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Time−Course Study and Effects of Drying Method on Concentrations of γ-Aminobutyric Acid, Flavonoids, Anthocyanin, and 2″-Hydroxynicotianamine in Leaves of Buckwheats

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Concentrations of γ-aminobutyric acid (GABA), rutin, minor flavonoids (such as orientin), anthocyanin, and 2″-hydroxynicotianamine (2HN) were quantified in the leaves of common and tartary buckwheat (*Fagopyrum esculentum* Moench and *Fagopyrum tataricum* Gaertn., respectively), at 14, 28, and 42 days after sowing (DAS). GABA and rutin concentrations peaked at 42 DAS, whereas anthocyain, 2HN, and minor flavonoid concentrations declined with the age of the plants. However, at 42 DAS, anthocyanin concentrations in the leaves of tartary buckwheat Hokkai T10 leaves were at least 10-fold greater than in the other buckwheats tested. In addition, the effects on target compound concentrations and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of three different drying methods (20 h at 40 °C, 7 h at 70 °C, or lyophilization) were investigated. In general, the drying method had no significant effect on the parameters tested. These results indicate that, in terms of GABA, rutin, and anthocyanin concentrations, leaf powder from 42 day old Hokkai T10 has the potential to be a useful food ingredient, such as Ao-jiru juice.

KEYWORDS: Buckwheat; leaf; γ-aminobutyric acid; 2″-hydroxynicotianamine; rutin; anthocyanin

INTRODUCTION

There are two main species of cultivated buckwheat, common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.). Both of them contain rutin, a kind of flavonol that has antioxidative (*1*, *2*), antihypertensive (*3*), and anti-inflammatory activities (*2*). Especially, tartary buckwheat, in particular, has received attention as a healthy food because it contains large quantities of rutin in its seeds (*4*) and sprouts (*5*). However, tartary buckwheat seed contains very potent enzymes that rapidly decompose rutin when the buckwheat flour is mixed with water (*6*, *7*). On the other hand, tartary buckwheat sprouts are suitable as a food ingredient, particularly in Ao-jiru juice, whose ability to decompose rutin is very low. Ao-jiru is a juice made of dried leaf powder, which is promoted as a health drink in Japan. However, as sprouts are very small, the greater quantity of leaves available from more developed tartary buckwheat would afford a more convenient source of food ingredient, should they maintain high target nutrient levels. Fabjan et al. (*8*) have reported that the vegetative portion of the tartary buckwheat plant contains about 3% rutin on a dry weight basis (dwb), while Suzuki et al. (*9*) have reported that rutin decomposition activity in leaves was very low as compared to that in the seed. However, their studies have been limited to rutin, whereas tartary buckwheat also contains other health-enhancing compounds.

Recently, an anthocyanin-rich tartary buckwheat variety, Hokkai T10, has been developed at our institute. Its sprouts contain about 1.7- and 37-fold higher rutin and anthocyanin levels, respectively, than its parent varieties. Anthocyanins identified in the sprouts of Hokkai T10 include cyanidin 3-*O*-glucoside (C3gl) and cyanidin and 3-*O*-rutinoside (C3r). These compounds have many antioxidative and anti-inflammatory activities in vitro (*10*) and prevent obesity and ameliorate hyperglycemia in mice (*11*). The compound C3r, found in

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blueberry (*Vaccinium corymbosum* L.) fruit, exhibits inhibitory effects on the migration of human lung cancer cell lines (*12*). Therefore, attention has been focused on its use as a food ingredient. However, levels of rutin and anthocyanins in leaves of plants at more advanced growth stages have not been studied.

Recently, γ-aminobutyric acid (GABA) and 2″-hydroxynicotianamine (2HN) have been found to serve as functional compounds in buckwheat. GABA has been reported to reduce blood pressure in humans (*13*, *14*). Buckwheat contains GABA in its seeds (*15*) and sprouts (*16*). The compound 2HN, shown to inhibit the enzymic conversion of angiotensin I and recently identified in buckwheat flour (*17*), is also found in buckwheat sprouts. However, GABA and 2HN concentration changes in leaves at more advanced growth stages have not been studied. In addition, to use buckwheat leaf powder as a food ingredient, it is important to establish which drying method would least affect concentrations of the above-mentioned functional compounds.

