Characterisation of odorant compounds and their biochemical formation in green tea with a low temperature storage process

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Abstract

We produced low temperature (15 °C) processed green tea (LTPGT) with higher aroma contents than normal green tea (Sencha). Normal temperature processed green tea (NTPGT), involved storing at 25 °C, and Sencha had no storing process. Sensory evaluation showed LTPGT had higher levels of floral and sweet odorants than NTPGT and Sencha. Aroma extract dilution analysis and gas chromatography-mass spectrometry-olfactometry indicated LTPGT had 12 aroma compounds with high factor dilution values (FD). Among LTPGT's 12 compounds, indole, jasmine lactone, *cis*-jasmone, coumarin, and methyl epijasmionate contributed to floral, fruity and sweet characters. In particular, indole increased initially, peaking at 16 h, then gradually decreased. Feeding experiments suggested [¹⁵N]indole and [¹⁵N]oxygenated indoles (OX-indoles) were produced from [¹⁵N]anthranilic acid. We proposed the increase in indole was due to transformation of anthranilic acid during the 16 h storage and the subsequent decline in indole level was due to its conversion to OX-indoles.

Key words: odorant compounds, *Camellia sinensis*, tea leaves, indole, [¹⁵N]indole, [¹⁵N]anthranilic acid, metabolome analysis.

Abbreviation list (as footnote)

LTPGT: low temperature processed green tea

NTPGT: normal temperature processed green tea

FD: factor of dilution value

GC-MS/O: gas chromatography-mass spectroscopy-olfactometry determination

AEDA: aroma extraction and dilution analysis

HR-MS: high resolution mass spectroscopy

49 ESI: electrospray ionisation

50 HIPV: herbivore-induced plant volatiles

51

52 1. Introduction

53 The tea plant is of great economic importance, and tea infusions are some of the
54 most consumed beverages around the world. According to the Food and Agriculture
55 Organization of the United Nations (2011), world tea production is 4.6 million tons per
56 annum, and the main producers are China (35.1%), India (20.7%), Kenya (8.0%), and Sri
57 Lanka (7.0%). Tea extracts are used as flavourings in sweets, bakery products, and bottled
58 tea beverages.

59 Teas can be divided into three major categories: unfermented green teas,
60 semi-fermented oolong teas, and fully-fermented black teas, which account for about 20%,
61 some 2%, and nearly 80% of the total world tea production, respectively (Flaten, 2002).
62 Around 300 types of teas have been described, of which only a very small number are known
63 and consumed in non-tea-producing countries.

64 Tea quality is important for its market value and is defined by colour, freshness,
65 strength, and aroma. To date, approximately 600 volatiles have been described in black tea,
66 with fewer numbers in oolong and green tea, due to the lesser degree of fermentation when
67 producing these teas, and thereby tea quality influences a certain market percentage. Fresh
68 tea leaves of *Camellia sinensis* are steamed immediately after plucking to produce Japanese
69 green tea (Sencha). Endogenous enzymes involved in aroma formation are inactivated by
70 the steam treatment, producing low aroma contents. The commercial value of Sencha is
71 mainly evaluated by umami (taste) and fresh green odour, whereas flowery and fruity odour
72 is essential for black tea or semi-fermented tea (oolong tea). The volatile compounds in
73 green tea, black tea and semi-fermented tea have been intensively analysed (Takei, Ishiwata,

& Yamanishi, 1976; Kawakami, Ganguly, Banerjee, & Kobayashi, 1995; Shimoda, Shigematsu, Shiratsuchi, & Osajima, 1995a, b). The potent odorants of Japanese and Chinese green teas and black tea have been investigated based on aroma extract dilution analysis (AEDA) (Guth & Grosch, 1993; Kumazawa & Masuda, 1999, 2002; Kumazawa, Wada, & Masuda, 2006).

To enrich green tea with more aroma attributes, a selection of raw materials (tea cultivars) can be employed. Among several types of Sencha aroma-rich green teas, made from *C. sinensis* cultivars, Kohshun and Shizu 7132 have a sweet odour (Yang, Kinoshita, Tanida, Sayama, Morita, & Watanabe, 2009), and are becoming popular in Japan.

Modification of the manufacturing process is an alternative approach. It is known that floral and fruity aroma-rich green tea is produced from leaves harvested in cool mountainous areas, followed by storing at ambient temperature prior to processing. (Luo, Jin, Han, & Wen, 2009) However, neither sensory evaluation nor examination of the odorant compounds have been performed for green tea made from stored fresh tea leaves. The manufacturing process of oolong tea includes withering and bruising processes prior to a pan-frying step for inactivation of the endogenous enzymes. Black tea processing includes withering, rolling, cutting, and oxidation steps. During tea processing, many odorant compounds are produced by the action of glycohydrolases and oxidative enzymes. Thus, tea leaves are exposed to numerous mechanical and environmental stresses after plucking to yield higher amounts of volatile odorant compounds (Wang, Kubota, Kobayashi, & Juan, 2001).

