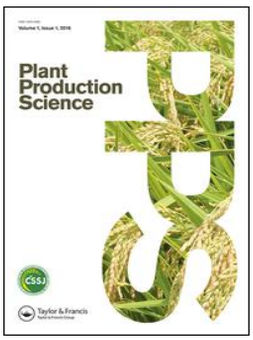

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Development of a High-Performance Liquid Chromatography Method to Determine the Fagopyrin Content of Tartary Buckwheat (*Fagopyrum tartaricum* Gaertn.) and Common Buckwheat (*F. esculentum* Moench)

Kentaro Eguchi[1], Tomohiro Anase[2] and Hideji Osuga[2]

([1]National Agricultural Research Center for Kyushu Okinawa Region, Koshi, Kumamoto 861-1192, Japan; [2]Faculty of Systems Engineering, Wakayama University, 930 Sakaedani, Wakayama 640-8510, Japan)

Abstract : Buckwheat contains fagopyrin, which induces photosensitization in light-skinned livestock when exposed to sunlight. Here, we developed a high-performance liquid chromatography (HPLC) method to measure the fagopyrin content of buckwheat. The HPLC profile of the fagopyrin extract purified from Tartary buckwheat 'Rotundatum' had 3 apparent peaks. The ultraviolet-visible (UV-vis) absorption spectrum of each peak yielded absorbance maxima (λ_{max}) at 547 nm and 591 nm, indicating that these peaks corresponded to fagopyrin and unidentified fagopyrin derivatives. We considered the total content reflected by the 3 peaks to be the fagopyrin content of buckwheat. We determined the fagopyrin content in the leaves of Tartary buckwheat 'Rotundatum' and common buckwheat 'Miyazakiootsubu' both by UV-vis photometric analysis and the newly developed HPLC method. The fagopyrin content is overestimated by UV-vis photometry because the extracts contain a considerable amount of chlorophyll. Thus, HPLC analysis is more efficacious for fagopyrin-content measurements than UV-vis photometric analysis. The HPLC analysis of fagopyrin is easy, quick and efficacious for screening buckwheat varieties with trace or no fagopyrin. There are only a few reports on the accumulation sites of fagopyrin in buckwheat. We revealed that in Tartary and common buckwheat, fagopyrin is present mainly in the leaves and flowers and slightly in the stems, hulls, and groats. The fagopyrin contents of the leaves and flowers of Rotundatum were approximately 2.6 and 2.8 times higher than those in Miyazakiootsubu, respectively.

Key words : Fagopyrin, Fagopyrism, High-performance liquid chromatography, Thin-layer chromatography.

Fagopyrin is a toxic polyphenol present in buckwheat (Fig. 1), and can induce photosensitization, also termed fagopyrism, in light-skinned livestock when exposed to sunlight (Johnson, 1983). However, there are only a few reports about the accumulation site of fagopyrin in buckwheat (Brockmann, 1957; Johnson, 1983; Joshi et al., 1991). Tartary buckwheat (*Fagopyrum tartaricum* Gaertn.) and common buckwheat (*F. esculentum* Moench) belong to the family *Polygonaceae* and have been recognized as healthful foods because they contain the antioxidant rutin (Morishita et al., 2007). However, buckwheat is considered to exert physiological effects of fagopyrism when consumed by livestock as forage.

The structure of fagopyrin is very similar to that of hypericin, which is present in St. John's wort (*Hypericum perforatum* L.). Fagopyrin contents were determined with a UV-vis spectrometer at 590 nm, i.e., the wavelength used for evaluating hypericin concentrations (Hinneburg and Neubert, 2005; Ožbolt et al., 2008). However, these extracts contain a considerable amount of chlorophyll; therefore, the fagopyrin content is overestimated by the UV-vis photometer. In this study, we attempted to develop a high-performance liquid chromatography (HPLC) screening method for determining the fagopyrin content of buckwheat. Further, we determined the accumulation site of fagopyrin in buckwheat and the fagopyrin content

