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# Identification of Nonbiological Turbidity in Beer by Fourier Transform Infrared Spectroscopy and Scanning Electron Microscopy with Energy-dispersive X-ray Spectroscopy

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**Hisanori Higurashi*, Kaori Kikuchi, Masayuki Omote, Susumu Masuda**
Quality Control Center, Asahi Breweries, LTD.
hisanori.higurashi@asahibeer.co.jp

## Summary


- Analytical methods based on Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS), and microscopic observation were developed to identify the components of permanent haze and frozen precipitates in beer. These components contribute to the typical nonbiological turbidity, which is important for beer quality.
- There were many beer brands that contained frozen precipitates in which protein is the main component instead of β-glucan.
- The proposed cause of the protein rich frozen precipitates was the use of exogenous enzymes (e.g., β-glucanase) that improve the filtration in a beer brewing process.[1]
- The protein species found in permanent haze and frozen precipitates were similar. Proteins (e.g., BDAI-1, CMb, and CMe) that have been reported as beer haze proteins were detected in both turbidity substances.[2]


## Introduction

Nonbiological turbidity important for beer quality is *permanent haze* and *frozen beer precipitates*. Permanent haze is formed by the association of proteins and polyphenols.[3] Frozen precipitates are turbidity substances, which are formed when a beer is frozen inadvertently. The main component of frozen precipitates has been shown to be β-glucan.[4] However, we have been detecting other proteins aside form β-glucan as the main component of frozen precipitates. It is important to investigate the cause of the change in frozen precipitates and to discuss the difference between permanent haze and frozen precipitates.

**Objectives**
- To develop methods to identify the components of nonbiological turbidity in beer.
- To investigate and discuss the differences in the composition of frozen precipitates in beer from all over the world.
- To examine whether there is any difference between the protein species of permanent haze and frozen precipitates.

## Qualitative analysis of permanent haze and frozen precipitates

**Materials & methods**

Twenty-two local and international beer brands were analyzed. Permanent haze samples were collected from beer samples that were >1 year past its production. Frozen precipitate samples were collected from beer samples that were made turbid by 10 cycles of freeze thawing. Turbidity substances were separated from a beer sample by filtration, washed with purified water, and identified by FT-IR and SEM-EDS. β-Glucan was detected by microscopic observation with a Congo red stain.

**Instruments:** FT-IR, SEM, and EDS were conducted with IR Tracer-100 (Shimadzu Corporation, Japan), SU3800 (Hitachi High-Technologies, Japan), and Ultim Max (Oxford Instruments, UK), respectively.

**Results**

### Permanent haze

FT-IR spectrum of brand D confirmed the presence of proteins (Fig. 1). SEM-EDS of brand D detected nitrogen and sulfur that were derived from proteins (Fig. 2). In the microscopic observation of brand G, few Congo red stained substances and brown film-like or string-like substances were observed (Fig. 3).

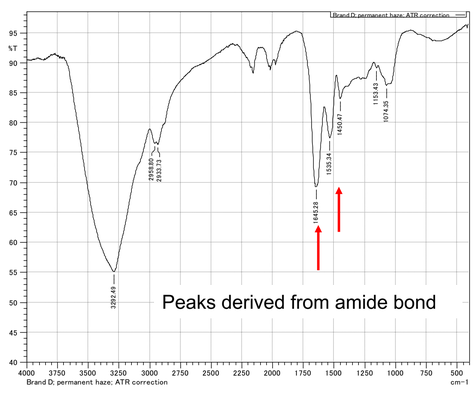


Fig. 1 FT-IR (brand D)

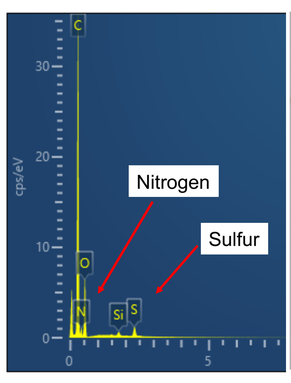


Fig. 2 SEM-EDS (brand D)

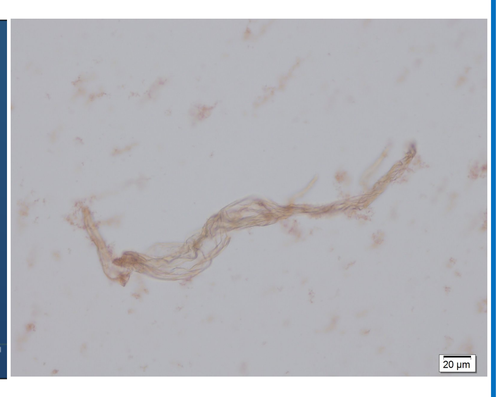


Fig. 3 (brand G)

### Frozen precipitate

FT-IR spectra of frozen precipitate samples were divided into two types. The protein type was similar to the permanent haze spectrum (Fig. 4). The β-glucan type showed a peak derived from β-glucan (Fig. 5). SEM-EDS analysis detected calcium derived from calcium oxalate (Fig. 6). In samples with the β-glucan peak in FT-IR, a reddish stained substance was observed by Congo red (Fig. 7).