The process of aroma production is similar to the defence response of plant leaves. As a defence response to attacks by herbivores, such as the smaller tea tortrix (*Adoxophyes honmai Yasuda*), tea leaves emit numerous volatiles such as (Z)-3-hexen-1-ol, linalool, α -farnesene, benzyl nitrile, indole, nerolidol, and ocimenes, in large amounts. Attack by

Kanzawa spider mites (*Tetranychus kanzawai* Kishida), another major pest insect of tea crops, can induce the emission of α -farnesene and ocimenes (Yang, Baldermann, Watanabe, 2013) from tea leaves. The exogenous application of jasmonic acid to tea leaves induced a volatile blend that was similar, though not identical, to that induced by the smaller tea tortrix. Most of the herbivore-induced plant volatiles (HIPV) were not stored in the tea leaves but emitted after herbivore attack.

As mentioned above, leaves have molecular responses to abiotic and biotic stresses. After plucking, the storing process may be similar to withering to affect a metabolic change in the green tea leaves, as clarified in oolong tea manufacturing. (Wang et al., 2001) We examined the effects of temperature and the processing period on the aroma profiles of the final green tea products. Fresh green tea leaves were kept at 15 °C or 25 °C for 24 h, and were processed in the usual manner to yield aroma-endowed green tea. We investigated the characteristics of aroma by sensory evaluations and identified potent odorants using the AEDA technique. Furthermore, we examined the change in the amount of indole, a volatile compound contributing to the floral aroma, during the storage of green tea leaves, and analysed the metabolites of indole using metabolome analyses. The conversion of anthranilic acid to indole and its metabolites was also investigated using a stable isotope labelled precursor.

We describe the aroma characteristics, contributing volatile compounds, and the metabolic features which accumulate during the storage of green tea leaves.

2. Materials and Methods

2.1 Tea samples

Tea (*Camellia sinensis* cv Sayamakaori) leaves comprising a bud with three or four new leaves were harvested by a tea plucker in May 2012 and 2013 from the tea field of Tea

Research Center, Shizuoka Prefectural Research Institute of Agriculture and Forestry, Shizuoka, Japan. The leaves (2 kg fresh weight) were spread with 10 cm thickness in a bamboo colander (90W × 60L × 20H cm) and stored at different temperatures (25 °C and 15 °C, relative humidity 70%) for 16 h under dark conditions, and they were processed according to the traditional green tea (Sencha) manufacturing process (Fig. 1). The green tea samples were then designated NTPGT (green tea stored at normal temperature, 25 °C, prior to processing) and LTPGT (green tea processed at low temperature, 15 °C, prior to processing), whereas the tea sample manufactured just after plucking was designated as Sencha.

2.2 Sensory evaluations by panellists

Sensory evaluation was performed in a sensory room by 15 panellists skilled in the evaluation of green tea or fragrance working at Takasago (Kanagawa, Japan) and the Local Tea Research Institute (Shizuoka, Japan). Tea samples (3 g) were extracted with hot water (90 °C, 150 mL). The tea infusion (25 mL) was presented to the panellists for orthonasal evaluation in a plastic cup (odour free, 90 mL, Asahi Kasei Pax Corporation). The panellists were first requested to freely describe the smell of the extracts. Seven types of aroma attributes were determined on the basis of the descriptions. The panellists scored the intensities of seven aroma attributes on a scale from 0 to 4 (0: no detection, 1: weak detection, 2: moderate, 3: intense, 4: very intense).

2.3 Preparation of aroma extracts from tea samples

The aroma compounds of each sample were concentrated using the adsorptive column method (Shimoda et al., 1995a, b). Tea samples were powdered using a mill (Iwatani Corporation) and the powders (8 g) were suspended in hot distilled water (160 mL, 80–90 °C)

for 5 min. The suspension was centrifuged for 10 min at 3,000 g at ambient temperature to separate supernatant from the residue. The supernatant (120 mL) was applied to a Porapak Q cartridge (2 mL of volume, 50-80 mesh; Waters Corporation, Milford, MA) conditioned with diethyl ether (5×4 mL), methanol (4 mL), and distilled water (4 mL). The cartridge was washed with 3 mL of water before the aroma compounds were eluted with 3 mL isopentane:diethyl ether (1:1, v/v). Ethyl decanoate (10 μ g/10 μ L) was added as an internal standard to the eluent. The eluate was dried over anhydrous sodium sulfate, and concentrated to 100 μ L in a stream of nitrogen to give a sample for GC-MS and GC-MS/olfactory analyses.

