On Kakishibu, II.[1]

By

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(Received September 17, 1924)

I. THE VOLATILE ACIDS.

When kakishibu is prepared freshly, it gives forth an aromatic odour which changes on standing in the open air, gradually to a peculiar one resembling soured milk, and this is usually regarded as the characteristic odour of kakishibu, owing to the presence of some organic acids. As to the volatile acid of shibu, Ishikawa[2], in determining the content of barium in the salt, has decided it to be composed of butyric acid.

On Sept. 15, Oct. 12, and Nov. 26, 1923, sp. gr., total acidity, content of the total sugars and shibuol in 100 c.c. of the shibu were determined.

Table I.

Date.	Sept. 15.	Oct. 2.	Nov. 26.
Sp. gr.	1·04	1·02	—
Total acid in 100 c.c.	0·2880 grm. as succinic acid.	0·6516 grm. volatile acid 0·3067 grm. as butyric acid, or 0·409 grm. as succinic acid, non-volatile acid, 0·189 grm.	1·033 grm.
Total sugars in 100 c.c.	3·794 grm.	0·338 grm.	no reducing sugars.
Shibuol.	2·84 "	2·65 "	—

1 The expenses of this investigation were shared by the Government Department of Education.

2 J. Chem. Soc. Japan., **3**, 12(1882).

In consequence of the fact that the development of the acid in shibu was accompanied with a decrease of the sugar content in almost inverse proportion as shown in above Table I, the authors came to believe that the acids should be formed from carbohydrates by the fermentation which occurs in the kaki-fruit, and extracted with water, and actually the phenomenon was noticed 24 hours after it was prepared, and the brisk evolution of gas ended in a few days.

Therefore, the composition of volatile acids on the one side and on the other of carbohydrates in the shibu somewhat extensively investigated, whereby to get some evidence for the explanation of the chemical change in the formation of the volatile acids in the fluid.

The shibu prepared on the 14th of Sept., 1922, was subjected to distillation on March 29, 1923, and the acidic distillate was neutralized with barium hydroxide. The dry barium salts were heated in a flask with syrupy phosphoric acid, to get free acids from them.

The free acids distilled from 117° to 162°, were transformed into their isoamylesters as usual, and the esters were then subjected to fractional distillation and were divided into the following 4 portions;

	Fraction.	Yield.
1.	to 135°	2 grm.
2.	135 – 145°	1·5 "
3.	145 – 175°	2·5 "
4.	175 – 178°	7·2 "

The last fraction, B. p. 175–178° indicating $d_{4^\circ}^{23^\circ}=0{\cdot}846$; $n_D^{23^\circ}=1{\cdot}4068$, was analysed with the following results:

C=68·41; 68·31; H=11·72; 11·42. which agree quite well with these C=68·27; H=11·48 for isoamylbutylate. The second fraction, B. p. 135–145°, gave C=65·50; H=11·49 on analysis, which show slight difference with theoretical value C=64·483; H=10·85 for isoamyl acetate, and accordingly it was hydrolysed with barium hydroxide, and barium acetate, thus formed, was extracted with absolute alcohol at ordinary temperature and the content of barium after dried at 100° to constant weight, was determined to be 53·11% which agrees quite well with that of pure barium acetate (53·78).

It was thus confirmed that the main part of the volatile acids is composed of butyric acid, as noticed by Ishikawa, accompanied by small quantity of acetic acid.

II. SUGARS.

On admitting the hypothesis that the volatile acids are produced in the kakishibu during its preservation, at the expense of some of the carbohydrates extracted from it, to be correct, the authors have undertaken the detection and the isolation of carbohydrates in the shibu in the various stages of its preservation, in the following manner.

The fresh kakishibu, clarified with basic lead acetate to get rid of the shibuol and pectin in it, and an excess of lead salt removed by means of hydrogen sulphide gas, was concentrated under reduced pressure to syrup and dissolved in glacial acetic acid and then left to stand in a cold place, where d-glucose was crystallized, and separated, and identified by the determination of the melting point (143–144°) and of the optical rotatory power in an aqueous solution, $[\alpha]_D^{23} = \frac{1 \cdot 108 \times 100}{2} = +55 \cdot 40^\circ$ after 20 hours it was prepared.

From the mother liqour separated from the crystals of d-glucose, d-fructose was isolated in a crystalline state melting at 93–94°.

No cane sugar could be detected in the solution. The content of these sugars in the shibu at various stages of preservation was determined as mentioned in Table I.

