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DEVELOPMENT OF A PRACTICAL METHOD TO PRODUCE GABA RICH GREEN TEA BY VAPOR TREATMENT WITH trans-2-HEXENAL

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ABSTRACT

A practical method to produce GABA (γ-aminobutyric acid) rich green tea was developed using vapor treatment of *trans*-2-hexenal from 2017 to 2018 in Kanto district, Japan. Although the treatment with *trans*-2-hexenal in a closed plastic bag at 5℃ for 4 hours failed to enhance GABA concentration in tea leaves, *trans*-2-hexenal vapor treatment in a ventilation system succeeded in enhancing GABA concentrations up to 8 to 10 times higher, or around 2 μmol/g. Flavor profiling by GC-O (gas chromatography-olfactometry) and GC-MS (gas chromatography-mass spectrometry) showed GABA rich tea produced by this method was found to possess slight strawberry and bean-like odors in addition to the original green tea flavor. Since *trans*-2-hexenal is a ubiquitous green odor generated from plant leaves and originally rich in tea leaves, this method can be regarded as a highly safe and environmentally friendly technique and might well contribute to the tea industry and tea market of Southeast Asian countries. It could also play an important role in promoting local public health as a functional tea to cope with stressful mental conditions.

Key words: amino acid composition, functional tea, metabolome analysis, ventilation system

INTRODUCTION

GABA (γ-aminobutyric acid) is known as one of the major inhibitory transmitters of the central nervous system (Bowery and Smart 2006) and has the potential to inhibit diabetic brain abnormality (Huang et al. 2013), promotes sleep (Cheng, et al. 2009), regulates blood pressure (Abe et al. 1995) among others. In Southeast Asian countries, tea cultivation has been one of the main industries historically, and easy and effective GABA rich tea production technique has long been earned, because GABA rich product market is now expanding all around the world (Horie et al. 2019).

Gabaron tea has been developed as a GABA rich semifermented tea produced by anaerobic treatment (Tsushida and Murai 1987), but it needs refinement to suppress unfavorable offensive odor (Hakamata et al. 1988). The vapor treatment of *trans*-2-hexenal treatment could enhance the amount of GABA in tomato fruit, thus the chemical was used to develop a new method to produce GABA rich green tea. Since *trans*-2-hexenal is a main natural constituent of tea leaf volatiles (Hatanaka and Harada 1973), it is quite acceptable to use this plant odor to produce GABA rich green tea.

MATERIALS AND METHODS

Treatment of tea leaves with *trans*-2-hexenal. Fresh leaves of tea, Yabukita cultivar of *Camelia sinensis* L., were harvested at Ishigami tea garden (112 Yoshikawa, Shimizu ward, Shizuoka city, Shizuoka prefecture) on May 2, 2017 at 10 o'clock in the morning and immediately treated with 0, 1, 10 and 100 ppm *trans*-2-hexenal vapor in an air-tight 600 ml glass jar which was prepared by evaporation from a filter paper on which a calculated amount of *trans*-2-hexenal was applied. After 1, 3, and 6 hours of incubation, tea leaves were exposed to liquid nitrogen and GABA was measured by GC-MS.

Method development. Two application methods were assessed to develop a practical method to produce GABA rich green tea with external application of *trans*-2-hexenal. This sought to determine whether GABA content in the tea leaves was enhanced, using a GC-MS. This was conducted at Okutomi-en on August 4, 2017 (36 Kazashi, Sayama, Saitama prefecture), and another experiment was conducted at Yoshida Cha-en on June 23, 2018 (1181 Õtsutsumi, Koga, Ibaraki prefecture).

Fresh harvested tea leaves were placed inside a 70 liter polyethylene plastic bag and 7 g *trans*-2-hexenal were applied on paper filter to make the inside *trans*-2-hexenal concentration to set 100 ppm (w/v). After incubation for 3 or 4 hours, which was carried out at 5 ℃ to avoid browning, leaves were processed by steam heating, cooling, pressing, rolling and twisting, and drying. GABA content was determined by GC-MS with 5 replications.

