Studies on the Fluorescence of Saké

Part 1. Fluorescence Spectrum of Saké and Identification of Harman

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Received February 28, 1966

By spectrofluorophotometric investigation on various kinds of Saké it was found that they have at least two kinds of fluorescent colors, the one is blue, the other yellowish green. The former is always more dominant than the latter, but is unstable although the intensities of both color decrease remarkably by treatment of active charcoal. Ferulic acid and harman as the blue fluorescent components are isolated, the former from Saké in young, the latter from Saké kept for a long time under direct sun light.

INTRODUCTION

Study on fluorescence of various kinds of Japanese foods and drinks was initiated by H. Hiratsuka and R. Sasaki¹⁾ who reported that fluorescent color of Saké and soy-sauce is indigo blue and that of vinegar is yellowish orange with green shade. F. Oana²⁾ found later that the fluorescent color of Saké varied from yellowish to greenish milk white, and to greenish blue by an addition of acetaldehyde and to indigo-violet by an addition of salicylic acid, and it acquired blue in acid side and golden yellow with some green shade in alkaline side after treatment with active charcoal followed by decolorization of its original color. K. Kurono et al.³⁾ observed indigo-violet fluorescent color in Saké in young as well as in rice-koji, but yellowish milk white in aged Saké which they considered to be caused by protein, oil and fats of rice and some melanin materials. They also have found a substance named "Saprogenine"⁴⁾ as the most commonly associated substance with the fluorescent color in Saké, as yet nothing is known of chemical

structure. H. Katsume et al.⁵⁾ studied the changes of fluorescence throughout the process in Saké brewing. S. Teramoto⁶⁾ and D. Miwa⁷⁾ have reported that Saké usually increases its fluorescent intensity in storage or aging, does sometimes two-fold although the components of fluorescent color are different before and after aging. F. Inukai⁸⁾ proved the existence of "Flavine" in Saké and Saké-cake by the animal feeding experiment and T. Higashi⁹⁾ obtained a green fluorescent substance named "Flazine" both from Saké-cake and vinegar-cake. About 20 years later, T. Ando et al.¹⁰⁾ compared the fluorescence of synthetic Saké with that of Saké fluorophotometrically.

The fluorescence of Saké may be caused by fungi *Aspergilli* used as koji, but no one could ascertain it until K. Sakaguchi and his co-workers¹¹⁾ proved that many kinds of fluores-

1) H. Hiratsuka and R. Sasaki, *J. Agr. Chem. Soc. Japan*, **3**, 1226 (1927).

2) F. Oana, *Rep. Res. Inst. Brew. Japan*, **102**, 85 (1929).

3) K. Kurono and H. Katsume, *ibid.*, **106**, 30 (1930).

4) K. Kurono, H. Katsume and H. Fujita, *ibid.*, **115**, 1 (1932).

5) H. Katsume, K. Shiba and I. Niimi, *Rep. Res. Inst. Brew. Japan*, **124**, 33 (1936).

6) S. Teramoto, *J. Ferm. Technol. Japan*, **14**, 201, 671, 854, 990 (1936).

7) D. Miwa, *ibid.*, **15**, 9, 14 (1937).

8) F. Inukai, T. Higashi and W. Nakahara, *Sc. Pap. I. P. C. R.*, **24**, 113 (1934).

9) T. Higashi, *Rep. Inst. Phys. Chem. Res. Japan*, **15**, 1060 (1936).

10) T. Ando, K. Kuriyama and S. Akamisaka, *J. Ferm. Technol. Japan*, **33**, 522 (1955).

11) K. Sakaguchi, Y. Kihara and H. Murooka, *J. Agr. Chem. Soc. Japan*, **22**, 34 (1947).

cent substances with diversity of such colors as yellow, yellowish green, blue, purplish blue and bluish white were produced by the various strains of the genus *Aspergillus*. Y. Kihara et al.¹²⁾ proved that yellow fluorescent substance was flavine, and Y. Kaneko¹³⁾ reported that a purple fluorescent substance was "Pterin" and found some other fluorescent substances such as $C_{19}H_{16}N_3O_4$,¹⁴⁾ in the culture filtrates of *Aspergilli*.