The carbohydrates occurring in the fruit, were also found in the shibu when fresh, but not the volatile acids. The latter subtances, however, are found in old shibu, and sugars are not.

It is, therefore, concluded that these carbohydrates, as we anticipated, were transformed into organic acids during the preservation by the action of micro-organisms.

Can we ascribe the formation of these acids to the butyric fermentation of the carbohydrates as in the souring of milk?

It was generally believed that the butyric fermentation by microbes was always accompanied by the evolution of carbon dioxide and hydrogen gases and especially of the chemical action of the latter gas, C. Neuberg has stated:[1] "Wie man sieht, trifft der Wasserstoff hier auf keinen organischen Acceptor, und schon früher habe ich darauf hingewiesen, dass Erregern von Butyl-Gärungen die Fähigkeit eigen ist, den atmosphärischen Stickstoff zu binden. Vielleicht ist die Vorstellung nicht zu kühn, das gerade der diesen Lebewesen der H zu Verfügung stellt, der im atomeren Entstehungszustande eine "Harber-Synthese" herbei führt."

1 Ber. D. Chem. Ges., **55**, 3635 (1923).

In the present case, however, the products of fermentation are much complicated, and we have in addition K. Oshima's[1] report that a compound having the same melting point as mannite was isolated from old shibu.

The authors have also succeeded in getting mannite from the fermented shibu as shown in the following statement.

III. MANNITE.

3600 c.c. of the fermented shibu after treatment with basic lead acetate and hydrogen sulphide successively to remove pectin, shibuol and lead salts from the solution, were concentrated under reduced pressure to syrup and were left to stand over-night at a temperature of 5–6°, and needle crystals were deposited, separated by filtration, washed with 94% alcohol and recrystallized from a 90% alcohol solution. The yield was 6 grm. It melted at 160°, and gave on analysis:

C = 39·32;	H = 7·83,	and the theory requires
C = 39·53;	H = 7·68	for $C_6H_{14}O_6$.

Although optically inactive, it shows dextro-rotatory when dissolved in a solution of 1·28 grm. of borax in 10 c.c. of water:

$$[\alpha]_D^{20} = \frac{2{\cdot}25 \times 100}{10} = +22{\cdot}5^\circ$$

As a matter of fact, mannite was found in the fermented kakishibu but not in fresh or in the kaki-fruit. Such facts can easily be explained by assuming that the sugars in the shibu are partly consumed in the building up of butyric, acetic acids, carbon dioxide and hydrogen, in the process of fermentation, and mannite has formed from another part of the sugars by secondary reaction taking place in the fluid, since the nascent hydrogen generated by the fermentation effects the process of reduction among the various bodies present in it.

Now, the authors were perplexed to decide whether the mannite formation should be attributed to the secondary reaction of the butyric fermentation or otherwise.

According to Pasteur,[2] a particular organism acts upon some sugars transforming them into a kind of dextrin or gum, with mannite and carbon

1 J. Pharm. Japan., No. 221, 1 (1900). And also Prof. R. Majima has confirmed this in his private communication to one of the authors (S. K.).

2 L. Pasteur: Bull. Soc. Chim., 1861. 35; V. P. Lippmann: Zuckerarten, I, 423 (1904).

dioxide being formed at the same time.

Admitting that mannite was formed in consequence of the so-called viscose or mucilage fermentation of sugars by organisms, the authors' attention was naturally directed to the examination of mucilage in the shibu, and fortunately the very striking phenomenon was noticed, which occurred in kakishibu during preservation, and was never mentioned previously by any investigators.

The liquor on exposure to the air becomes viscous, and forming there a white jelly-like translucent mass. This membrane gradually increases in size and in thickness, so that the veil or pellicle appears laminated.

IV. FILM.

The white jelly-like membrane formed in the fluid, separated from the solution washed, with cold water to remove the acidic mother-liquor adhering to it, and analysed after drying to constant weight:

C=51·60; 51·52; H=5·04; 4·98; Ash=4·23.

The film was then treated with boiling 75% alcohol for 5 hours, repeating the operation twice, and the insoluble residue was dried at 110° and analysed:

C=47·94; H=5·56; Ash=3·68.

It seems from the analytical figures that the residue was composed of a mixture of mucilage and pectin. The soluble part of the film in 75% alcohol was confirmed, by chemical reaction and also by analysis, to consist of shibuol:

C=57·39; 56·90; H=4·51; 4·38; Ash=4·90.