Fresh tea leaves were harvested and treated with 0, 10 and 100 ppm *trans* -2-hexenal for 1, 3, or 6 hours inside a 3 m^3 steel container with ventilation system and covered with a blue plastic sheet. After the treatments, green tea leaves were processed in the same manner. The metabolome analysis including GABA was conducted with a GC-MS with 3 replications and flavor profiling was done with GC-MS and GC-O system.

GABA measurement and metabolome analysis with GC-MS. Metabolome analysis including GABA was conducted with GCMS-QP2010 Plus (Shimadzu, Japan), using an electron ionization, on a nonpolar phase column (DB-5, Agilent Technologies, USA) according to Yin et al. (2010) and Ijima and Aoki (2009) with some modifications. Each sample (0.1 g of frozen tissue powder) was extracted with 250 µl of methanol and chloroform, one after another. After adding 50 µl of 2.0 mg/ml ribitol solution as an internal standard and 175 µl of ultrapure water, the samples were vigorously mixed. These samples were centrifuged at 12000 rpm for 10 min at room temperature. Then 80 µl of the supernatant fluid of each sample was corrected into a 1.5 ml plastic tube. These samples were evaporated to dryness for 3 hours in a centrifuge evaporator (CVE-200D, Tokyo Rikakikai Co, Ltd, Japan). These samples were freeze-dried overnight using a lyophilization container (Modulyo 4K, Edwards, USA). For methylation, 40 µl of methoxylamine (20 mg/ml pyridine) was added to the samples and incubated for 90 min at 37℃ using a dry block bath (EB603, AS ONE company, Japan). Trimethylsilylation was performed by adding 50 µl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) solution for 30 min at 37 ℃. Helium gas was used as carrier gas at 2.0 mL/min. The initial column oven temperature was set at 100 ℃ for 4 minutes, then increased by 4 ℃ per minute until 320 ℃ for 10 minutes. Metabolites can be identified by comparing fragment patterns and retention indices with those of standard compounds in databases. The principal compound analysis (PCA) was done using the software Pirouette (GL science)

GC−O (gas chromatography-olfactometry) analysis. This analysis was performed using an Agilent 7890 A gas chromatograph (Agilent Technology, CA, USA) equipped with an olfactory port (OP275, GL Science). DB-FFAP and DB-5 columns (30 m x 0.32 mm i.d., 0.25 µm film thickness, J&W Scientific) were used. The samples (1.0 µL) were injected by the cold on-column method. The column

temperature was held at 40 °C for 2 min, increased at a rate of 6°C/min to 230 °C, and then held at 230 °C for 5 min. Helium was used as carrier gas at a flow rate of 2.0 mL/min. The flow split ratio between the flame ionization detector and the olfactory port was 1:1. Linear retention indices (RI) of each odorant were calculated using n-alkanes C6−C26. The odors were determined by retention time and each separated odor was individually characterized by a trained specialist.

GC−MS (gas chromatography−mass spectrometry) analysis. An Agilent 7890 A gas chromatograph equipped with a 5975C mass spectrometer was used to identify each odorant. DB-FFAP and DB-5 columns (30 m x 0.32 mm i.d., 0.25 µm film thickness, J&W Scientific) were used. Samples (1.0 µL) were injected by the cold on-column method at 40 °C. The column temperature was held at 40 °C for 5 min, increased at a rate of 6 °C/min to 230 °C, and then held at 230 °C for 5 min. Helium was used as carrier gas at a flow rate of 2.0 mL/min. The ion energy for electron impact was 70 eV. The mass scan range was m/z 33–450. The compounds were identified by their GC RIs, which were calculated from the retention times in relation to those of a series of C6–C26 n-alkanes on DB-FFAP and DB-5 columns capillary column. The identification of compounds was performed by comparing their fragmentation patterns and RIs and co-injection with authentic compounds, if available.