2.4 Gas chromatography-mass spectrometry (GC-MS) and GC-MS/olfactometry (GC-MS/O)

GC/MSD (5975C, Agilent Technologies Inc., Santa Clara, CA) was used for mass spectrometric identification. The GC was equipped with a DB-WAX capillary column (60 m $\times$ 0.25 mm I.D., 0.25 μ m film thickness, Agilent Technologies Inc.). Helium was used as a carrier gas with a flow rate of 2 mL/min. The injector temperature was 240 °C. The GC oven was maintained at 40 °C for 2 min. The temperature of the oven was programmed to increase by 5 °C/min to 240 °C, and was kept at this temperature for 25 min. The mass spectrometer was operated in full scan mode with a mass range of m/z 20–280. For GC-MS/O, the effluent was split into the olfactory detection port (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany.) and MS using two deactivated, uncoated fused silica capillaries (106 cm $\times$ 0.15 mm I.D., 139 cm $\times$ 0.1 mm I.D.) at the end of the capillary.

2.5. Identification of volatile compounds in the tea infusion based on GC-MS and AEDA

Volatile compounds in LTPGT, NTPGT, and Sencha were identified based on their Kovats GC retention indices and their mass spectra. The relative ratio of each volatile compound

was based on the total ion traces to that of the internal standard. The data are summarised in supplemental Table 1.

The original odour concentrate of the sample infusion was step wisely diluted with isopentane:diethyl ether (1:1, v/v) 1:4 = 4¹, 1:16 = 4², 1:64 = 4³, 1:256 = 4⁴, and 1:1024 = 4⁵, and aliquots (2 µL) of each sample were analysed by GC-MS/O. The flavour dilution (FD) factors of the odorants were determined by AEDA.

Volatile compounds were identified by direct comparison with the Kovats GC retention indices, mass spectra, and odour quality with those of authentic specimens. Odorant compounds with FD factors ($\geq 4^5$) were analysed to determine each compound. The identification of odorant compounds was based on direct comparison of the retention index, MS data, and the aroma profile with those of authentic specimens. The data are summarised in Table 1.

2.6 Analysis of odorant compounds accumulated in green tea during the storage period at 15 °C and 25 °C

Fresh tea leaves (2 kg of the first flush plucked in Kikugawa, Shizuoka, in May, 2012) were spread in a bamboo colander and stored at 15 °C and 25 °C. Every 4 h at 0, 4, 8, 12, 16, 20, and 24 h, a portion (10 g) of the leaves was treated in triplicate as mentioned above (section 2.4) to obtain aroma concentrates. The concentrates were subjected to GC-MS, as mentioned in section 2.5, to analyse the change in the concentration of 5 odorant compounds: indole, jasmine lactone, *cis*-jasnone, linalool, and methyl-jasmonate.

2.7. Analysis of indole emitted from fresh tea leaves during the storage period at 15°C and 25 °C

Three sets of fresh tea leaves (20 g of the first flush plucked in Kikugawa, Shizuoka, in May,

2013) in sample bags (TedlarTM bag, GL Sciences Inc., Tokyo, Japan) were kept at either 15 °C or 25 °C for 24 h. Through a gas purifier, with active carbon (GL Sciences Inc.), air was pumped into the sample bag at a flow rate of 300 mL/min. A Tenax TATM column (Gerstel) was attached at the outlet of the sample bag and the columns were exchanged every 4 h during the 24 h. Aroma compounds absorbed on the Tenax TATM column were analysed using a TDU (Twister Desorption Unit, Gerstel) and CIS4 (Cooled Injection System, Gerstel) installed on a 5975C GC/MSD (Agilent Technologies Inc.). Each of the columns were thermally desorbed by programming the TDU from 40 °C to 250 °C (held for 2 min) at 720 °C/min with 20 mL/min desorption flow. Volatile compounds were desorbed by cryo-focusing on a liner in the CIS4 at 60 °C. After desorption, the CIS4 was programmed from 60 °C to 250 °C (held for 5 min) at 720 °C/min to inject trapped compounds onto the GC/MSD. GC conditions were as described above. Amounts of indole were estimated based on the calibration curves for authentic indole.