This is the first report to investigate GABA, flavonoid, anthocyanin, and 2HN concentrations, along with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, in leaves of a tartary buckwheat variety (Hokkai T8), an anthocyanin-rich tartary buckwheat variety (Hokkai T10), and a widely grown conventional variety of common buckwheat (Kitawasesoba) at different plant growth stages. In addition, their stabilities under different drying methods have been studied.

MATERIALS AND METHODS

Solvents and Reagents. External high-performance liquid chromatography (HPLC) standards for GABA were purchased from Wako Pure Chemical Industries (Osaka, Japan), and other flavanoids such as chlorogenic acid, orientin, isoorientin, vitexin, isovitexin, cyanidin, C3gl, C3r, and cyanidin 3-*O*-garactoside (C3ga) along with those for rutin and quercetin were purchased from Extrasynthèse (Genay, France). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Wako Pure Chemical Industries. Partially purified 2HN was a gift from Dr. Aoyagi.

Preparation of Plant Materials. Tartary buckwheat varieties (Hokkai T8 and Hokkai T10) and common buckwheat variety (Kitawasesoba) were tested. They were grown at the experimental field of the National Agricultural and Food Research Organization for the Hokkaido region in Memuro, Hokkaido, Japan (longitude, 143° 03′; latitude, 42° 53′). Buckwheat seeds were sown on June 6, 2007, and leaves were harvested at 14 days after sowing (DAS), 28 DAS, and 42 DAS. Leaves harvested at 14 and 28 DAS (about 80 g fresh weight each) were directly dried with an incubator under ventilation (model DKM600, Yamato Co. Ltd., Tokyo, Japan) just after harvesting. The thickness of the layer was about 0.5 cm. Leaves harvested at 42 DAS (about 80 g fresh weight each) were lyophilized directly just after harvesting. Dried leaves were ground with IFM-180G Millser (Iwatani International Co., Tokyo, Japan) individually to a fine powder and then stored at −30 °C until chemical analysis.

Determination of GABA. Samples were homogenized with an Ultra-Turrax instrument (T25 Ika Works, Selangor, Malaysia) for 1 min in 5 mL of 80% (v/v) ethanol. The homogenate was allowed to stand for 1 h at ambient temperature. After it was filtered through two layers of #2 filter paper (Advantec, Tokyo, Japan) using an aspirator, the filtrate was concentrated by a vacuum evaporator at 37 °C. It was then poured into a volumetric flask and adjusted to 5 mL with a 0.2 mol/L sodium citrate buffer solution (pH 2.2; Wako Pure Chemical Industries). After it was filtered through a Sep-Pak C18 cartridge (Waters, MA) and a 0.45 μm olefine-polymer filter (Kurabo, Osaka, Japan), the filtrate was analyzed by HPLC (LC-10A amino acid analysis system, Shimadzu, Kyoto, Japan) equipped with a Shim-pack Amino-Na column (6 mm × 10 mm, Shimadzu) and an ISC30/S0504 precolumn (4 mm × 50 mm). Detection was achieved at 450 nm, the flow rate was 0.6−0.7 mL/min, and the column oven temperature was set at 60 °C. The quantification was performed using an external amino acid standard solution (type H) with GABA reagent.