RESULTS AND DISCUSSION

Vapor applications of *trans*-2-hexenal in a glass jar enhanced GABA content, around 8 -10 times, when treated with 10 or 100 ppm for 3 or 4 hours; the control tea leaves contained less than 0.1 µmol/g but 100 ppm *trans*-2-hexenal treatment achieved up to 2.0 µmol/g (Fig. 1). According to the PCA analysis (Fig.2), GABA, alanine, pyruvic acid and α-ketoglutaric acid were positively enhanced by *trans*-2-hexenal, while glutamic acid, glutamine and aspartic acid were decreased (proportion of the variance showed 86.4%). The metabolome shift (Fig. 3) indicated a similar tendency with the case of tomato fruit maturation under low O_2 conditions (Mae et al. 2012) and tea leaves treated by repeated anaerobic and aerobic incubation (Sawai et al. 2001). GABA is biosynthesized from α-ketoglutaric acid via glutamate by the activities of glutamate dehydrogenase and glutamate decarboxylase and catalyzed by the activity of GABA transaminase which is coupled with the formation of alanine. The enhancement of GABA by *trans*-2-hexenal vapor treatment might be derived by the activation of glutamate dehydrogenase, glutamate decarboxylase and the deactivation of GABA transaminase (Mei et al. 2016).

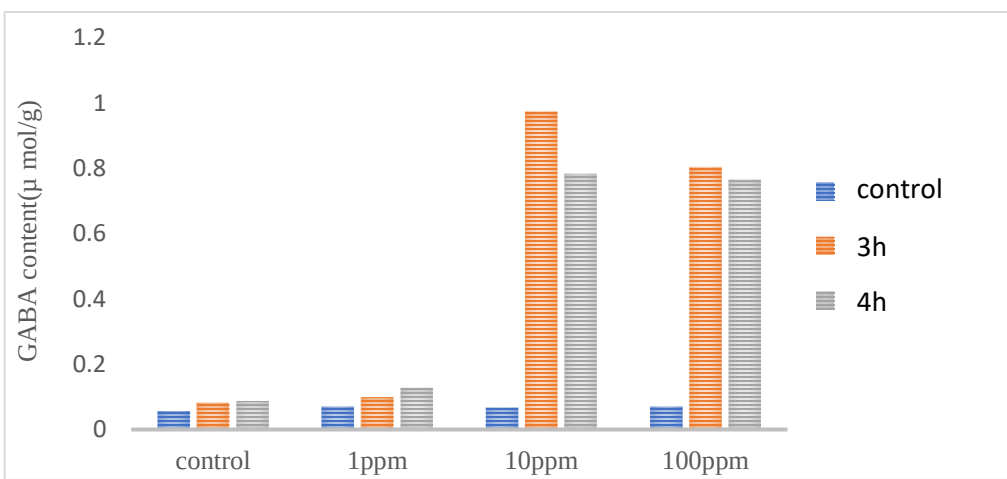

Fig. 1. Effect of *trans*-2-hexenal vapor treatment on GABA content in green tea leaves.

