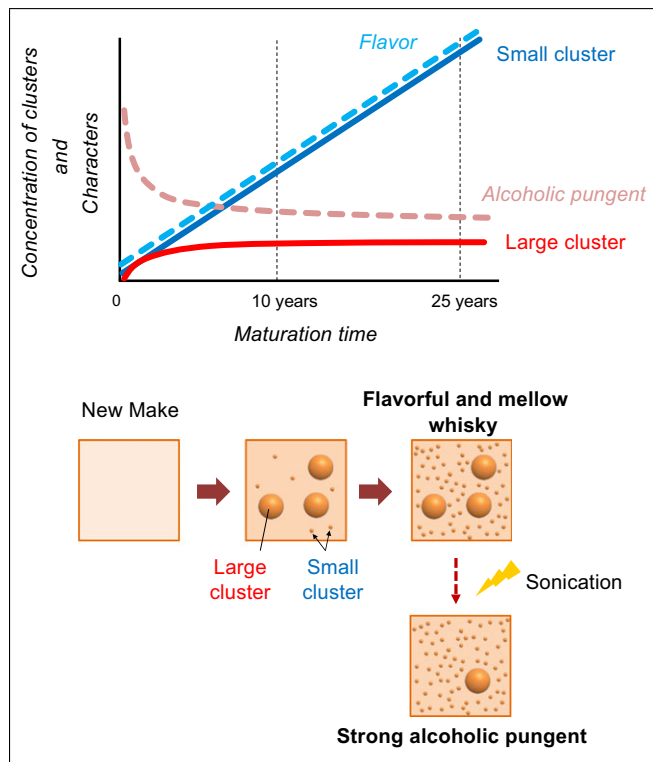


Figure 4—Schematic illustration of the maturation time dependence of the cluster concentrations and texture of whiskies.

conducted diffusion-ordered spectroscopy (DOSY) using NMR to determine the diffusion coefficient of carbon-13 in ethanol in whiskies. The diffusion coefficient of ethanol tended to decrease with  $t_{\text{mat}}$  (Figure S4), and this decrease corresponded to an increase in the viscosity with  $t_{\text{mat}}$ . The SAXS results suggest an increase in the small components, which may increase in the viscosity. On the other hand, NMR showed a decrease in the diffusion coefficient. Hence, the increase in the viscosity corresponds to the decrease in the diffusion coefficient. Therefore, we conclude that the concentration of the small clusters is significantly related to the mellow flavor produced by maturation while the large clusters are not.

A sonication test was performed to investigate the role of large clusters. Sonication has been reported to accelerate the maturation process, although the mechanism remains unknown (Delgado-González et al., 2017). In this study, we sonicated whiskies with the expectation that their qualities would be improved. However, we observed that sonication drastically increased in the alcoholic irritation (alcohol-burn and acrid odor of alcohol) the whiskies; therefore, the sonication in this study degraded the whiskies rather than helping them to mature.

Figure 3(A) displays the sonication time  $t_{\text{sonic}}$  dependence of the SAXS profile of a whisky ( $t_{\text{mat}} = 10$  years). The intensity of the upturn in the low- $q$  region decreases with sonication, whereas the profile in the high- $q$  region is independent of  $t_{\text{sonic}}$ . That is, sonication only affects the large clusters. Because the  $R_{\text{H,Large}}$  obtained from DLS is independent of  $t_{\text{sonic}}$  (Figure S5), we found that the sonication decreased the concentration  $c_{\text{Large}}$ . The decrease of  $c_{\text{Large}}$  by sonication was characterized by model fitting analysis using Eq. (5), as shown in Figure 3(B). Based on the decrease in  $c_{\text{Large}}$  and the increase in alcoholic irritation resulting from sonication, we conclude that large clusters are related to the alcoholic irritation.

## Conclusion

Maturation process of whisky was investigated by two scattering methods, one small-angle X-ray scattering (SAXS) and the other dynamic light scattering (DLS). We revealed the existence of two types of clusters in matured whiskies and characterized their sizes and concentrations. The flavor of whisky is primarily governed by small clusters which increase with maturation process. In contrast, large clusters most likely have relationship with the alcoholic irritation of the whisky (Figure 4). Nose *et al.* proposed an association model involving water and ethanol molecules with the assistance of organic acids (e.g., gallic acid and protocatechuic acid) (Nose & Hojo, 2006). If the large clusters consist of organic acids, then their hydrophilic groups, such as carboxylic group, should exist on the surfaces of the clusters. Thus, ethanol molecules can be trapped on the surfaces of these large clusters, reducing the alcoholic irritation of the whisky.