The fluorescent materials,¹⁻¹⁴⁾ for a long time, have been of chemical interest as components of Saké but recently they began to be recognized as physiologically important agents produced by fungi. Recognizing that the range of fluorescent color in Saké is often considerable and sometimes it has been expressed with somewhat obscurity, the authors by using the spectrofluorophotometer have investigated the fluorescence spectra of Saké, their changes under physical and chemical conditions, and then found a substance "Harman" (1-methyl 9-H-pyrid 3,4-b indol) as strongly blue fluorescent material.

MATERIALS AND METHODS

Eight kinds of Saké, A, B, C, D, E, F, G and H as shown in Table I, were taken as sample. The spectro-

fluorophotometer used is one of Hitachi MPF-2 type.

Value of the intensity of fluorescent energy of 1 p.p.m. quinine sulfate solution in 0.1 N H_2SO_4 was decided as 2000 at excitation wave length (represented with E. below) $352 m\mu$ incident to emission wave length (represented with A. below) $450 m\mu$ on this apparatus, and these values of the samples were compared with this standard value and was represented with R.E. (Relative Value of Fluorescence Energy) below.

EXPERIMENTAL

Fluorescence Spectra of Various Kinds of Saké

Twenty-five peaks appear in the fluorescence spectra of whole samples, each of the peaks has a pair of its own E. and A. All peaks were classified into 6 groups such as Group I, II, III, IV, V and VI by their similarities of E. and A. and they were exemplified by the data of Table II and their representative spectra were shown in Fig. 1, 2, 3 and 4. In these peaks, peak I is invisible with the naked eye, peak II, III, IV, and VI show blue fluorescent color and peak V shows yellowish green.

Sample A has following three peaks: the first, E. $300 m\mu$ incident to A. $365 m\mu$; the second, E. $343 m\mu$ to A. $408 m\mu$; the third, E. $468 m\mu$ to A. $522 m\mu$. Sample E has also three peaks, but two of them differ from those of sample A in their locus accompanied by the disappearance of two peaks such as E. $300 m\mu$ to A. $365 m\mu$ and E. $343 m\mu$ to A. $408 m\mu$. The appearance of new two peaks such as E. $308 m\mu$ to A. $434 m\mu$

TABLE I. SAMPLES OF SAKÉ USED

Sample	Saké	Treatment
A	Ordinary Saké brewed in 1965	Keeping in cool and dark place.
B	Sample A	Keeping in white and transparent bottle under direct sun light for 1 hour.
C	Sample A	" for 4 hours.
D	Sample A	" for 21 hours.
E	Sample A	" for 132 hours.
F	Ginjō Saké* brewed in 1965	Keeping in cool and dark place.
G	Ordinary Saké brewed in 1962	Keeping at room temperature in dark place.
H	Sample A	Addition of salicylic acid (25 g per 100 liters).

* Saké brewed with highly polished rice.

12) Y. Kihara, H. Murooka and K. Sakaguchi, *J. Fer. Technol. Japan*, **26**, 297 (1948).

13) Y. Kaneko, *J. Agr. Chem. Soc. Japan*, **31**, 118, 122 (1957).

14) Y. Kaneko and H. Umemoto, Ordinary Meeting of Kansai and Chūbu Branch of Agricultural and Chemical Society of Japan (1961).

TABLE II. THE PEAKS FOUND IN FLUORESCENCE SPECTRA OF VARIOUS KINDS OF SAKÉ

Peak Location of peak	I			II			III			IV			V			VI		
	E 296~ 300	A 360~ 367		E 306~ 313	A 410~ 430		E 343~ 346	A 407~ 408		E 368~ 376	A 426~ 437		E 467~ 468	A 521~ 524		E 326	A 406	
Sample	Em μ	Am μ	R.E.	Em μ	Am μ	R.E.	Em μ	Am μ	R.E.	Em μ	Am μ	R.E.	Em μ	Am μ	R.E.	Em μ	Am μ	R.E.
	A	300	365	2016	*		343	408	1536	*			468	522	36	*		*
	B	298	364	1872	306	412	1152	*		368	435	1920	468	523	40	*		*
	C	300	367	1440	308	428	1440	*		372	434	3216	468	523	53	*		*
	D		*		308	430	3168	*		374	432	12672	468	523	78	*		*
	E		*		308	434	2160	*		376	437	16128	468	524	128	*		*
	F	296	360	1920	*		346	407	1152	*			468	523	62	*		*
	G		*		313	410	1680	*		370	426	4032	467	521	296	*		*
	H		*		*		*			*			468	523	66	326	406	17280

* No peaks are found.

