Gas chromatographic Analysis of Organic Acids in Japanese Green Tea Leaves

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Abstract: Herein, gas chromatography is used to determine and quantify organic acids in Japanese green tea leaves, and the established method is employed to profile the acid components of Matcha, Gyokuro, Sencha green teas, and green tea varieties and thus acquire data needed to ensure the high quality and safety of green tea. The tea leaves were esterified with 10 vol% sulfuric acid in 1-butanol at 100°C for 2 h. Oxalic acid contents were high in Asatsuyu and Okuyutaka samples and were low in Sofu, increasing in the order of Sencha < Gyokuro < Matcha, while citric acid content increased in the order of Sencha < Matcha < Gyokuro. Moreover, the oxalic acid content of Gyokuro only slightly increased with increasing tea grade. The relative contents of the different fatty acids did not strongly vary between the different green tea varieties. However, the n-3 to n-6 ratio was found to be low in Sofu. The progressing maturity increased the n-3 to n-6 ratio of Yabukita. The n-3 to n-6 ratio was low in high-grade Matcha, Gyokuro, Sencha green teas, and was related to the green tea quality. The developed method was concluded to be suitable for the evaluation of green tea quality.

Key words: fatty acid, gas chromatography, green tea, oxalic acid, organic acid

1 Introduction

Green tea enjoys global popularity and includes several varieties of tea. For example, Sencha green tea (hereafter referred to simply as Sencha) is a popular and general green tea in Japan. Gyokuro green tea (hereafter referred to simply as Gyokuro) is a higher quality tea than Sencha, and requires more time to prepare than Sencha, including a long shading period. Matcha green tea (hereinafter referred to simply as Matcha) is made by grinding Tencha green tea, which is cultivated using more than 20 days of shading. These green teas contain catechins and amino acids, which are believed to benefit human health\(^1\)\(^-\)\(^2\). Moreover, high-grade green tea is characterized by a high content of amino acids, which, alongside with other components, influence tea taste. Kaneko et al. reported that succinic acid and theogallin enhance the taste of glutamic acid in Matcha\(^3\), while Horie et al. suggested that the taste of green tea may be influenced by citric acid\(^4\). However, succinic acid makes up a very low fraction of organic acids found in green tea leaves\(^5\), whereas the fraction of oxalic acid is usually high. Kamiya et al. indicated that high-grade Matcha is rich in oxalic acid, i.e., consumption of this tea leads to excessive oxalic acid intake\(^6\), which, according to some studies, may increase the risk of kidney stone formation\(^7\)\(^-\)\(^10\). However, the intake of Sencha is not expected to induce kidney stone formation, as (i) this tea has a lower oxalic acid content than Gyokuro and Matcha and (ii) epigallocatechin gallate contained in Sencha may actually suppress kidney stone formation\(^11\)\(^,\)\(^12\). The growing export of Japanese green tea needs to be backed up by high tea quality, and high oxalic acid content should therefore be avoided. Analysis of the organic acids in tea leaves is carried out via GC or capillary electrophoresis. Kamiya et al. measured the oxalic acid content of green tea using methyl esterification\(^10\). However, this method is cumbersome, and only oxalic acid can be measured. Capillary electrophoresis is not general equipment compared to GC or high performance liquid chromatography. Organic acid analysis using GC-MS is often carried out via trimethylsilylation. Organic acids contain carboxyl groups; therefore, these components can be easily analyzed using esterification. However, the reported butanol method has a large interday variation, and a stable value cannot be obtained\(^10\). In view of the above, one should clarify the organic acid contents of green tea to improve its quality. Herein, we identify and quantify organic acids in Japanese green tea.

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leaves (including commercial Matcha, Gyokuro, and Sencha) using gas chromatography to ensure high tea quality and safety.

2 Materials and Methods

2.1 Samples

Crude tea leaves were collected in the field of the Kanaya Tea Research Station in April and May (2016 or 2018). Matcha, Gyokuro, and Sencha tea leaves were purchased (online or directly) in 2018. Gyokuro, and Sencha samples were powdered using a mill.