It has been a difficult problem to qualify the alcoholic irritation even by chemical approaches. Hence, it has been hardly expected that physical characterization, such as scattering measurements (DLS and SAXS), can be used for characterization of whisky maturation. It is rather surprising that the clusters observed by DLS and SAXS suggest strong correlation with alcoholic irritation of a whisky. This knowledge provides a new criterion for the evaluation of alcoholic irritation of whiskies. This point of view can be a hint to solve the mystery of the maturation of a whisky.

## Conflict of Interest

The authors declare no conflict of interests and no competing financial interest.

## Author Contribution

Conceived and designed experiments: K.M., N.N., K.M., H.M., and M.S. Performed DLS experiments and analysis: K.M., X.L., and M.S. Performed SAXS experiments and analysis: K.M., N.N., K.M., Y.T., H.M., and M.S. Performed HPLC, GC and UV-vis experiments and analysis: N.N. Performed NMR experiments and analysis: S.M. and T.I. Wrote the paper: K.M., N.N., K.M., Y.T., T.I., and M.S.

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Vanillin, 8. Lyoniresinol, 9. Syringaldehyde, 10. Sinapaldehyde, 11. Scopoletin, 12. Coniferyl aldehyde

**Table S1.** HPLC results for the 12 major compounds in Hakushu. The unit of concentration is mg/L.

**Figure S2.** Maturation time dependence of the total concentration  $c_{\text{Total}}$  of Hakushu.

**Figure S3.** (a) and (b): Vertical shifted SAXS profiles and fitting curves. These data are identical to Fig 1(b) and Fig 3(a), respectively. (c) and (d): The residuals of the fits, i.e.,  $DI(q)/I(q) = (I_{\text{exp}}(q) - I_{\text{model}}(q))/I_{\text{exp}}(q)$  and  $I_{\text{model}}(q)$  means the experimental and model  $I(q)$ , respectively.

**Figure S4.** Maturation time dependence of the diffusion coefficient and viscosity. The diffusion coefficients were determined by DOSY. CD3OD (30  $\mu\text{L}$ ) was added to a whisky sample (570  $\mu\text{L}$ ), and the sample was then transferred to Wilmad 5-mm NMR tubes. Eighteen glass capillaries (0.8-mm OD, 0.4-mm OD, 210-mm length; Nippon Electric Glass) were inserted into each tube to suppress thermal convection[1]. All NMR spectra were acquired at 298 K using a Bruker AVANCE III HD 800 spectrometer equipped with a 5-mm TCI cryogenic probe and Z-axis gradient (Bruker Biospin AG, Switzerland). DOSY spectra were collected using stimulated echo pulse sequences with the longitudinal-eddy current delay[2] and composite pulse decoupling. We observed the carbon-13 of the methyl groups of ethanol. Data analyses were performed using the Bruker TopSpin 3.2 software (Bruker Biospin AG, Switzerland). The signal intensities of the DOSY spectra were fit using the Stejskal-Tanner equation[3].

**Figure S5.** Sonication time  $t_{\text{sonic}}$  dependence of RH, Large for whiskies with various maturation times.

**Figure S6.** Gas chromatogram (GC) of Hakushu 10 years treated by sonication. Peak identifications: 1. 2-Methylpropanol, 2. Isoamyl alcohol, 3. Ethyl lactate, 4. Ethyl octanoate, 5. Ethyl decanoate, 6. Internal standard (Benzyl acetate), 7. Ethyl dodecanoate, 8. 2-Phenylethanol.

**Figure S7.** UV-visible spectra for the sonicated whiskies. The lines which are results for various sonication times (0h: red, 2.5h: blue, and 5h: green) are overlapping. The spectra have been measured by SpectraMax i3 (Molecular Devices).

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1(a).** HPLC chromatograms for Hakushu.

**Figure S1(b).** Chemical structures of the 12 major compounds. Peak identifications are 1. Gallic acid, 2. Protocatechuic acid, 3. Furfural, 4. Vanillic acid, 5. Syringic acid, 6. Ellagic acid, 7.