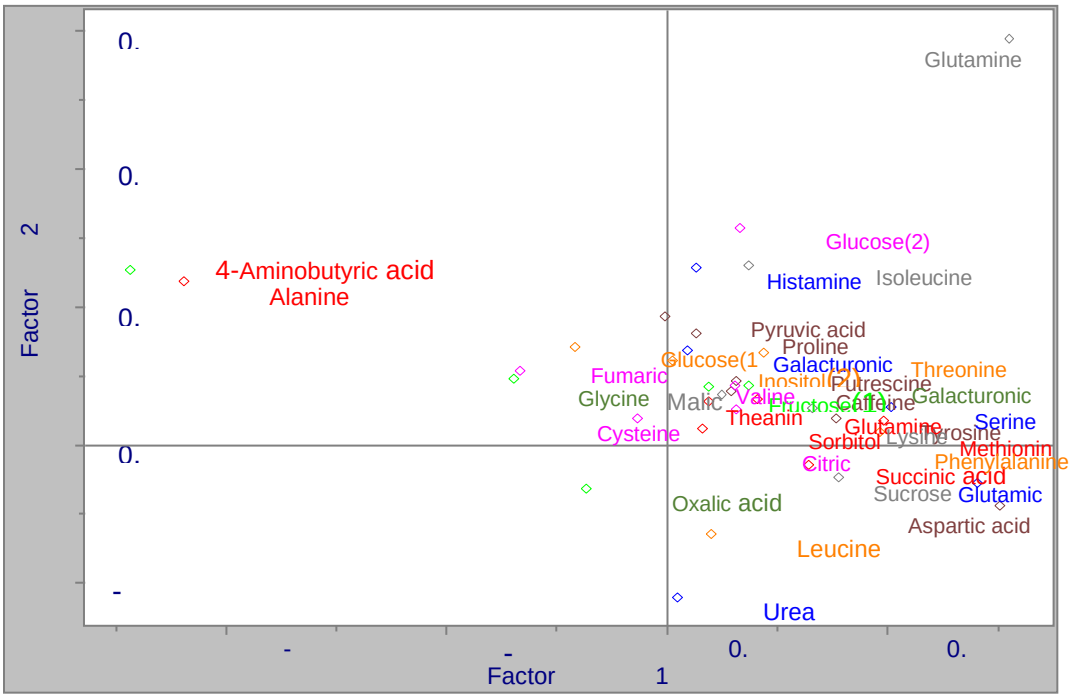

Fig. 2-1. Score plots on principal component analysis (PCA) of physicochemical parameters derived from metabolome analysis

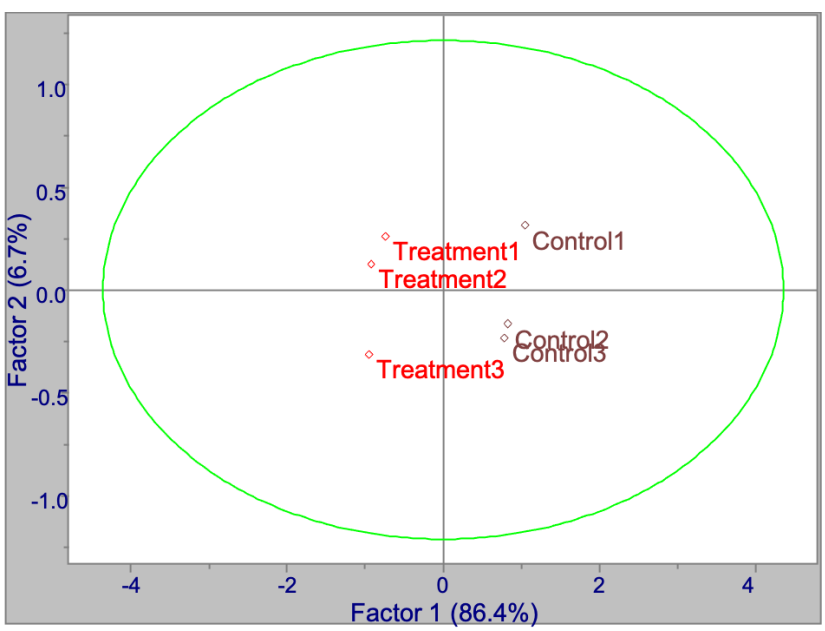

Fig. 2-2. Principal component analysis of control and 100 ppm *trans*-2-hexenal treated green tea leaves.