2.2 Chemicals

Sulfuric acid, 1-butanol, n-hexane, sodium carbonate, 2,2-dimethoxypropane, oxalic acid, malic acid, succinic acid, and citric acid were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Glutaric acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.3 Esterification

Typically, a 15 mL screw cap test tube was sequentially charged with a 50 mg sample, 2,2-dimethoxypropane (20 μL), 1-butanol (50 μL) containing glutaric acid as an internal standard, and 10 vol% sulfuric acid in 1-butanol (1 mL), and heated at 100°C for 2 h. During the reaction, the test tube was agitated every 15 min using a Vortex mixer. The test tube was cooled to room temperature, charged with n-hexane (3 mL), and agitated again using the Vortex mixer. Saturated sodium carbonate solution (1 mL) was added, and the test tube was agitated again and left to stand to allow the two phases to separate. The upper phase was collected and transferred to another test tube, which was charged with distilled water (5 mL), strongly shaken, and left to stand to achieve phase separation. The lower phase was discarded and washing with distilled water was performed two more times. The wet organic phase was subjected to centrifugation, and the upper phase was collected and analyzed by gas chromatography.

2.4 Gas chromatographic analysis

Organic acid analysis was performed on a gas chromato-

![Chromatograms](image)

Fig. 1 Chromatograms of Matcha (A) and rapeseed oil (B).

a: oxalic acid, b: succinic acid, c: malic acid, d: citric acid, I: palmitic acid, II: stearic acid, III: oleic acid, IV: linoleic acid, V: linolenic acid
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3 Results and Discussion

Organic and fatty acid butyl esters of Matcha and rapeseed oil were well separated (Fig. 1). Figure 2 shows the changes of organic and fatty acid contents of Matcha with reaction time, revealing that malic, succinic, and citric acid contents were low during the first 30 min and stabilized after 60 min. The oxalic acid content was maximal after 120 min. The 16:0 percentage slightly increased, and the 18:3n-3 percentage decreased with increasing time. These changes of fatty acids might be due to oxidation with sulfuric acid. Therefore, 120 min was chosen as the optimal reaction time. The contents of oxalic, malic, succinic, and citric acids relative to that of glutaric acid (100%) equaled 107.2 ± 1.2, 106.8 ± 0.3, 58.1 ± 0.5, and 73.8 ± 2.4%, respectively. The recoveries of malic and citric acids were low, as these acids contain a hydroxyl group and 1-butanol was used as the reaction solvent. The slightly higher recovery of citric acid compared to that of malic acid was ascribed to tributyl ester formation. These results imply that the established method may underestimate malic and citric acid contents. However, the developed method featured small relative standard deviations (oxalic acid: 2.1%, succinic acid: 4.2%, malic acid: 2.4%, citric acid: 1.6%; see Fig. 2, 120 min) and was therefore sufficiently suited for comparative acid content analysis. A highly reproducible analytical value of for the organic acid in green tea was obtained because dimethoxypropane was substituted for anhydrous sodium sulfate. Furthermore, the peak of 1-butanol observed in sample chromatograms was ascribed to the solubility of this alcohol in n-hexane. However, washing with distilled water removed 1-butanol from n-hexane without affecting organic acid contents. The fatty acid content of tea leaves could not be measured using the developed method because of esterification ratio differences between fatty acids and glutaric acid used as the internal standard. Therefore, another internal standard is necessary for fatty acid quantitation.

Table 1 lists the organic and fatty acid contents of green tea samples, revealing that oxalic acid content was high for
Asatsuyu and Okuyutaka and low for Sofu. Succinic and malic acid contents did not strongly vary with green tea varieties. The highest citric acid content was observed for Okuyutaka, and the major fatty acids were identified as 16:0, 18:2\(\text{n-6}\), and 18:3\(\text{n-3}\). The relative contents of different fatty acids did not strongly vary with green tea varieties. The \(\text{n-3}\) to \(\text{n-6}\) ratio was lowest in Sofu. Increased maturity increased the \(\text{n-3}\) to \(\text{n-6}\) ratio of Yabukita, in agreement with a previous report\(^{15}\).