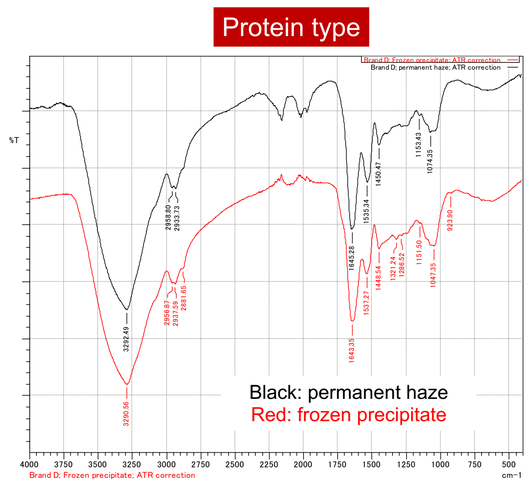


Fig. 4 FT-IR (brand D: Overlay)

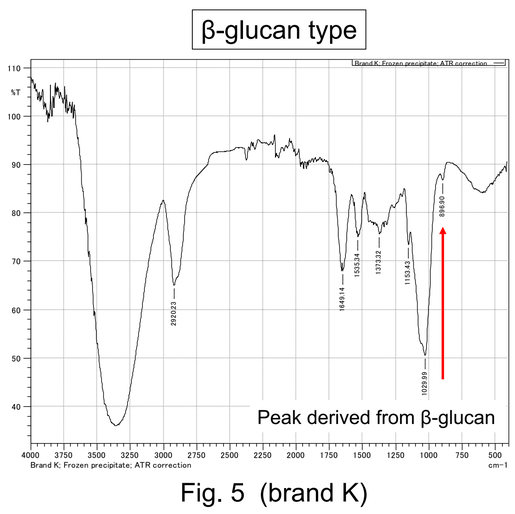


Fig. 5 (brand K)

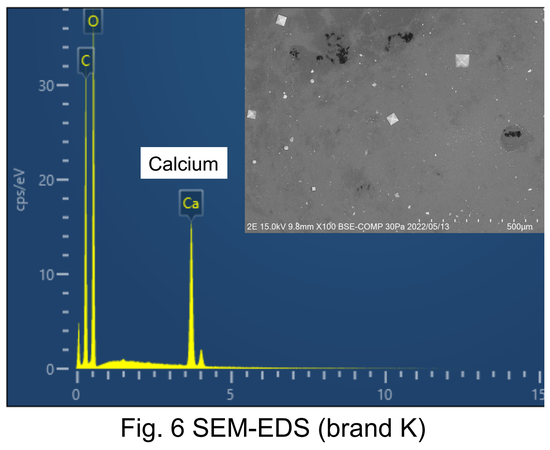


Fig. 6 SEM-EDS (brand K)

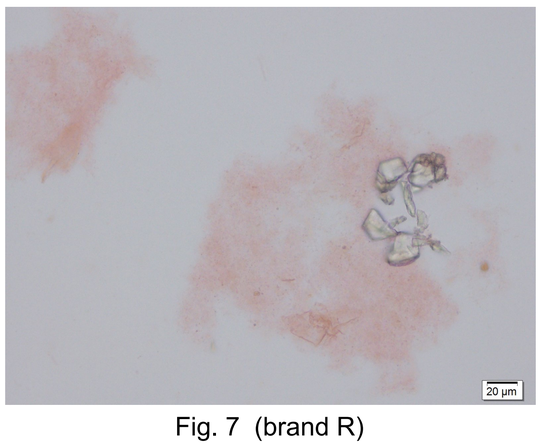


Fig. 7 (brand R)

**Discussion**
- The presence of proteins, β-glucan, and calcium oxalate, which are turbidity substances in beer, was confirmed by examining the results obtained from FT-IR, SEM-EDS, and microscopic observation.
- Proteins were the main component of frozen precipitates instead of β-glucan in many beer brands.
- Frozen precipitates were characterized by the presence of large amounts of calcium oxalate, which was detected easily by SEM-EDS.