FIG. 1. Fluorescence Spectra of Sample A

FIG. 2. Fluorescence Spectra of Sample E

FIG. 3. Fluorescence Spectra of Sample G

FIG. 4. Fluorescence Spectra of Sample H

and E. 376 $m\mu$ to A. 437 $m\mu$, and one peak such as E. 468 $m\mu$ to A. 524 $m\mu$ remained invariably. These changes of peaks were considered to be caused by keeping the sample A for about ten days under direct sunlight. Sample G has peaks similar to those of the sample E. Sample H has a new peak such as E. 326 $m\mu$ to A. 406 $m\mu$ followed by the disappearance of two peaks such as E. 300 $m\mu$ to A. 365 $m\mu$ and E. 343 $m\mu$ to A. 408 $m\mu$, and has one invariable peak similar to that of sample E. These changes of peaks were caused by an addition of salicylic acid (additional amount 25 g per 100 liters) and specialized by the loss of the various kinds of peaks found in ordinary Saké.

Among other samples, B and C have the peaks of intermediate form between those of A and E; D and

F have the peaks similar to those of E and A, respectively. On the basis of these data, above six groups of spectral peaks can be gathered up as follows: Peak I and III—in Saké in young but not in that aged or kept under direct sunlight; Peak II and IV—not in Saké in young but in that aged or kept under direct sunlight; Peak V—always in every samples tested; Peak VI—only in Saké added with salicylic acid.

Changes in Fluorescence of Saké Treated with Active Charcoal

To 50 ml of a sample, 20 to 100 mg of active charcoal was added and after 24 hours R.E. of peaks of the filtrate was measured. The results are shown in Fig. 5. By the charcoal treatment, Saké has increased more its R.E. of peak I or II than that of the untreated Saké, because the charcoal may adsorb any substance which arrests the appearance of peak I or II. On the contrary, main peak IV and the peak V remarkably decreased.

At any rate it is true that the blue and the yellowish green fluorescent materials are adsorbed very easily with active charcoal.

Ferulic Acid as a Blue Fluorescent Component

Experiment was carried out with paper- and column-chromatography on sample A and E. Two hundred ml of each sample was concentrated to about 30 ml under reduced pressure at 35°C, then 50 μ l of the concentrate was spotted on paper (Toyo Roshi No. 50) and developed with butanol saturated with water. One spot

Fig. 5. Change in Intensity of Fluorescence of Saké Treated with Active Charcoal

○—○ Sample A ●—● Sample B △—△ Sample E ▲—▲ Sample F

with blue fluorescence under U.V. ray of 3650 Å was found for one sample, and their R_F values were different with each other according to sample taken, and the spot of sample A which gave a reddish violet color by spraying diazobenzene sulfonic acid in Na_2CO_3 solution, seemed to be of a phenolic substance. The sample A passed through the column of IR-4B lacks its spot and the sample E through that of IR-120 lacks its spot, therefore, it seems that a spot of sample A has an acid property and that of sample E a basic one. The component containing these spots can be obtained from Saké by the following fractional extraction.

The ether extraction was first carried out, and ethereal layer was subjected to the subsequent extraction with 5% NaHCO_3 , 2N Na_2CO_3 , 2N NaOH and 2N HCl successively. Each solution was neutralized with HCl or NaOH , and stirred with ether. Each ethereal layer obtained was evaporated to dryness and dissolved in 2 ml of absolute ethanol.