In another experiment, the film was soaked in cold water for two days to extract the water-soluble substance or mucilage as far as possible, and then the residue was treated with boiling 70% alcohol until the extract indicated no more colour reaction with ferric chloride.

V. MUCILAGE IN THE FILM.

The aqueous extract of the film was found to contain some polysaccharide resembling mucilage in many respects. It was thrown out of the viscous solution by the addition of alcohol, and was separated, washed with alcohol and ether successively, and then analysed after drying to constant weight. The yield was 1·6 grm.

C=47·52; 47·57; H=5·63; 5·82; Ash=2·91; 3·21.

In another experiment, the mucilage isolated from the film, gave on

analysis the following results:

C=48·02; 47·60; H=5·64; 5·44; Ash=4·47; 4·50.

It shows a characteristic property in forming a slimy mass when moistened with water, and analytical figures as shown above agree fairly well with those described by many investigators in plant mucilage.[1]

VI. SHIBUOL IN THE FILM.

The 70% alcohol extract of the film after treatment with water showing a colour reaction with ferric chloride which resembled shibuol, was concentrated under reduced pressure to a small volume, and a mixture of absolute alcohol and ether was added, whereby shibuol was precipitated, and separated from the solution, dried to constant weight. The yield was 5 grm.

The chemical reactions and analytical results of the substance agree well with those of shibuol described by the authors' first communication on this subject.[2]

VII. PECTIN IN THE FILM.

The insoluble residue in cold water and also in boiling 70% alcohol, amounting to 2·5 grm., was analysed after drying at 110° to constant weight.

C=52·20; 51·72; H=5·72; 5·71; Ash=1·75; 1·34.

The purified sample, however, by precipitation from a hot aqueous solution with alcohol, gave on analysis the following results:

C=50·47; 49·57; H=5·19; 5·24; Ash=1·9; 1·3.

These results agree well with those of the pectin substance from fresh kakishibu obtained in the following way, and also from the kaki-fruit by one of the authors (S. K.) and H. Ueda.[3]

3250 c.c. of the fresh kakishibu were concentrated under reduced pressure to 1400 c.c. and an equal volume of 94% alcohol were added to precipitate the pectin-like substance only, the clear liquor was decanted off, and 70% alcohol added, and centrifuged off the solution.

The washing of the precipitate with 70% alcohol was repeated 4 times to remove shibuol which adhered to it as much as possible, and the

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1 W. Kirchner and B. Tollens: Lieb. Ann., **175**, 215 (1875); Mulder: J. prak. Chem., **37**, 340 (1888); B. Gans and B. Tollens: Lieb. Ann., **249**, 245 (1888); Schimidt: Ibid., **51**, 29 (1844), and also refer S. Komatsu and H. Ueda: On mucilage: These Memoirs.

2 These Memoirs, **7**, 15 (1923).

3 J. Biochem., I, 191 (1922).

residue washed with 94% alcohol and ether successively and then dried. The yield was 4·5 grm. which corresponded to 0·13% of the kakishibu. On analysis, it gave the following results:

$$C = 51{\cdot}96\,; \quad H = 5{\cdot}29\,; \quad Ash = 3{\cdot}78\,; \quad CH_3O = 1{\cdot}87.$$

In conclusion, it was obvious that the mucilage was produced by the carbohydrates occurring in kakishibu, whatever the exact nature of the chemical changes for its formation may be, and subsequently was absorbed by the pectin with shibuol in the acidic medium forming the film, and the latter two substances (pectin and shibuol) occur in the fruit, and were extracted in solution in making shibu.

The shibu clarified by filtration through an asbestos mat and also by treating with alcohol, yields no film on being exposed to the air for a long time, neither could pectin be detected in it. Such evidences are in favour of the authors' view that mucilage, pectin and shibuol combine loosely and form the film; the ratio of these substances in the film is 1 : 1·5 : 3.

These facts, so far as we have studied, manifest the purely chemical aspects of the fate of simple and polysaccharides in the kakishibu, which they are liable to undergo, in which it would appear that one part was consumed in the process of fermentation in forming destructive organic acids, while another part in building mannite and a complex carbohydrate or film.

The exact nature and the mechanism for the formation of these compounds will be discussed in an another article after the microbes in the fluid have been investigated.

April, 1924. Laboratory of Organic- and Bio-Chemistry.