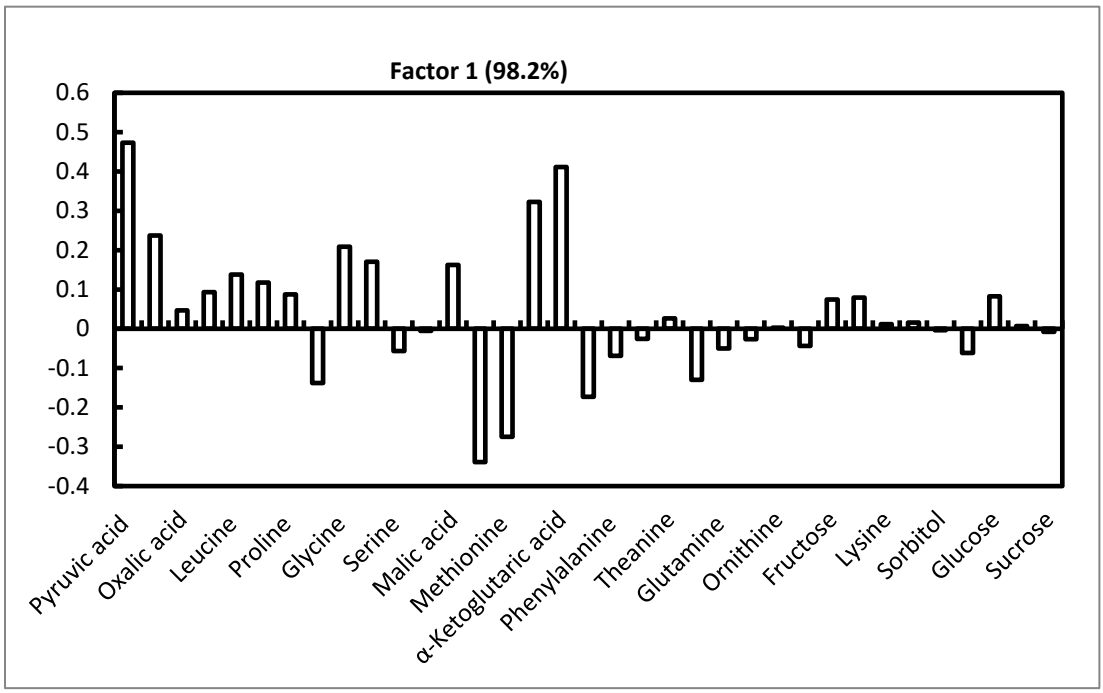

Fig. 3. Effect of 1 hour vapor treatment with 10 ppm *trans*-2-hexenal on metabolome of green tea leaves

As for the practical method to make GABA rich tea, the use of a closed system under low temperature failed to increase GABA (Fig. 4). The data suggested that a closed system might increase GABA content like a procedure to make Gabaron tea by repeated anaerobic and aerobic treatments. The failure might be due to the low temperature treatment which inhibited enzyme activities involved in GABA accumulation. When stored at ambient conditions, harvested tea leaves immediately lost water and started to brown, thus the closed method was not found to be practical.

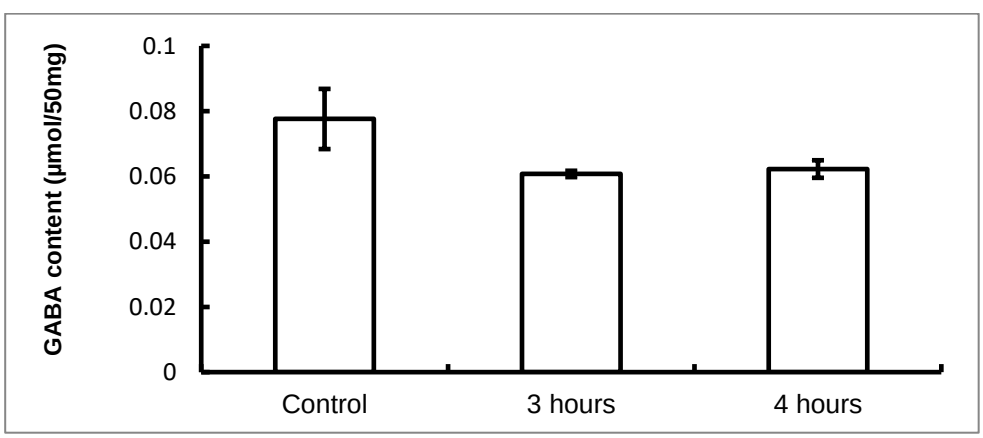

Fig. 4. GABA content of green tea in closed polyethylene plastic bag exposed to 10 ppm *trans*-2-hexenal vapor treatment.

On the other hand, the vapor treatment of 10 ppm *trans*-2-hexenal in a closed ventilation system succeeded in enhancing GABA content up to 2µmol/g, in which enzyme activities could be maintained (Fig. 5).