2.8. Metabolome analyses targeting with compounds derived from anthranilic acid

Tea leaves were kept at 15 °C and 25 °C for 24 h under dark conditions. The tea samples (100–120 mg) were crushed in liquid N₂ and extracted with three volumes of MeOH, and the combined extracts were subjected to LC-MS analyses. The LC-MS analyses were performed at the KAZUSA DNA Research Institute, according to a previous method (Iijima, et al., 2008). An Agilent 1200 system coupled to an LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) was used for LC-Orbitrap-MS analysis. The data were acquired and browsed using X-calibur software, version 2.0 (Thermo Fisher Scientific). The methanol extract was applied to a TSKgel column ODS-100V (3 × 50 mm, 5 µm; TOSOH Corporation, Tokyo Japan). Water (HPLC grade; solvent A) and acetonitrile (HPLC grade; solvent B) were used as the mobile phase with 0.1% v/v formic acid added to both solvents.

224 The gradient program was as follows: 3% B to 97% B (5 min), 97% B (5 min) and 3% B (5
 225 min). The flow rate was set to 0.5 ml min⁻¹, and the column oven temperature was set at
 226 40 °C; 5 µl of each sample were injected. To monitor HPLC elution, a photodiode array
 227 detector was used in the wavelength range 190–600 nm. The ESI setting for positive
 228 ionisation mode was as follows: spray voltage 4.0 kV and capillary temperature 300 °C.
 229 Nitrogen sheath gas and auxiliary gas were set at 40 and 15 arbitrary units, respectively. A
 230 full MS scan with internal standards was performed in the *m/z* range 100–600 at a resolution
 231 of 60,000 (at *m/z* 400). For the internal calibration standard, 5 µM Prochloraz (*m/z*
 232 376.03809 [M + H]⁺; AccuStandard Inc., New Haven, CT) dissolved in 50% v/v acetonitrile
 233 was introduced by a post-column method at a flow rate of 20 µl min⁻¹. MS/MS fragmentation
 234 was carried out at a normalised collision energy of 35.0% and an isolation width of 4.0 (*m/z*),
 235 and spectra were obtained in ion trap mode. The identification of each metabolite was
 236 based on the HPLC retention time and the HR-MS trace, i.e., indole (*t_R* = 5.58 min, HR-MS
 237 (ESI⁺), *m/z* 118.0561 [M + H]⁺, calcd. for C₈H₈N₁, 118.0657, +0.4 mmu); **oxygenated indole**
 238 **(designated as OX-indole) 1** (*t_R* = 4.41 min, *m/z* 134.0600 [M + H]⁺, calcd. for C₈H₈N₁O₁,
 239 134.0604, -0.4 mmu); **OX-indole 2** (*t_R* = 3.88 min, *m/z* 134.0600 [M + H]⁺, calcd. for
 240 C₈H₈N₁O₁, 134.0604, -0.4 mmu); **OX-indole 3** (*t_R* = 3.70 min, *m/z* 134.0600 [M + H]⁺, calcd.
 241 for C₈H₈N₁O₁, 134.0604, -0.4 mmu); anthranilic acid (*t_R* = 4.38 min, *m/z* 138.0549 [M + H]⁺,
 242 calcd. for C₇H₈N₁O₂, 138.0555, -0.6 mmu); L-tryptophan (*t_R* = 3.58 min, *m/z* 205.0971 [M +
 243 H]⁺, calcd. for C₁₁H₁₃N₂O₂, 205.0978, -0.7 mmu). [¹⁵N] labelled metabolites were detected
 244 as follows, [¹⁵N]indole (*t_R* = 5.58 min, *m/z* 119.0627 [M + H]⁺, calcd. for C₈H₈¹⁵N₁,
 245 119.0621, +0.6 mmu); [¹⁵N]**OX-indole 1** (*t_R* = 4.41 min, *m/z* 135.0571 [M + H]⁺, calcd. for
 246 C₈H₈¹⁵N₁O₁, 135.0576, -0.5 mmu); [¹⁵N]**OX-indole 2** (*t_R* = 3.88 min, *m/z* 135.0570 [M + H]⁺,
 247 calcd. for C₈H₈¹⁵N₁O₁, 135.0576, -0.6 mmu); [¹⁵N]**OX-indole 3** was not detected.;
 248 [¹⁵N]anthranilic acid (*t_R* = 4.37 min, *m/z* 139.0519 [M + H]⁺, calcd. for C₇H₈¹⁵N₁O₂,

139.0525, -0.6 mmu); L- ^{15}N]tryptophan ($t_R = 3.58$ min, m/z 206.0944 $[\text{M} + \text{H}]^+$, calcd. for $\text{C}_{11}\text{H}_{13}\text{N}_1^{15}\text{N}_1\text{O}_2$, 205.0948, -0.4 mmu). The amount of each metabolite was expressed based on the ion intensity of each compound.