Determination of Flavonoids. Flavonoids were extracted with 1 mL of MeOH containing 10% phosphoric acid [0.1% (v/v)] per 10 mg dry weight (DW), and the mixture was stored for 3 h at 37 °C in an incubator (model IJ 200, Yamato Co. Ltd.). After centrifugation at 10000*g* for 5 min, the supernatant was passed through a disposable syringe filter (PTFE, 0.5 mm, hydrophobic; Advantec). The filtrate was then analyzed by HPLC (class-VP chromatography data system; Shimadzu) in a Capcell PAK ODS column (250 mm, 4.6 mm, particle size 5 mm; Shiseido, Tokyo, Japan). Detection was achieved at 350 nm, the flow rate was 1.0 mL/min, and the column oven temperature was 40 °C. The mobile phase consisted of (A) MeOH:water:acetic acid (v/v/v, 5:92.5:2.5) and (B) MeOH:water:acetic acid (95:2.5:2.5). The initial mobile phase composition was 0% B, followed by a linear gradient to 80% B in 48 min and isocratic conditions with 0% B for 10 min (*18*). The quantification was performed using commercial external standards (Extrasynthèse), which were passed through the same extraction process.

Determination of Anthocyanins. HPLC/electrospray ionization−mass spectrometry (ESI-MS) analysis was carried out according to the method of Suzuki et al. (*19*). The anthocyanins were extracted overnight at 22 °C with 1 mL of extraction solvent (MeOH/AcOH/H_2O, 80:0.2:19.8) per 60−80 mg of samples. The extracts were passed through a 0.45 μm filter and applied to an HPLC/ESI-MS system consisting of an Agilent 1100 Series LC (Agilent Technologies) column coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The positive-ion ESI-MS was performed at a capillary temperature of 365 °C and voltage of 3.5 kV, at a nebulizing pressure of 50 psi; N_2 was used as the sheath gas. The ion trap MS analysis was carried out with helium as the collision gas. The normalized collision energy was set to 30%. HPLC was carried out on a 150 mm × 2 mm i.d. Cadenza CD-C_{18} column (Imtakt Corp., Kyoto, Japan) at a flow rate of 0.3 mL/min. To determine anthocyanin profiles, these compounds were separated using a 0−50 min linear gradient of 0−100% solvent A (CH_3CN/H_2O/TFA, 7.5:92.5:0.1) to solvent B (CH_3CN/H_2O/TFA, 55:45:0.1). The anthocyanin concentrations were determined by the peak areas of the extracted ion chromatogram (C3gl *m/z* 449, $[M + H]^+$; C3r, *m/z* 595, $[M + H]^+$; C3ga *m/z* 449, $[M + H]^+$; cyanidin 3-*O*-galactosyl-rhamnoside (C3ga-r), *m/z* 595, $[M + H]^+$) using a standard curve derived from commercial anthocyanins (Extrasynthesè). As C3ga-r was not commercially available, we used C3r instead.

Determination of 2HN. An HPLC/ESI-MS analysis was carried out for 2HN. The 2HN was extracted overnight at 37 °C with 1 mL of extraction solvent (EtOH/H_2O, 70:20) per 60−80 mg of sample. The extracts were passed through a 0.45 μm filter and applied to an HPLC/ESI-MS system consisting of an Agilent 1100 Series LC (Agilent Technologies) column coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics). The positive-ion ESI-MS was performed at a capillary temperature of 365 °C, voltage of 3.5 kV, and nebulizing pressure of 50 psi; N_2 was used as the sheath gas. The ion trap MS analysis was carried out with He as the collision gas. The normalized collision energy was set to 30%. HPLC was carried out on a 150 mm × 2 mm i.d. TSK-gel amide 80 (TOSOH Kyoto, Japan) column at a flow rate of 0.2 mL/min. The 2HN was separated using a 0−50 min linear gradient of 0−100% solvent A (CH_3CN) to solvent B (DW). The 2HN relative concentrations were determined by the peak areas of the extracted ion chromatogram (*m/z* 320 $[M + H]^+$) using a standard curve derived from partially purified 2HN.