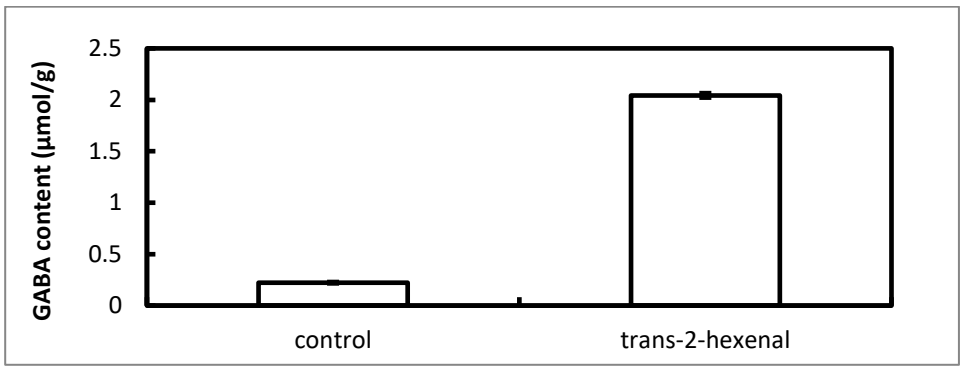

Fig. 5. GABA content of green tea exposed to10 ppm *trans*-2-hexenal vapor treatment in a closed ventilation system.

About the sensory characteristics of GABA rich green tea produced by this method, flavor profiling by GC-MS and sensory data are listed in Table 1 and Table 2. With the treatment with *trans*-2-hexenal, the odors like aldehyde, ketone, ester and acid groups increased, adding slight strawberry and bean-like odor on the basic green tea aroma, which might be acceptable to consumers judging from our taste test, judging from the specialists' reaction during the GC-O olfactory test.

The closed ventilation system produced more aldehydes than control which might be derived from the deactivation of alcohol dehydrogenase. This enzyme converts a variety of aldehydes into correspondent alcohols, but in cases when a substantial amount of *trans*-2-hexenal exists, other aldehydes might not be catalyzed, since the affinity of the enzyme is specifically strong for *trans*-2-hexenal (Eriksson 1968). Among such prominent aldehydes, *trans*-nonadienal might be the main attribute to exert bean-like smell (Kaneko et al. 2011). On the other hand, the strawberry-like odor might be attributed to γ-decalactone which only exists in *trans*-2-hexenal treated tea (Larsen et al. 1992).

Table 1. Flavor profile of GABA rich green tea exposed to 10 ppm *trans*-2-hexenal vapor treatment using ventilation method.

Retention time	**Compound**	**Control**	**10 ppm *trans*-2-hexenal**
12.886	undecane	○	○
14.956	1-methylethylbenzene	n.d.	○
15.976	2-hexenal	n.d.	○
17.442	tridecane	n.d.	○
17.756	1,2-diethyl-benzene	n.d.	○
17.978	1,4-diethyl-benzene	n.d.	○
19.178	1-methyl-2-benzene	n.d.	○
19.241	tetradecane	○	○

Retention time	Compound	Control	10 ppm *trans*-2-hexenal
19.763	(*E*)-2-hexen-1-ol	n.d.	○
20.784	1-ethenyl-4-ethyl-benzene	n.d.	○
21.209	pentadecane	○	○
23.076	hexadecane	○	○
24.851	heptadecane	○	○
25.712	4-ethyl-benzaldehyde	n.d.	○
26.268	4-ethyl-benzaldehyde	n.d.	○
26.539	octadecane	○	○
	heptadecane	n.d.	○
27.453	4-ethyl-benzoic acid	n.d.	○
27.618	hexanoic acid	n.d.	○
27.749	1-(4-ethylphenyl)-ethanone	n.d.	○
27.869	4-ethylbanzoic acid	n.d.	○
	3.5-dimethyl-benzoic acid	n.d.	○
28.160	eicosane	○	n.d.
	nonadecane	○	n.d.
28.406	1-(4-ethylphenyl)-ethanone	n.d.	○
28.948	phenylethylalcohol	n.d.	○
29.320	(*E*)-2-hexenoic acid	○	○
	2-hexenoic acid	n.d.	○
29.698	eicosane	○	○
30.738	*E*-15-heptadecenal	n.d.	○
33.020	14-methyl-pentadecanoic acid	○	n.d.
	hexadecanoic acid	○	○
33.717	2-(tetradecyloxy)-ethanol	n.d.	○
	2-(hexadecyloxy)-ethanol	n.d.	○
35.318	2-methoxy-4-(1-propenyl)-phenol	n.d.	○
36.101	16-methyl-heptadecanoic acid	○	○
	octadecanoic acid	○	○
36.377	19-methyl-heptadecanoate	n.d.	○
36.808	*trans*-13-octadecanoic acid	n.d.	○
	cis-13-octadecanoic acid	n.d.	◯
36.808	16-octadecanoic acid	n.d.	◯
36.812	indole	◯	◯
37.103	4-methyl-benzonitrile	n.d.	◯
37.291	2H-1-benzopyran-2-one	○	○