2.9. Administration of ^{15}N]anthranilic acid into tea leaves and analysis of ^{15}N]indole and its metabolites

Young leaves (1 g = 6 leaves) of *C. sinensis* Sayamakaori were immersed in a solution of ^{15}N]anthranilic acid ($^{15}\text{N}\% = 98\%+$, 10 mM; Cambridge Isotope Laboratories Inc., Cambridge, MA) under reduced pressure (-75 Pa) for 1 min. The leaves were then taken out from the solution and rinsed with water. After wiping off the water, they were incubated for an appropriate period (2, 4, 8, 12, 16, and 24 h) under dark conditions, either at 15°C or 25°C . The leaves were homogenised in liquid N_2 for the metabolome analyses mentioned in section 2.8.

3. Results and discussion

3.1 Sensory characterisation of tea samples

The orthonasal aroma impressions of tea samples (LTPGT, NTPGT, and Sencha) were screened by the panellists with descriptive sensory evaluation. The following aroma attributes were determined based on the descriptors evaluation of tea samples: roast, green, fresh green, sweet, fruity, floral, and fresh floral. The major aroma impressions were green for Sencha and floral for LTPGT and NTPGT. Seven aroma attributes were evaluated on a scale from 0 to 4 by panellists (Fig. 2). For Sencha, green and fresh green were strongly detected, roast and sweet were detected whereas scores for fruity, floral and fresh floral were low. The fruity and fresh floral and fresh green aroma characteristics of NTPGT were evaluated to be higher than Sencha. For LTPGT, the score of sweet was similar to Sencha,

but the scores for roast, green and fresh green were lower than in Sencha. In LTPGT, fruity, floral, and fresh floral were dominant and strongly detected among the three tea samples. The aroma characteristics became remarkably fruity and sweet after storing for 16 h at 15 °C before the manufacturing process: Sencha and NTPGT samples showed lower scores for fruity and sweet than the LTPGT samples.

3.2 Comparison of the volatile compounds in each tea sample

GC-MS analyses revealed that more than 4.6 times more volatile compounds were detected in LTPGT than in Sencha, whereas only 1.4 times of those compounds were detected in NTPGT (Supplemental Table 1).

Indole and jasmine lactone were remarkably higher in LTPGT than Sencha, suggesting their contribution to the floral and sweet odours. Changes in the concentration of dominant volatile compounds, indole, jasmine lactone, linalool, *cis*-jasmonone, and methyl jasmonate, were analysed during storage at 15 °C and 25 °C. Indole, jasmine lactone, and *cis*-jasmonone gradually increased and reached their maximum levels at 16 h after 15 °C storage, whereas maximum levels were reached at 4 and 8 h at 25 °C which was lower than levels in LTPGT. (Supplemental Figs. 1-a, b, c). Levels of linalool and methyl jasmonate changed differently to each other and their maxima were not reached at 16 h after 15 °C storage (Supplemental Figs. 1-d, e). The change in the levels of indole, jasmine lactone and *cis*-jasmonone may represent the differences between LTPGT and NTPGT, whereas the accumulation levels of linalool and methyl jasmonate did not represent the differences based on the sensory evaluation in each tea sample. To confirm the contribution of each odorant to the sensory characteristics of the three tea samples, we have analysed the contribution based on AEDA and GC-O-MS.

3.3 GC-O-MS and AEDA of odorants contributing to the characteristic aroma in each tea

sample

GC-O-MS and AEDA revealed that a total of 20 odour-active peaks were detected in samples from 15 °C, 18 from 25 °C, and 9 in Sencha, with FD factors in the range of 4^2 – 4^5 (Table 1). In LTPGT, 12 peaks (numbers 2, 5, 7, 10, 13, 15, 17, 18, 19, 20, 21, and 22) showed high FD factors ($\geq 4^3$), whereas only 6 and 3 peaks showed FD factors of $\geq 4^3$ in NTPGT and Sencha, respectively. Furthermore, these potent odorants were floral, fresh floral and geranium-like among the three samples. The data strongly suggested that 12 odorant compounds contribute to the floral character of LTPGT. Among the odour-active compounds in these teas, 13 peaks (5, 7, 8, 10, 11, 13, 15, 16, 17, 18, 19, 21, 22, and 23) were identified by direct comparison with Kovats indices, MS spectra, and odour qualities of authentic specimens (Supplemental Fig. 2). Six peaks (1, 2, 4, 6, 12, and 14) were not compared with mass spectra, because they were present in too small amounts. We were not able to identify 3 peaks (3, 9, 20), even after further concentration of the volatile extracts.