Table 2 shows the composition of Sencha, revealing that the contents of oxalic, succinic, malic, and citric acids equaled 10–12, 0.3, 1.1–1.6, and 1.7–2.3 mg/g, respectively. High-grade Sencha was rich in citric acid, while malic and citric acid contents were slightly higher in Kyushu products than in Honshu and Shikoku products. However, no marked differences were observed between Honshu/Shikoku and Kyushu products in terms of fatty acid composition. The 18:2\(\text{n-6}\) percentage was low and the 18:3\(\text{n-3}\) percentage was high in low-grade Sencha, and the \(\text{n-3}\) to \(\text{n-6}\) ratio was thus also high.

Table 3 shows the composition of Gyokuro, revealing that its oxalic, succinic, malic, and citric acid contents were 16–18, 0.2, 1.4–1.6, and 2.7–3.5 mg/g, respectively. The oxalic acid content of Gyokuro increased with its increasing grade, while citric acid content was highest in middle- and high-grade Gyokuro. Moreover, the oxalic and citric acid contents of Gyokuro exceeded those of Sencha. As in the case of Sencha, the 18:2\(\text{n-6}\) percentage was low, and the 18:3\(\text{n-3}\) percentage was high in low-grade Gyokuro. Hence, the corresponding \(\text{n-3}\) to \(\text{n-6}\) ratio was also high.

Compared to low- and middle-grade Sencha, Gyokuro featured a low 18:1\(\text{n-9}\) percentage. However, the 18:3\(\text{n-3}\) percentage and the \(\text{n-3}\) to \(\text{n-6}\) ratio of Gyokuro were slightly higher than those of Sencha.

Table 4 presents the composition of Matcha, revealing that its oxalic, succinic, malic, and citric acid contents equaled 19–21, 0.2, 1.4–1.6, and 2.6–3.1 mg/g, respectively. Although oxalic acid content was not affected by tea grade, the citric acid content of low-grade Matcha was slightly lower than that of high-grade Matcha. Moreover, the oxalic acid content of Matcha exceeded those of Sencha and Gyokuro, and the citric acid content of Matcha exceeded that of Sencha. In Matcha, the 18:2n-6 percentage was low, and the 18:3\(\text{n-3}\) percentage was high in low-grade Matcha. Hence, the \(\text{n-3}\) to \(\text{n-6}\) ratio was also high in the latter tea. Finally, Matcha featured a lower 18:2\(\text{n-6}\) percentage and higher 18:3\(\text{n-3}\) percentage than Sencha and Gyokuro.

Kamiyama's reports indicate that high-grade Gyokuro and Matcha are rich in oxalic acid\(^{12}\). However, this tendency was only observed for Gyokuro and not for Matcha in the