DPPH Radical Scavenging Activity. The DPPH superoxide scavenging activity was evaluated as described below. The scavenging activity was expressed as micromoles of Trolox not reduced by superoxide. A dried sample was extracted in 70% EtOH at 75 °C for 10 min so as to result in a final sample concentration of 12.5 mg DW/mL. To 0.2 mL of sample solution, 0.8 mL of Tris-HCl buffer (pH 7.4, 0.1M) and 1 mL of 0.1 mM DPPH-EtOH solution were added. The mixture was shaken, and the absorbance of the resulting solution was measured at 517 nm. The solution was then allowed to stand for 20 min in the dark at room temperature, the absorbance was again measured, and the decrease in absorbance was calculated.

Statistical Analysis. Bonferroni's multiple range test was applied for multiple comparisons and used to determine statistically significant differences ($P \leq 0.05$).

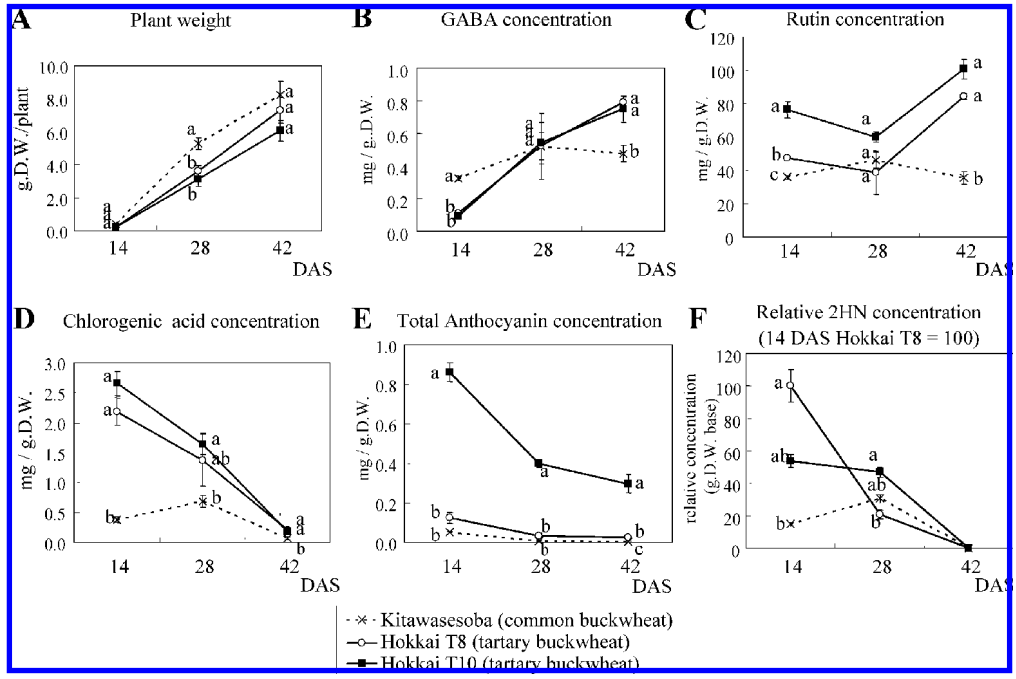

Figure 1. Time−course analysis of plant weight and functional compounds in buckwheat cultivars. (**A**) Plant weight, (**B**) GABA concentration, (**C**) rutin concentration, (**D**) chlorogenic acid concentration, (**E**) total anthocyanin concentration, and (**F**) relative 2HN concentration (14 DAS 'Hokkai T8' = 100). Bars indicate SD (n = 3). Within the same DAS, values followed by the same letters are not significantly different at the 5% level, using Bonferroni's multiple range test.

RESULTS AND DISCUSSION

Time−Course Analysis of Plant Dry Mass, GABA, Rutin, Anthocyanin, Chlorogenic Acid, and 2HN Concentrations. In all buckwheat varieties, plant dry mass increased linearly as DAS at sampling increased (**Figure 1A**). At 28 DAS, Kitawasesoba plants were significantly heavier than Hokkai T8 or Hokkai T10 plants. However, at 14 and 42 DAS, the mass of each of the buckwheat varieties was not significantly different. These results indicate that late stage harvesting is suitable for obtaining greater dry mass.