Peaks 7 and 22 were identified as 3-methylnonane-2,4-dione and coumarin, respectively. They are rare constituents for a green tea aroma. The odour characteristic of 3-methylnonane-2,4-dione was reported to be fruity or green (Guth et al., 1993; Kumazawa et al., 1999; Hattori, Takagaki, & Fujimori, 2003, 2005), and coumarin was reported to have a cherry flower-like character (Yang et al., 2009). In addition, (5Z)-1,5-octadien-3-one (peak 2) had a high FD factor (FD_f) of 4^4 in LTPGT with a geranium-like, green, metallic smell (Guth et al., 1993; Kumazawa et al., 1999; Hattori et al., 2003, 2005). (5Z)-Octa-1,5-dien-3-one may contribute to the green aroma character; however, the green character was lower than fruity or floral in LTPGT, based on the sensory evaluation, suggesting a masking effect of the other odorants, reducing the green aroma in this tea sample.

Among the three tea samples, geraniol (peak 8, $FD_f=4^3$) and an unknown compound

(peak 3, $FD_f=4^3$, geraniumu-like) were present at their highest concentrations in NTPGT, suggesting a large contribution to the aroma character of NTPGT. (Z)-Methyl *epi*-jasmonate (peak 19, $FD_f=4^4$) was high in both LTPGT and NTPGT. This compound is produced when the plant is wounded and acts as a plant hormone. (Creelman, Tierney, & Mullet, 1992). The increase in this compound may be caused by plucking and storing the fresh tea leaves, whilst its contribution in Sencha was low.

We were unable to characterise a compound occurring in both LTPGT and NTPGT (peak 20, $FD_f=256$) due to low levels of MS ion intensities. We suggest this compound must be contributing to the fresh floral character in these tea samples, and identification and characterisation of this compound is under investigation.

Indole (peak 21) highly contributed ($FD_f=4^5$) to the floral aroma and strengthened the overall green tea odour (Hattori et al. 2003, 2005). Moreover, there was a large difference in the amount of indole between LTPGT and NTPGT. Jasmine lactone (sweet, peak 17) had an $FD_f=4^4$, suggesting a high contribution to the floral aroma in LTPGT. The amount of indole increases during the manufacturing of oolong tea (Tokitomo, Ikegami, Yamanishi, 1984). In the oolong tea manufacturing process, withering increased the indole content, and induced hydrolysis of glycoconjugates of the alcoholic aroma, such as linalool, geraniol, (Z)-3-hexenol, benzyl alcohol and 2-phenylalcohol, which have flowery and sweet characters (Wang et al., 2001).

Potent odorants of green tea made in China and Japan were meaty, metallic, fatty, honey-like, floral, and animal-like (Guth et al., 1993; Kumazawa et al. 1999, Hattori et al. 2003, 2005). In this study, we confirmed the potent characters of Sencha were green and fresh green. 4-Methoxy-2-methyl-2-butanethiol and 4-mercapto-4-methyl-2-pentanone were reported as key compounds of green tea (Kumazawa et al. 1999); however, these compounds were not detected in our study. We suggest this is due to differences in the processing

methods. Normally, green tea is roasted for 30 min after the crude green tea is processed, resulting in the production of roasted flavours, such as 4-methoxy-2-methyl-2-butanethiol and 4-mercapto-4-methyl-2-pentanone. Sencha, LTPGT, and NTPGT were not roasted in this study.

We analysed the change in the concentration of indole and related metabolites. Storing fresh tea leaves prior to the steaming process produced positive effects in increasing the concentration of volatile compounds. The increase is suggested to be due to the induction of biochemical reactions. We investigated why larger effects were recorded at the lower storage temperature, 15 °C compared with 25 °C.

3.4 Emission amounts of indole during the storing process at 15 °C and 25 °C

Among the odorant compounds, we focused our research on clarifying the mechanisms of the increase in the indole level in LTPGT compared with NTPGT. During the storage of green tea leaves, they were exposed to physiological stress resulting in metabolic changes, such as the NAD(P)⁺-NAD(P)H dependent process (Selmar, & Kleinwaelchter, 2013; Wilhelm, & Selmar, 2011). The oxidative cleavage of α -linolenic acids by the action of a series of enzymes, producing oxylipins, methyl jasmonate, jasmine lactones, and short chain aliphatic aldehydes and ketones, may have occurred during the storage process (Christensen et al., 2013). We believe the accumulation is dependent on the emission amounts of volatile compounds during the storage period. The amount of indole emitted from the tea leaves from 25 °C storage was high during the initial 4–8 h, and then gradually decreased, with a sharp decrease during 8th–12th h. In contrast, at 15 °C storage, emission amounts of indole increased until the 12th h and was then maintained at relatively high levels (Supplemental Fig. 3). Total amounts emitted from green tea leaves were less than 1% for 25 °C, and 2% for 15 °C, suggesting that the emission does not influence the

amounts of indole accumulated in each green tea sample, and that indole biosynthesis and catabolism must be dependent on the temperature during the storing process.