Ethanol solutions were chromatographed on paper by using butanol saturated with water. The chromatogram is shown in Fig. 6, the spot seen in the concentrated sample A described above was a substance which was contained more abundantly in the ethereal layer extracted with 5% NaHCO_3 than in that extracted with 2N NaOH , and the spot seen in the concentrated sample E was a substance which was contained in that extracted with 2N HCl . The spot in sample A, when being developed on paper with 2% acetic acid, separated into two spots. And 3 mg of crude crystal was obtained by the method shown in

Fig. 6. Paperchromatogram of Five Fractions of Ether Extracted Substances in Saké

Solvent: Butanol satd. with water.
 Flour. spot under U.V. ray
 B: Blue LB: Light blue
 YG: Yellowish green
 Positive spot to diazobenzene sulfonic acid — Na_2CO_3 reagent.

Fig. 7 from the spot which gave the R_F value of 0.35. Ferulic acid also gave two spots on paper chromatogram developed with 2% acetic acid, and both the crystal and ferulic acid gave the same fluorescent spectra and U.V. absorption spectra as shown in Table III. From these results, it seemed that the crystal obtained was ferulic acid which had been found in Saké by Yamamoto.¹⁵⁾

15) A. Yamamoto, K. Sasaki and R. Saruno, *J. Agr. Chem. Soc. Japan*, 35, 715 (1961).

Fig. 7. Separation and P. C. of Ferulic Acid in Saké

TABLE III. SPECTRAL PROPERTIES OF FERULIC ACID
OBTAINED FROM SAKÉ

Fluor. spectrum (EtOH)	E 356 mμ	A 407 mμ
U.V. spectrum (EtOH)	$\lambda_{\min}$ 262 mμ	$\lambda_{\max}$ 323 mμ

Separation and Identification of Harman

Ten liters of sample E was concentrated to 1.2 liters under the reduced pressure at 35°C, and after an addition of NaOH to give blue color with B.T.B. test paper, it was extracted continuously for ten days with ether. The extract was concentrated to about 200 ml, stirred with 2 N NaOH and the acid part was removed. Substance contained in the residual ethereal solution comes into HCl solution by stirring with 2 N HCl, to which was added NaOH to give blue color with B.T.B. test paper and the mixture extracted again with ether. The ethereal solution was dehydrated with anhydrous Na₂SO₄, concentrated to dryness and dissolved in 2 ml of absolute ethanol. By paperchromatography of the alcohol solution with solvent of 2% acetic acid, one main spot of R_F 0.5~0.7 and three weak spots of R_F 0.13, 0.20, and 0.80 were detected under U.V. ray.

The sections of many papers, on which were observed the main fluorescent spots, were cut into pieces and extracted with 2 N HCl. The extract was adjusted to alkaline and extracted with ether; then ethereal solution was dehydrated and solvent removed to give a pale yellow needles crystal. The crystal was dissolved in a small amount of absolute ethanol, and on addition of one drop of conc. HCl white crystal was obtained. Further purification was carried out by means of sublimation under the reduced pressure at 180~200°C. M.p. 235~237°C. Yield 15 mg. *Anal.* Found: C, 78.99; H, 5.34; N, 15.27. *Calcd.* for C₁₂H₁₀N₂: C, 79.09; H, 5.53; N, 15.37%.

The paper chromatogram of this substance always gives one spot but showing different R_F values according to the kinds of solvents used, e.g., R_F 0.08 to 5% NH₄OH, R_F 0.33 to 3% NH₄Cl and R_F 0.33 to butanol-acetic acid-water (4:1:4). Aromatic ring, methyl, and imino-group are found in the crystal by infrared absorption spectroscopy. W. H. Perkin et al.¹⁶ reported that harman (1-methyl 9-H-pyrid 3,4-b indole), one of alkaloids, had a violet fluorescence and m.p. 238°C. The authors compared the substance obtained from Saké with harman prepared by Harvey's method¹⁷ in their chemical and physical properties as shown in Table IV, and no depression in m.p. was observed in admixture of these two substances. The two samples had identical infrared absorption spectra as shown in Fig. 8. Thus the substance obtained is identified with harman.