In the common buckwheat variety Kitawasesoba, the GABA concentration increased from 14 DAS to a peak at 28 DAS (**Figure 1B**). On the other hand, GABA concentrations in tartary buckwheat cultivars Hokkai T8 and Hokkai T10 increased linearly from 14 to 42 DAS. At 14 DAS, the GABA concentration of the common buckwheat cultivar Kitawasesoba was significantly greater than that of tartary buckwheat cultivars Hokkai T8 and Hokkai T10. On the other hand, at 42 DAS, GABA concentrations in tartary buckwheat cultivars Hokkai T8 and Hokkai T10 were significantly greater than those in the common buckwheat cultivar Kitawasesoba. These results indicate that to obtain GABA-rich material, 42 day old leaves of Hokkai T8 and Hokkai T10 are suitable. In common buckwheat flour, the GABA concentration is around 0.124 mg/g DW (*15*). In common buckwheat sprouts, the GABA concentration was found to reach 0.66 mg/g DW (*16*). As compared to these data, the GABA concentration of common and tartary buckwheat leaves at 14 DAS was a little lower than that in flour or sprouts but higher after 28 DAS. The difference may be related to varietal differences and growth conditions; samples in this report were grown in an experimental field, whereas others' were grown in a greenhouse.

Recently, a study of the hypotensive effects of fermented GABA-containing milk revealed that systolic blood pressure was significantly lowered by a diet including the treated milk (corresponding to 12.5 mg GABA/day) as compared to a placebo group (*14*). In the present study, GABA concentrations in tartary buckwheat leaves were shown to reach about 0.8 mg/g DW. If dried tartary buckwheat leaf powder is added to Ao-Jiru juice, drinkers can take up 2.4−3.2 mg GABA per day (people generally consume 3−4 g of leaf powder in Ao-Jiru juice per day). If the dried powder of tartary buckwheat leaves were to be added to buckwheat noodles, at 10% of the dough weight, consumers could take in 8 mg GABA per 100 g of flour used in making the noodles. By these two means, GABA uptake could be increased by 20−60% and contribute to lowering systolic blood pressure.

In tartary buckwheat cultivars Hokkai T8 and Hokkai T10, the rutin concentration slightly decreased from 14 to 28 DAS but then rose to a maximum at 42 DAS (**Figure 1C**). In the common buckwheat cultivar Kitawasesoba, the rutin concentration increased slightly from 14 DAS to 28 DAS and then decreased slightly from 28 to 42 DAS. At 42 DAS, rutin concentrations from highest to lowest occurred in the following order: Hokkai T10 > Hokkai T8 > Kitawasesoba, with the rutin concentration in the tartary buckwheats Hokkai T8 and Hokkai T10 being significantly higher than that in the common buckwheat cultivar Kitawasesoba. Hokkai T10 had the highest rutin concentration at every growth stage. Remarkably, at 42 DAS, Hokkai T10 contained about 100 mg/g DW of rutin. Suzuki et al. reported that rutin contents in leaves and enzyme activities related to rutin synthesis were highest in fully expanded leaves as compared to young or senescent leaves (*20*, *21*). This is consistent with the fact that buckwheat plants have many more fully expanded leaves at 42 DAS than at either 14 or 28 DAS (**Figure 1C**). These results indicate that rutin-rich material can be successfully obtained from 42 DAS leaves of Hokkai T10.

In tartary buckwheats, the chlorogenic acid concentration decreased from 14 to 42 DAS (**Figure 1D**). In contrast, in common buckwheat, the chlorogenic acid concentration reached a maximum at 28 DAS and then slightly decreased by 42 DAS.