Retention time	Compound	Control	10 ppm *trans*-2-hexenal
38.268	1,4,7,10,13,16-hexaoxacyclooctadecane	○	n.d.
39.187	vanillin	○	n.d.
39.889	1,4,7,10,13,16-hexaoxacyclooctadecane	○	○
42.370	tetradecanoic acid	n.d.	○
42.853	1.2-benzenedicarboxylic acid	n.d.	○

○ = detected; n.d. = not detected

Table 2. Sensory characteristics of GABA rich green tea exposed to 10 ppm *trans*-2-hexenal vapor treatment using the ventilation method.

Retention Index	Compound	Control	Treated	Aroma description
975	methylbutanoate	n.d.	○	yogurt
1110	methyl-3-methylbutanoate	n.d.	○	sweet
1130	2- methyl-pentane-1-thiol	n.d.	○	green
1240	(*E*)-6-decenal	n.d.	○	green, Matcha
1275	unidentified	○	n.d.	peanut
1290	1-octene-3-one	n.d.	○	mushroom
1300	propyl-2-methylhexanoate	n.d.	○	lemon
1330	Hexyl-2-methylpropanoate	○	n.d.	green
1360	dimethyltrisulfide	n.d.	○	pungent
1380	(*Z*)-3-hexenol	n.d.	○	green cut grass
1440	2-methyl-2,3-dimethylpyrazine	○	○	earthy
1440	hexyl-3-methylbutanoate	○	○	sweet
1470	5-nonene-7-one	n.d.	○	green, green tea
1510	2-isobutyl-3-methoxypyrazine	n.d.	○	cucumber, green pepper
1530	(*Z*)-4-decenal	○	○	green
1570	(*E*,*Z*)-2,6-nonadienal	n.d.	○	cucumber, green
1635	1-terpinene-4-ol	○	n.d.	green tea
1665	3-methylbutanoicacid	n.d.	○	sweaty
1700	geranial	n.d.	○	black tea
1735	(*E*)-2-undecenal	n.d.	○	green, oily
1800	(*E*,*E*)-2,4-decadienal	n.d.	○	green, fatty
1887	unidentified	n.d.	○	green tea
1900	2-phenylethanol	○	n.d.	honey, rose
1927	β-ionone	n.d.	○	rose, flowery

Retention Index	Compound	Control	Treated	Aroma description
1947	(*E*)-2-tridecanal	○	n.d.	green
1967	maltol	○	n.d.	sweet, cotton candy
1980	1-mercaptoctane-3-ol	n.d.	○	sweaty
2033	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	○	○	sweet, cotton candy
2120	4-hydroxy-5-methyl-3(2*H*)-furanone	○	○	sweet
2147	γ-decalactone	n.d.	○	peach, coconuts
2180	γ-(Z)-decenolactone	○	○	peach
2240	3-propylphenol	○	○	phenol, leather
2280	δ-undecalactone	○	n.d.	flowery,
2313	α-sinensal	n.d.	○	green, metallic
2333	2-furfuryl-2-methyl-3-furyldisulfide	○	n.d.	smoky
2461	coumarin	○	○	sakuramochi
2500	unidentified	○	n.d.	sweet
2550	phenylacetate	○	○	honey, rose
2580	vanillin	n.d.	○	vanilla
>2600	unidentified	n.d.	○	flowery
>2600	unidentified	n.d.	○	green tea

CONCLUSION

A method for GABA rich tea production was developed using vapor treatment with *trans*-2-hexenal. The vapor application of *trans*-2-hexenal could enhance GABA content 8-10 times higher if treated in a closed system with ventilation. GABA rich green tea produced by this method exerted slight strawberry and bean-like flavor. It has the potential acceptability to the market in Southeast countries.