### Table 1 Organic acid contents and fatty acid profiles of green tea varieties.

| Year | Organic acid (mg/g) | Fatty acid (area%) |
|------|---------------------|-------------------|
|      | Oxalic  | Succinic | Malic  | Citric  | 16:0 | 18:0 | 18:1\(\text{n-9}\) | 18:1\(\text{n-7}\) | 18:2\(\text{n-6}\) | 18:3\(\text{n-3}\) | \(\text{n-3}\)/\(\text{n-6}\) |
| 2016 | Asatsuyu | 13      | 0.4    | 1.5    | 1.8  | 20.2 | 2.4  | 6.9   | 0.7   | 23.0  | 46.9  | 2.0   |
|      | Okumidori | 9       | 0.3    | 1.1    | 1.6  | 22.6 | 2.0  | 8.7   | 1.0   | 22.1  | 43.6  | 2.0   |
|      | Okuyutaka | 12      | 0.3    | 1.2    | 2.5  | 22.3 | 1.6  | 6.7   | 0.8   | 23.6  | 45.1  | 1.9   |
| 2017 | Kanayamidori | 12     | 0.4    | 1.3    | 1.7  | 20.9 | 1.5  | 8.2   | 0.8   | 23.2  | 45.3  | 2.0   |
|      | Saeakari  | 10      | 0.3    | 1.6    | 1.6  | 21.8 | 2.2  | 6.9   | 0.8   | 23.8  | 44.5  | 1.9   |
|      | Saemidori | 12      | 0.4    | 1.4    | 1.6  | 20.1 | 2.0  | 6.9   | 0.7   | 23.6  | 46.8  | 2.0   |
| 2018 | Soufu     | 8       | 0.3    | 1.4    | 1.7  | 21.3 | 1.5  | 7.1   | 1.2   | 26.0  | 42.9  | 1.7   |
|      | 10       | 0.2    | 1.4    | 1.9    | 20.5 | 1.5  | 7.1  | 1.2   | 26.8  | 42.9  | 1.6   |
|      | Harumidori | 9    | 0.3    | 0.7    | 1.5  | 21.7 | 1.6  | 6.6   | 0.7   | 24.0  | 45.4  | 1.9   |
|      | Yabukita (Younger new) | 10 | 0.4    | 1.2    | 1.7  | 21.8 | 1.8  | 7.8   | 0.8   | 25.3  | 42.4  | 1.7   |
| 2019 | Yabukita (Normal) | 9     | 0.3    | 1.5    | 1.6  | 21.2 | 2.0  | 8.7   | 0.6   | 24.7  | 43.7  | 1.8   |
|      | Yabukita (Over matured) | 10 | 0.3    | 1.6    | 1.7  | 21.2 | 2.2  | 7.7   | 0.5   | 24.0  | 44.4  | 1.8   |
|      | 10       | 0.3    | 1.3    | 1.6    | 21.3 | 2.2  | 7.4   | 0.3   | 23.4  | 45.3  | 1.9   |

\(\text{tr.}:\text{trace}\)
The theanine content of green tea is known to increase with increasing tea grade\(^{16}\). Moreover, the theanine and oxalic acid contents of green tea can increase upon shading cultivation\(^{17, 18}\), as confirmed by the high oxalic acid contents of shading-cultivated Matcha and Gyokuro. Thus, oxalic acid content was concluded to be mainly influenced by the use of shading. The citric acid contents of Gyokuro, Matcha, and Sencha increased with increasing grade. According to some reports, the use of nitrogenous fertilizers increases the citric and oxalic acid contents of tea leaves\(^{4, 19}\). Although our study does not provide evidence that tea grade affects the oxalic acid content of Sencha and Matcha, high-grade Gyokuro showed the highest oxalic acid content. The high consumption of oxalic acid increases the risk of urolithiasis\(^{6-10}\). Furthermore, oxalic acid easily binds calcium, interfering with its absorption and decreasing its bioavailability\(^{20}\). Thus, fertilization and shade, which are necessary for the production of high-grade green tea, also relate to its high oxalic acid content. Therefore, it is difficult to reduce the oxalic acid

### Table 2

Organic acid contents and fatty acid profiles of different-grade Sencha.