Table 1. Time−Course Analysis of Minor Flavonoid Concentrations in Buckwheat Cultivars[a]

	μg/g DW				
	orientin	isoorientin	vitexin	isovitexin	quercetin
			DAS 14		
Kitawasesoba	529 a[b] ± 61	1430 a ± 160	606 a ± 96	2040 a ± 316	15.5 a ± 7.4
Hokkai T8	50.7 b ± 1.0	143 b ± 3	504 a ± 20	1480 a ± 74	34.1 a ± 5.9
Hokkai T10	19.9 c ± 5.2	118 b ± 19	731 a ± 107	1620 a ± 237	106 a ± 31
			DAS 28		
Kitawasesoba	trace	74.8 ± 7.2	67.1 a ± 32.3	210 a ± 67	62.6 a ± 6.6
Hokkai T8	trace	trace	trace	36.0 a ± 3.5	47.4 a ± 15.3
Hokkai T10	trace	trace	15.1 a ± 13.1	25.0 a ± 4.3	44.9 a ± 4.0
			DAS 42		
Kitawasesoba	ND[c]	ND	ND	ND	76.7 a ± 7.3
Hokkai T8	ND	ND	ND	ND	18.6 a ± 4.0
Hokkai T10	ND	ND	ND	ND	41.9 a ± 2.9

[a] Data are means ± SD ($n = 3$). [b] Within each column, values followed by the same letters are not significantly different at the 5% level, using Bonferroni's multiple range test. [c] Not detectable.

At all sampling times, Hokkai T10 showed statistically greater chlorogenic acid concentrations than common buckwheat.

Table 1 shows the concentrations of minor flavonoids in leaves of common and tartary buckwheats. Generally, minor flavonoids concentrations decreased from 14 to 42 DAS. By 42 DAS, except for quercetin, no flavonoids were detectable. Therefore, 42 DAS leaves are poorly suited as a source of chlorogenic acid and minor flavonoids. At 14 DAS, orientin and isoorientin concentrations in common buckwheat were statistically greater than in tartary buckwheats. During seed maturation of common and tartary buckwheat, concentrations of the minor flavonoids (**Table 1**) were at trace levels (*7*), whereas in young sprouts, these were at high levels (*5, 18*). These results indicate that the minor flavonoids are mainly accumulated in the young stages of sprouts.

In common and tartary buckwheats, total anthocyanin concentrations decreased from 14 to 42 DAS (**Figure 1E**). Cultivar Hokkai T10 showed a statistically greater total anthocyanin concentration (about 10-fold greater) than the other cultivars tested. In tartary buckwheat sprout, Hokkai T10 contained about 5.0 mg/g DW of anthocyanins (*22*). On the other hand, the total anthocyanin concentration of Hokkai T10 in this report at every DAS was lower than that of sprout. However, the anthocyanin content per plant was much higher, especially at 42 DAS, than that of sprout. Therefore, the leaves of 42 DAS Hokkai T10 show promise as an anthocyanin-rich material.

Table 2 shows the composition of the anthocyanins detected in the cultivars under study. In all buckwheats tested, the most common anthocyanin was C3r. At every growth stage, common buckwheat variety Kitawasesoba contained the four anthocyanins: C3r, C3gl, C3ga, and C3ga-r, whereas tartary buckwheats contained only two anthocyanins: C3r and C3gl. Anthocyanins identified in the present study were also identified in buckwheat sprouts (*22*) and flowers (*19*). Among them, C3gl had many antioxidative and anti-inflammatory activities in vitro (*10*) and was shown to prevent obesity and improved hyperglycemia in mice (*11*). Also found in blueberry fruit, C3r exhibited inhibitory effects on the migration of human lung cancer cell lines (*12*). Therefore, anthocyanins in buckwheat leaves may have similar functionality.

In tartary buckwheats leaves, the relative dry weigh basis 2HN concentration decreased as plant development progressed (**Figure 1F**). However, in common buckwheat, it increased from 14 to 28 DAS but decreased thereafter, such that it was not detectable at 42 DAS. Therefore, late stage harvesting (i.e., 42 DAS) is not suitable if one wishes to obtain a 2HN-rich material.