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REFERENCES CITED

Abe, Y., Umemura, S., Sugimoto, K.I., Hirawa, N., Kato, Y., Yokoyama, N., Yokoyama, T., Iwai, J. and M. Ishii. 1995. Effect of green tea rich in γ-aminobutyric acid on blood pressure of Dahl salt-sensitive rats. American Journal of Hypertension, 8(1):74-79.

Cheng, T.C. and J.F. Tsai. 2009. GABA tea helps sleep. The Journal of Alternative and Complementary Medicine. 15(7): 697-698.

Eriksson, C. E. 1968. "Alcohol: NAD oxidoreductase (EC 1.1. 1.1.) from peas." Journal of Food Science. 33(1): 525-532.

Hakamata, K., Nakada, N., Mukai, T., Fukushima, H., Yamaguchi, R., and T. Nakada. 1988. Improvements of manufacturing process of anaerobically treated tea (Gabaron tea). Chagyokenkyuhoukoku. 68:8-13.

Hatanaka, A. and T. Harada. 1973. Formation of cis-3-hexenal, *trans*-2-hexenal and *cis*-3-hexenol in macerated *Thea sinensis* leaves. Phytochemistry. 12(10): 2341–2346.

Horie, K., Watanabe, K., Sakashita, M. and A. Yamatsu. 2019. Establishment of GABA production technology and market development of the GABA-containing functional foods: How was the unique functional food material "GABA" born ? Kagaku to Seibutsu. 57: 207-212.

Huang, C.H., W. Kuo, H. Wang, Y. Lin, J. Chen, C. Kuo, P. Chen and J. Lin. 2014. GABA tea ameliorates cerebral cortex apoptosis and autophagy in streptozotocin-induced diabetic rats. Journal of Functional Foods. 6: 534-544.

Iijima, Y.; Aoki, K. 2009. Application of metabolomics to improve tomato fruit productivity and quality. J. Japan. Soc. Hort. Sci. 78:14-22.

Kaneko, S., Kumazawa, K. and O. Nishimura. 2011. Studies on the key aroma compounds in soy milk made from three different soybean cultivars. Journal of Agricultural and Food Chemistry. 59(22): 12204-12209.

Larsen, M., Poll, L. and C.E. Olsen. 1992. Evaluation of the aroma composition of some strawberry (*Fragaria ananassa* Duch) cultivars by use of odour threshold values. Zeitschrift für Lebensmittel-Untersuchung und Forschung. 195(6): 536-539.

Mae, N., Makino, Y., Oshita, S., Kawagoe, Y., Tanaka, A., Aoki, K., Kurabayashi, A., Akihiro, T., Akama, K., Koike, S. and M. Takayama. 2012. Accumulation mechanism of γ-aminobutyric acid in tomatoes (*Solanum lycopersicum* L.) under low O_2 with and without CO_2. Journal of Agricultural and Food Chemistry. 60, 1013-1019.

Mei, X., Chen, Y., Zhang, L., Fu, X., Wei, Q., Grierson, D., Zhou, Y., Huang, Y., Dong, F. and Z.Yang. 2016. Dual mechanisms regulating glutamate decarboxylases and accumulation of gamma-aminobutyric acid in tea (*Camellia sinensis*) leaves exposed to multiple stresses. Scientific Reports. 6, 1-11.

Sawai, Y., Yamaguchi, Y., Miyama, D. and H. Yoshitomi. 2001. Cycling treatment of anaerobic and aerobic incubation increases the content of γ-aminobutyric acid in tea shoots. Amino Acids. 20: 331-334.

Tsushida, T. and T. Murai. 1987. Conversion of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic conditions. Agricultural and Biological Chemistry. 51, 2865-2871.

Yin, Y.G., Tominaga, T., Iijima, Y., Aoki, K., Shibata, D., Ashihara, H., Nishimura, S., Ezura, H. and C. Matsukura. 2010. Metabolic alterations in organic acids and gamma-aminobutyric acid in developing tomato (*Solanum lycopersicum* L.) fruits. Plant Cell Physiol. 51:1300–1314.