| Grade (Yen/100 g) | <999 | 1,000 | 1,001-3,000 | 3,001< |
|-------------------|------|------|-------------|-------|
| Locality          | Honshu & Shikoku | Kyushu |             |       |
| Localities        | n = 5 | n = 8 | n = 6 | n = 6 | n = 3 |
| Organic acid (mg/g) | | | | |
| Oxalic            | 11 ± 2 | 11 ± 1 | 12 ± 2 | 12 ± 2 | 10 ± 2 |
| Succinic          | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.0 |
| Malic             | 1.2 ± 0.1 | 1.3 ± 0.2 | 1.6 ± 0.4 | 1.3 ± 0.2 | 1.1 ± 0.2 |
| Citric            | 1.7 ± 0.2 | 1.8 ± 0.3 | 2.1 ± 0.4 | 2.0 ± 0.4 | 2.3 ± 0.7 |
| Fatty acid (area%) | | | | |
| 16:0              | 21.0 ± 0.3 | 21.1 ± 0.7 | 20.9 ± 0.5 | 21.3 ± 0.8 | 22.7 ± 0.6 |
| 18:0              | 2.2 ± 0.1 | 2.1 ± 0.3 | 2.0 ± 0.3 | 1.9 ± 0.2 | 1.6 ± 0.2 |
| 18:1n-9           | 7.3 ± 1.0 | 7.0 ± 0.6 | 7.5 ± 1.0 | 7.2 ± 0.5 | 6.5 ± 0.4 |
| 18:1n-7           | 0.5 ± 0.1 | 0.5 ± 0.2 | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.3 |
| 18:2n-6           | 21.9 ± 1.3 | 23.1 ± 1.4 | 23.1 ± 1.6 | 23.4 ± 1.1 | 24.3 ± 0.7 |
| 18:3n-3           | 47.1 ± 0.8 | 46.2 ± 1.6 | 45.9 ± 1.3 | 45.5 ± 1.6 | 44.0 ± 1.2 |
| n-3/n-6           | 2.2 ± 0.2 | 2.0 ± 0.2 | 2.0 ± 0.2 | 2.0 ± 0.2 | 1.8 ± 0.1 |

### Table 3

Organic acid contents and fatty acid profiles of different-grade Gyokuro.

| Grade (Yen/100 g) | <4,999 | 5,000-7,999 | 8,000< |
|-------------------|--------|-------------|--------|
| Localities        | n = 5 | n = 4 | n = 4 |
| Organic acid (mg/g) | | | |
| Oxalic            | 16 ± 1 | 17 ± 3 | 18 ± 1 |
| Succinic          | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 |
| Malic             | 1.6 ± 0.4 | 1.7 ± 0.4 | 1.6 ± 0.1 |
| Citric            | 2.7 ± 0.5 | 3.5 ± 0.6 | 3.4 ± 0.8 |
| Fatty acid (area%) | | | |
| 16:0              | 20.4 ± 0.1 | 20.2 ± 0.4 | 20.4 ± 0.9 |
| 18:0              | 2.0 ± 0.3 | 1.7 ± 0.1 | 2.0 ± 0.4 |
| 18:1n-9           | 6.8 ± 0.7 | 6.7 ± 1.4 | 6.1 ± 0.4 |
| 18:1n-7           | 0.7 ± 0.2 | 1.0 ± 0.3 | 1.1 ± 0.3 |
| 18:2n-6           | 21.3 ± 1.2 | 21.6 ± 0.5 | 22.9 ± 1.0 |
| 18:3n-3           | 48.7 ± 1.7 | 48.7 ± 2.1 | 47.5 ± 2.0 |
| n-3/n-6           | 2.3 ± 0.2 | 2.3 ± 0.1 | 2.1 ± 0.2 |
in tea leaves. However, in the opinion of the author, the oxalic acid content of green tea leaves should be reduced for their further use. High-grade Sencha was rich in citric acid. The oxalic acid content of Gyokuro and Macha exceeded those of Sencha. These results suggested that citric acid may be related to the green tea quality.

The \( n\)-3 to \( n\)-6 ratio was low in high-grade Sencha, Gyokuro, and Macha. Anan and Nakagawa reported that the total lipid and \( 18:3\) \( n\)-3 contents of tea leaves increased with increasing tea leaf maturity \( ^{15} \). Therefore, these results indicate that high-grade green tea may be harvested early. Polyunsaturated fatty acids are easily oxidized, and their derivatives are responsible for green tea aroma. Baba et al. showed that \( (Z)\)-3-hexenal, \( (Z,Z)\)-3,6-nonadienal, and \( (E,E)\)-2,4-decadienal showed higher flavor dilution factors in medium- and low-grade Matcha than in high-grade Matcha \( ^{21} \). In the present work, the \( n\)-3 to \( n\)-6 ratio was shown to be high in medium- and low-grade Matcha, and the change of this ratio was suggested to influence the aroma of this tea. In agreement with previous studies, which reported that low-grade Matcha features strong fatty notes due to the presence of polyunsaturated fatty acids, the present work shows that low-grade green tea is rich in easily oxidizable \( 18:3\) \( n\)-3 acids.