3.5 Biochemical change of indole precursor and change in the levels of indole and OX-indoles by storing fresh tea leaves

Indole is synthesised from chorismic acid and anthranilic acid via 1-(*O*-carboxyphenylamino)-1-deoxy-ribulose-5-phosphate and indole-3-glycerol phosphate in *Arabidopsis* (Radwanski, & Last, 1995). Indole thus produced is converted to either L-tryptophan (Radwanski et al., 1995) or several oxygenated indoles (OX-indoles), such as 4-hydroxy-, 5-hydroxy-, 6-hydroxy-indoles in higher plants. 2-Hydroxyindole and 2-oxindole (oxindole) were also produced in microorganisms (Yuan, Liu, & Xiao, 2011). Indigo-producing plants metabolised indole to 3-hydroxyindole (indoxyl), 3-oxindole (Horvath, 1977a,b; Medvedev, Korshikov, Bashkatov, & Tarabrin, 1977) (Fig. 3). The conversion of indole to 3-hydroxyindole (indoxyl) and 3-oxindole was also reported in pea seeds microsomes in the presence of hydroperoxide (Ishimaru & Yamasaki, 1977). P450 enzymes were involved in the oxidation process of indole (Gillam, Notley, Cai, De Voss, & Guengerich, 2000). We therefore examined the metabolic changes in LTPGT and NTPGT based on metabolome analyses, targeting indole, anthranilic acid, OX-indoles and L-tryptophan. The levels of L-tryptophan increased in both tea samples during the 16 h storage, whereas anthranilic acid rapidly decreased in NTPGT compared with LTPGT (Figs. 4-a,b). These data suggested that conversion of anthranilic acid to indole may contribute to indole accumulation, whereas conversion of indole to L-tryptophan may not be the primary factor for indole accumulation. Ions at m/z 119.0621 $[M + H]^+$ were monitored to detect OX-indoles (corresponding to C_8H_8NO) present in both tea samples. Three candidate OX-indoles: 1, 2, and 3 were detected at different retention times of 4.41, 3.88, and 3.70 min,

respectively, based on the metabolome analyses, suggesting the presence of hydroxyindoles or/and oxindoles (Fig. 3). As shown in Figs. 5-a, b, the levels of OX-indole 3 were low during the storage process at both temperatures, suggesting that conversion of indole to OX-indole 3 is not involved in the change in indole levels. OX-indoles 1 and 2 increased from 4 h and reached a peak at 12 h of storage at 25 °C. However these compounds were highest at 20th hour of storage at 15 °C and the levels of each OX-indole during the initial 16 h were changed within almost 2/3 of those detected in NTPGT. In addition, OX-indoles 1 and 2 increased during the period when indole decreased in NTPGT, whereas they did not increase until the 12th hour in LTPGT. These changes became evident at higher indole accumulation levels in LTPGT compared with NTPGT. Although the conversion of indole to OX-indoles is not directly confirmed, this conversion is suggested to be a key process in determining the accumulation level of indole in each tea sample during the storage process. LC-MS/MS is now underway to characterise each OX-indole

Administration of [¹⁵N]anthranilic acid to fresh green tea leaves revealed that [¹⁵N]anthranilic acid was converted to [¹⁵N]indole, and the highest levels were reached at 4 h for NTPGT and 12 h for LTPGT (Supplemental Fig. 4), suggesting that the conversion rate from [¹⁵N]anthranilic acid at 25 °C was higher than at 15 °C. [¹⁵N]OX-indoles 1 and 2 were also identified based on the metabolome analyses with HRMS which unambiguously revealed the conversion to [¹⁵N]OX-indoles from [¹⁵N]anthranilic acid as described in section 2.8.

4. Conclusion

We have successfully controlled the production of odorant compounds by storing fresh green tea leaves prior to the steaming process. Storing at a low temperature (15 °C) produced greater floral, sweet, and fruity characters compared with NTPGT and Sencha.

The odorant compounds contributing to the character of LTPGT were identified to be indole, jasmine lactone, methyl-epijasmone, coumarin, (5Z)-octa-1,5-dien-3-one, and 3-methylnonane-2,4-dione. The amounts of these compounds were much higher than in Sencha. However, less than 7.5% of indole and 4.0% of jasmine lactone were found in NTPGT, suggesting that these compounds contribute to the higher sweet and floral characters of LTPGT compared with NTPGT.

Quantitative analyses of indole during the storage process revealed that the higher accumulation of indole in LTPGT must be ascribed to the low conversion rate of indole into OX-indoles. We have also clarified that anthranilic acid was converted to indole during a 16-h storage process, even at 15 °C.