## Relationship of β-glucan content in beer and frozen precipitates

**Materials & methods**

To discuss the cause of the frozen precipitate change from β-glucan to protein type, the concentration of high molecular weight β-glucan in a beer was determined by the Megazyme International assay kit according to the European Brewing Convention (EBC method 8.12.1).

**Results**

A correlation was found between the concentration of β-glucan in the beer solution and IR spectrum type of the frozen precipitates.

Table 1 Quantitative results of β-glucan in beer solution and the type of frozen precipitate

| Brand | Production country | β-glucan content (mg/L) | Frozen precipitate Type |
|---|---|---|---|
| A | Belgium | <10 | protein |
| B | China | <10 | protein |
| C | China | <10 | protein |
| D | Japan | <10 | protein |
| E | Japan | <10 | protein |
| F | UK | <10 | protein |
| G | Japan | <10 | protein |
| H | USA | <10 | protein |
| I | Japan | 13 | protein |
| J | Japan | 26 | β-glucan |
| K | USA | 37 | β-glucan |
| L | Mexico | 52 | β-glucan |
| M | Germany | 62 | β-glucan |
| N | Thailand | 63 | β-glucan |
| O | Mexico | 70 | β-glucan |
| P | Japan | 72 | protein |
| Q | Italy | 85 | protein |
| R | Japan | 86 | β-glucan |
| S | Spain | 102 | β-glucan |
| T | Tahiti | 107 | β-glucan |
| U | Czech | 110 | β-glucan |
| V | Japan | 159 | β-glucan |

**Discussion**
- The lower content of high molecular weight β-glucan likely caused the high proportion of proteins in the frozen precipitates.
- The lower content of high molecular weight β-glucan was most likely caused by the use of an exogenous enzyme (e.g., β-glucanase), which improves filtration during a brewing process.

## Proteome analysis in permanent haze and frozen precipitates

**Materials & methods**

Proteomic analysis was conducted to determine the differences in the proteins between permanent haze and frozen precipitates. Samples were digested with trypsin and analyzed by high-resolution tandem mass spectrometry (MS/MS) with liquid chromatography. The product ion spectral data were searched in a protein sequence database.

**Results**

The protein species found in permanent haze and frozen precipitates were similar. Proteins (e.g., BDAI-1, CMb, and CMe) that have been reported as beer haze proteins were detected in both turbidity substances.

Table 2 Protein species in permanent haze and frozen precipitate from brand G

| Permanent haze | Frozen precipitate |
|---|---|
| alpha-amylase inhibitor BDAI-1 | alpha-amylase inhibitor BDAI-1 |
| alpha-amylase inhibitor BMAI-1 | alpha-amylase inhibitor BMAI-1 |
| alpha-amylase/trypsin inhibitor CMa | alpha-amylase/trypsin inhibitor CMa |
| alpha-amylase/trypsin inhibitor CMb | alpha-amylase/trypsin inhibitor CMb |
| alpha-amylase/trypsin inhibitor CMd | alpha-amylase/trypsin inhibitor CMd |
| avenin-like a precursor | avenin-like a precursor |
| barwin or pathogenesis-related protein 4 | barwin or pathogenesis-related protein 4 |
| D hordein | B1-hordein |
| gamma 3 hordein | D hordein |
| hordoindoline b | gamma 1 hordein |
| Non-specific lipid-transfer protein 1 LTP1 | gamma 3 hordein |
| trypsin inhibitor CMe | hordoindoline b |
| Eno2p | Non-specific lipid-transfer protein 1 LTP1 |
| Nca3p | trypsin inhibitor CMc |
| Scw4p | trypsin inhibitor CMe |
| Scw10p | Scw4p |
| | Scw10p |

**Discussion**
- The FT-IR spectra were similar for permanent haze and frozen precipitate; thus, it is important to focus on the components (e.g., polyphenols) with low content other than proteins.
- Further compositional analysis of the frozen precipitates as well as investigation on their formation mechanism are required.

**Literature cited:**
[1] J. M. Sendra, et al., *J. Inst. Brew., 95*, 327-332 (1989)
[2] T. Iimure, et al., *J. Cereal Sci., 49*, 141-147 (2009)
[3] E. Kahle, et al., *J. Am. Soc. Brew. Chem., 79*, 99-114 (2021).
[4] S. Takayanagi, et al., *J. Inst. Brew., 75*, 284-292 (1969).

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