Table 2. Time−Course Analysis of Anthocyanin Concentrations in Buckwheat Cultivars[a]

	μg/g DW			
	C3r	C3gl	C3ga	C3ga-r
		DAS 14		
Kitawasesoba	24.6 a ± 3.0	14.2 a ± 1.9	0.46 ± 0.08	13.4 ± 1.8
Hokkai T8	123 a[b] ± 29	trace	ND[c]	ND
Hokkai T10	671 b ± 33	193 b ± 24	ND	ND
		DAS 28		
Kitawasesoba	trace	trace	trace	5.35 ± 1.74
Hokkai T8	31.7 a ± 17.6	trace	ND	ND
Hokkai T10	343 b ± 12	56.6 ± 0.0	ND	ND
		DAS 42		
Kitawasesoba	trace	trace	trace	4.61 ± 2.05
Hokkai T8	26.9 a ± 3.7	trace	ND	ND
Hokkai T10	247 a ± 54	49.9 ± 14.0	ND	ND

[a] Data are means ± SD ($n = 3$). [b] Within each column, values followed by the same letters are not significantly different at the 5% level, using Bonferroni's multiple range test. [c] Not detectable.

While Aoyagi (*17*) demonstrated the presence of 2HN in some polygonaceous plants, including common buckwheat, there exists no such information regarding leaves of other cultivated buckwheat species, for example, tartary buckwheat leaf. Therefore, this is the first report to demonstrate that tartary buckwheat leaf contains 2HN. In this paper, extraction efficiency of 2HN was not high because 2HN was extracted more in a solution, which consisted of low ethanol concentration. In addition, we could not employ quantitative analysis because the standard of highly purified 2HN was not available. However, the objective of this study is to compare relative 2HN concentration among samples. Therefore, low extraction efficiency of 2HN is not a big problem. For the next step to study 2HN, experiments about extraction efficiency of 2HN and quantitative analysis using standard of highly purified 2HN are necessary.

Effects of Drying Method on Concentrations of Functional Compounds and DPPH Radical Scavenging Activity. To use buckwheat leaves as food ingredients, particularly in Ao-Jiru juice, a drying process is necessary. Our study having shown that 42 DAS leaves were suitable in terms of their mass and GABA and rutin concentrations, we investigated the effects of different drying methods on their concentrations. These drying methods were (i) lyophilization (LY), (ii) drying at 45 °C, and (iii) drying at 70 °C. In addition, we investigated the DPPH radical scavenging activity of powders generated by different drying methods.