We need to clarify the molecules, enzymes and genes involved in indole accumulation in green tea leaves during the period of storage.

In conclusion, oxidative biochemical pathways were activated during storage processes at 15 °C and 25 °C, however, further catabolism of odorant compounds was suppressed at the lower temperature, increasing the important odorant compounds in LTPGT.

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562

Legends to figures:

Fig. 1. Manufacturing process for Japanese green tea (Sencha), LTPGT, and NTPGT.

Fig. 2. Sensory characterization of tea samples.

Solid line (○), LTPGT; dashed line (●), NTPGT; solid line (▲), Sencha.

The following aroma attributes were determined based panelist's evaluation of tea samples: roast, green, fresh green, sweet, fruity, floral, and fresh floral.

Fig. 3. Biosynthetic pathways of indole from anthranilic acid and oxidation to **OX-indoles** from indole.

[¹⁵N]anthranilic acid was converted to [¹⁵N]indole and [¹⁵N]**OX-indoles 1 and 2**, whereas . [¹⁵N]**OX-indole 3** was not detected. The presence of OX-indoles **1,2, and 3** was confirmed in the NTPGT and LTPGT as shown in Fig. 5.

Fig. 4. Change in the amount of L-tryptophane (a), anthranilic acid (b), and indole (c) during the storage process at 15 °C (solid line ○) and 25 °C (dashed line ●).

Fig. 5. Change in the amount of **OX-indoles-1** (dashed line ●), -2 (solid line ○), and -3 (dashed line ▲) in tea leaves stored for 24 h at 15 °C (a) and 25 °C (b).

FIGURES

(a) Japanese green tea (sencha)

(b) LTPGT and NTPGT

Figure 1 Tsuyoshi KATSUNO

Figure 2 Tsuyoshi KATSUNO

Figure 3 Tsuyoshi KATSUNO

(a) L-Tryptophan

(b) Anthranilic acid

(c) Indole

Figure 4 Tsuyoshi KATSUNO

(a) 15 °C

(b) 25 °C

Figure 5 Tsuyoshi KATSUNO

TABLES

Table 1. Potent odorants in LTPGT, NTPGT and Sencha detected by AEDA.

No.	RI	compound	odor quality	FD factor			peak area ratio		
				LTPGT	NTPGT	Sencha	LTPGT	NTPGT	Sencha
1	1307	1-octen-3-one ^b	mushroom-like	<16	16	<16	nd	nd	nd
2	1380	(5Z)-octa-1,5-dien-3-one ^b	geranium-like	256	16	16	nd	nd	nd
3	1395	unknown	geranium-like	16	64	16	nd	nd	nd
4	1463	methional ^b	fruity	16	<16	<16	nd	nd	nd
5	1550	linalool ^a	fresh floral	64	16	<16	1.125	0.468	0.135
6	1594	(2E,6Z)-nona-2,6-dienal ^b	fresh green	16	16	16	nd	nd	nd
7	1726	3-methylnonane-2,4-dione ^a	floral	1024	64	64	0.297	0.238	0.310
8	1854	geraniol ^a	fresh floral	16	64	<16	0.200	0.645	0.232
9	1874	unknown	minty	16	nd	<16	nd	nd	nd
10	1957	β -ionone ^a	floral	64	16	64	0.059	0.031	0.043
11	1965	cis-jasmone ^a	floral	16	nd	<16	2.193	0.558	0.074
12	2051	furaneol ^b	sweet	16	<16	<16	nd	nd	nd
13	2180	bovolide ^a	fresh floral	64	16	<16	1.201	0.623	0.931
14	2184	sotolon ^b	spicy	<16	16	<16	nd	nd	nd
15	2222	δ -decalactone ^a	floral, sweet	64	16	16	0.282	0.055	0.026
16	2262	methyl anthranilate ^a	fruity	16	<16	<16	0.160	0.126	0.033
17	2291	jasmine lactone ^a	floral, sweet	256	16	<16	12.646	0.792	0.527
18	2359	methyl jasmonate ^a	floral	64	16	<16	0.672	0.224	0.152
19	2417	methyl epijasmonate ^a	fresh floral	256	256	16	0.380	0.110	0.104
20	2435	unknown	fresh floral	256	256	16	nd	nd	nd
21	2470	indole ^a	floral	1024	16	<16	204.894	15.078	2.210
22	2489	coumarin ^a	floral	1024	256	64	2.152	1.019	1.050
23	2589	vanillin ^a	sweet	<16	16	<16	0.369	0.197	0.290

^aIdentified by direct comparison with Kovats GC retention indices, mass spectra, and odor quality with those of the authentic specimens.

^bIdentified based on the Kovats GC retention indices and odor quality with those of the authentic specimens, because of their low ion intensities.