DISCUSSION

It is necessary to consider in fluorometric analysis that an appearance or disappearance

TABLE IV. PROPERTIES OF HARMAN*
OBTAINED FROM SAKÉ

	m.p. 237°C	M.W. 182
Fluor. spectrum (0.1 N H ₂ SO ₄)	E 384 mμ	A 430 mμ
U. V. spectrum (EtOH)	$\lambda_{\max}$ 235, 288, 336, 350 mμ

16) W. H. Perkin, jun., and R. Robinson, *J. Chem. Soc.*, **115**, 967 (1919).

17) D. G. Harvey and W. Robson, *J. Chem. Soc.*, **1938**, 91.

FIG. 8. Infrared Spectra of Harman (Chloroform)
(Top). Harman isolated from Saké (Bottom). Authentic harman

of fluorescence in mixed solution not always corresponds with that of the substance to be associated with fluorescence, although it has been considered to be one of the most sensible and sometimes very convenient methods in seeking for materials.

From 140 out of 200 kinds of compounds, so far as is known now, in Saké and less than those in other brewing products have been found, but so far no case of fluorescence has been substantially proved, except some kinds of compounds such as ferulic acid found in soy sauce¹⁸⁾ and Saké,¹⁵⁾ and flavine. Recently, T. Yokotsuka et al.¹⁹⁾ who attempted to detect any substances like aflatoxin in the culture filtrates of about sixty kinds of industrial strains of the *Aspergillus* in Japan, failed to find them but succeeded in isolation of such substances as flavacol, aspergillic acid and ergosterol as fluorescent matters. Therefore harman may be regarded as a component isolated for the first time from Saké.

O. Fisher²⁰⁾ found that harman was caused via harmol from harmine contained in *Peganum harmala* and showed blue to violet fluorescent color under U.V. ray. From this time onwards it has become to be found in

some other plants²¹⁻²³⁾ and in casein hydrolysates.²⁴⁾ The fact that harman is found limitedly in Saké aged or kept for a long time under direct sunlight, suggests the existence of a close correlation between its appearance and the aging in Saké.

SUMMARY

The fluorescence spectra of various kinds of Saké were measured by using spectrofluorophotometer (Hitachi MPF-2). At least two kinds of fluorescent color were observed in Saké, the one is blue and the other yellowish green. Blue color was dominant but its characters varied with kinds of Saké; on the contrary yellowish green color was weak but seemed to have always the same characters. By the addition of salicylic acid to Saké, the blue color increased and its spectrum varied distinctly from that of original Saké, and by the treatment with active charcoal, they decreased together remarkably.

From the observation on fluorescence spectra of Saké, it seemed that the components of fluorescent color of Saké aged for a long time was similar to those of Saké kept under direct sunlight.

18) Y. Asao and T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **32**, 622 (1958).

19) T. Yokotsuka, M. Sasaki, T. Kikuchi, Y. Asao and A. Nobuhara, Ordinary Meeting of Kantō Branch of Agricultural Chemical Society of Japan, (1966).

20) O. Fisher, *Chem. Centr.*, **1901**, i, 957.

21) E. Späth, *Monatsh.*, **40**, 351, **41**, 401 (1920).

22) V. A. Chatterjee, *J. Indian Chem. Soc.*, **28**, 29 (1951).

23) J. Lutomski, *Buil. Inst. Roślin Leczniczych*, **6**, 209 (1960).

24) R. Tschesche, H. Jenssen and P. N. Rangachari, *Chem. Ber.*, **91**, 1732 (1958).

Among the blue fluorescent components, ferulic acid was isolated only from Saké in young on the contrary a considerable amount of harman was found in Saké kept under direct sunlight. Harman was isolated by the ethereal extraction from Saké and the subsequent paperchromatography.

It was considered that an increment of blue fluorescent color in aging was caused by that of harman.

Acknowledgement. The authors wish to ex-

press their thanks to an Emeritus Prof. Dr. K. Sakaguchi, the University of Tokyo, and Dr. M. Suzuki, Research Institute of Brewing, Tokyo, for continued interest and encouragement; to Assistant Prof. Dr. N. Takahashi, the University of Tokyo, for analysis on infrared absorption spectra; to Mr. Y. Fukui, Research Institute of Brewing, for technical assistance, and to the Japan Saké Brewers Central Association, Tokyo, which afforded facilities for making spectrofluorophotometric analysis.