### 4 Conclusions

The developed method allowed us to quantify individual organic acids in green tea leaves and determine their fatty acid profiles despite the fact that the recoveries of citric and malic acids were low. The oxalic acid content and the \( n\)-3 to \( n\)-6 ratio were found to be low in Sofu. Importantly, our method allowed us to clarify the difference between tea varieties and was concluded to be well suited for the evaluation of green tea quality.

### Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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### References

1. Williams, J.; Sergi, D.; McKune, A.J.; Georgousopoulou, E.N.; Mellor, D.D.; Naumovski, N. The beneficial health effects of green tea amino acid L-theanine in animal models: Promises and prospects for human trials. *Phytother. Res.* **33**, 571-583 (2019).

2. Khan, N.; Mukhtar, H. Tea polyphenols in promotion of human health. *Nutrients* **11**, E39 (2018).

3. Kaneko, S.; Kumazawa, K.; Masuda, H.; Henze, A.; Hofmann, T. Molecular and sensory studies on the umami taste of Japanese green tea. *J. Agric. Food Chem.* **54**, 2688-2694 (2006).

4. Horie, H.; Ujihara, T.; Kohata, K. Contents of organic acids in green teas and their contribution to tea quali-
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1271-1277 (2019)

J. Oleo Sci. 68, (12) 1271-1277 (2019)

J. Oleo Sci. 68, (12) 1271-1277 (2019)

Mitchell, T.; Kumar, P.; Reddy, T.; Wood, K.D.; Knight, Bazyar, H.; Ahmadi, A.; Zare Javid, A.; Irani, D.; Wu, Z.B.; Jiang, T.; Lin, G.B.; Wang, Y.X.; Zhou, Y.; Kamiya, C.; Ogawa, M.; Ohkawa, H. Oxalic acid and kidney stone formation. J. Chromatogr. Sci. 23, 333-340 (1985).

Anan, T.; Nakagawa, M. Studies on the lipids of tea leaves. Part I. Changes in lipid content and fatty acid composition of tea leaves during growing period of the first and second flushes. Nippon Shokuhin Kogyo Gakkaishi 24, 305-310 (1977).

Goto, T.; Horie, H.; Ozeki, Y.; Masuda, H.; Warashina, J. Chemical composition of Japanese green teas on market. Tea Res. J. 80, 23-28 (1994).

Morita, A.; Tuji, M. Nitrate and oxalate contents of tea plants (Camellia sinensis L.) with special reference to types of green tea and effect of shading. J. Soil Sci. Plant Nutr. 48, 547-553 (2002).

Matsunaga, A.; Sano, T.; Hirono, Y.; Horie, H. Effects of various directly covered shading levels on chemical components in tea new shoots of the first flush. Tea Res. J. 122, 1-7 (2016).

Morita, A.; Oofa, M.; Yoneyama, T. Changes of the contents of nitrate, oxalic acid and polyamines in tea plants (Camellia sinensis L.) grown with different nitrogen forms and concentrations. Jpn. J. Soil Sci. Plant Nutr. 70, 107-116 (1999).

Swartman, J. A.; Hintz, H. F.; Schryver, H.F. Inhibition of calcium absorption in ponies fed diets containing oxalic acid. Am. J. Vet. Res. 39, 1621-1623 (1978).

Baba, R.; Amano, Y.; Wada, Y.; Kumazawa, K. Characterization of the potent odorants contributing to the characteristic aroma of Matcha by gas chromatography–olfactometry techniques. J. Agric. Food Chem. 65, 2984-2989 (2017).

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J. Oleo Sci. 68, (12) 1271-1277 (2019)