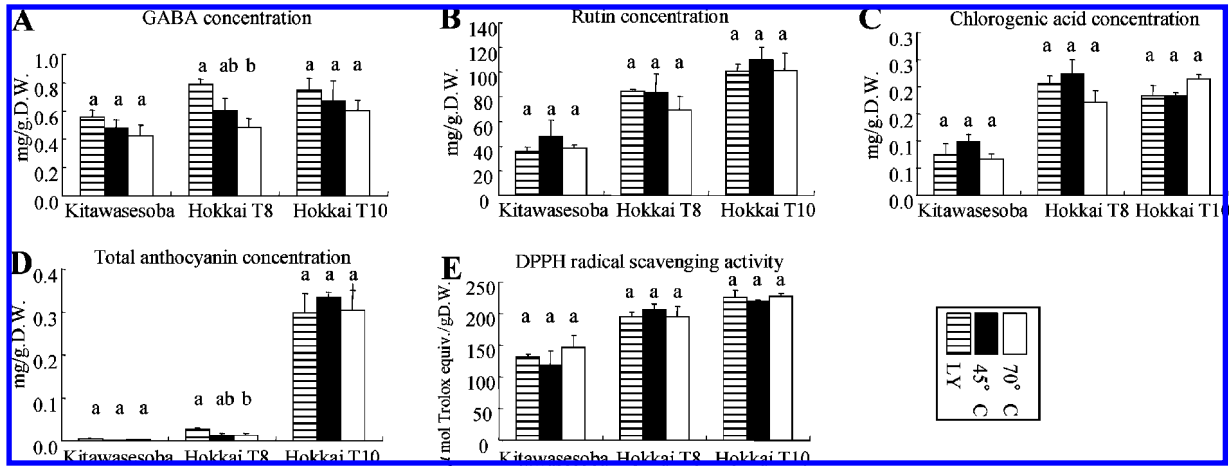

Figure 2. Effects of drying method on functional compound concentration and DPPH radical scavenging activity in buckwheat cultivars. (**A**) GABA concentration, (**B**) rutin concentration, (**C**) chlorogenic acid concentration, (**D**) total anthocyanin concentration, and (**E**) DPPH radical scavenging activity. Bars indicate SD ($n = 3$). Within the same varieties, values followed by the same letters are not significantly different at the 5% level, using Bonferroni's multiple range test.

Table 3. Correlation of Values for Measurements of Flavonoids, Anthocyanins, and DPPH Radical Scavenging Activity

	chlorogenic acid	orientin	isoorientin	vitexin	isovitexin	rutin	quercetin	C3r	C3gl
DPPH	0.413	0.173	0.018	0.014	−0.056	0.601[a]	−0.167	0.443	0.381

[a] Significant at the 5% level.

For all buckwheat species/cultivars, GABA concentrations followed the order LY > 45 °C > 70 °C (**Figure 2A**). However, differences between drying methods were not significant, except for LY and 70 °C for Hokkai T8. For other compounds, their concentrations were not significantly different between different drying methods (**Figure 2B−D**), except for LY and 70 °C for chlologenic acid in Hokkai T10. In general, high-temperature drying results in a deterioration in quality such as a decrease of functional compounds content. However, in the present study, the drying method had little effect on ultimate concentrations of these compounds. This may be related to the short length of the drying period and ventilation. Leaves were dried for the shortest period possible: 20 h at 40 °C and 7 h at 70 °C. On the basis of these results, a low-cost drying method such as 20 h at 40 °C would be suitable. In a future study, we will investigate effects of drying conditions such as ventilation, humidity, and ample thickness on dried leaves quality.

In addition, we investigated effects of the drying method on DPPH radical scavenging activity, using 42 DAS samples. DPPH radical scavenging activity was not significantly affected by drying method (**Figure 2E**). In the next step, to study which compounds measured in this report were best correlated with DPPH radical scavenging activity, we investigated correlations between measured flavonoid and anthocyanin levels and DPPH radical scavenging activity, using 42 DAS samples. Only rutin showed a significant positive correlation to DPPH radical scavenging activities (**Table 3**).

Correlations between antioxidant activity and rutin content have been studied in buckwheat materials (*23*−*25*). Generally, both flavonoids and anthocyanins have DPPH radical scavenging activities. In tartary buckwheat flour, rutin provides a greater contribution to DPPH radical scavenging activities than other compounds such as quercetin (*23*). In this case, the rutin concentration at 42 DAS was at least 1000-fold greater than that of the other compounds measured. Therefore, it is not unexpected that rutin would contribute predominantly to DPPH radical scavenging activities. On the other hand, Holasova (*24*) demonstrated a higher significant relationship than this report between rutin content and antioxidant activity. This may be caused by differences of materials; they used not only leaves but also seeds, husks, and strews (*25*).

From our results, we can assert that tartary buckwheat leaves at 42 DAS are a promising potential food ingredient, particularly in Ao-Jiru juice, in terms of leaf mass, GABA, and rutin. Cultivar Hokkai T10 was deemed particularly suitable because it contained higher anthocyanin concentrations than others. The drying methods tested showed no significant effects on the concentration of functional compounds or DPPH radical scavenging activity. On the other hand, if one focused solely on 2HN and minor flavonoids such as orientin and vitexin, leaves at 14 DAS were more suitable. To better establish the potential role of buckwheat leaves in human health, feeding experiments on animals and humans are required.

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