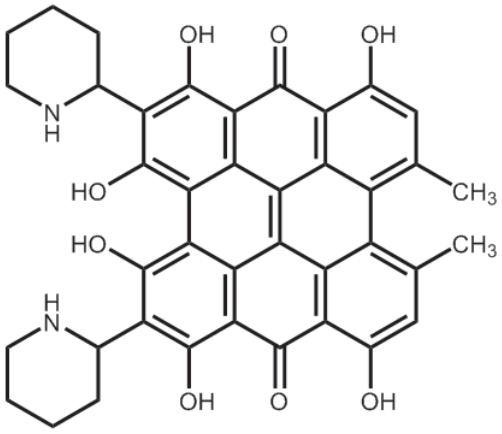

Fig. 1. Structure of fagopyrin.

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Abbreviations : DW, dry weight; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; TFA, trifluoroacteic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV-vis, ultraviolet-visible.

of Tartary buckwheat 'Rotundatum' and common buckwheat 'Miyazakiootsubu' by HPLC analysis.

Materials and Methods

1. Plant material

The cultivars Rotundatum of Tartary buckwheat and tetraploid common buckwheat Miyazakiootsubu (Nagatomo et al., 1982), were sown on 11 April, 2008, in a field at the National Agricultural Research Center of Kyushu Okinawa Region (Kumamoto, Japan). The size of the plots from which the seed samples were collected was 900 cm^2, and the seeding density was 125 plants per unit area (m^2). We collected 3 sets of samples each of leaves, stems, and flowers at flowering time, and those of hull and groats at maturation time.

2. Fagopyrin purification

Fagopyrin was purified according to the method of Samel et al. (1996) with some modifications. We sampled the leaves, stems, and flowers (40 g) of Rotundatum at flowering time. The fresh samples were immediately frozen in liquid nitrogen and then freeze dried in a freeze dryer (FDU-1200; Eyela, Japan). The dried samples were crushed using a food mill. The crushed material was packed in a glass beaker and macerated in 1 L of dichloromethane for 2 d. The mixture was then filtered through a filter paper (No. 2; Advantec Co. Ltd., Japan), and the residue was stirred 6 times in 0.5 L of dichloromethane until the dichloromethane filtrate turned pale yellow. The residue was then macerated in a 1-L mixture of acetone/acetic acid/water (80:10:10) for 2 d. A reddish-brown extract was obtained, which was filtered using filter paper No. 2 (Advantec Co. Ltd.) and 0.5 L of the abovementioned solvent mixture. The reddish-brown filtrate was collected, and the solvent was evaporated under reduced pressure. The solution obtained was concentrated to 100 mL. Next, the extract (5 mL) was examined using thin-layer chromatography (TLC) on silica gel (pore size, 150 Å; Whatman, Japan) according to the method of Samel et al. (1996), wherein a 50:40:10:20 solution of toluene/ethyl formate/formic acid/pyridine was used; the spots visualized under fluorescent lamps. The one red band (Rf=0.65) on the TLC plate was collected and dissolved in 5 mL of methanol and centrifuged at 10,700×*g* for 10 min at 4ºC. HPLC vials were exposed to a light for 24 h at 4ºC to convert protofagopyrin to fagopyrin. Thus, we obtained a solution containing a red pigment.

3. Identification of fagopyrin

The ultraviolet-visible (UV-vis) spectra of the red solution were recorded on a UV-vis spectrometer (V-560; Jasco, Japan). The red solution (2 μL) was monitored using TLC on Kieselgel 60 F_{254} (Merck Ltd., Japan) according to the method of Samel et al. (1996), wherein a 50:40:10:20 solution of toluene/ethyl formate/formic acid/pyridine was used; the spots visualized under UV light at 302 nm. Negative-ion electrospray ionization mass spectrometry (ESI-MS) analyses were performed using an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, USA). The samples were introduced by direct infusion into a solution of methanol at a flow rate of 5 μL min^{-1}. The capillary temperature was set at 220ºC, and the spray voltage, at 5.0 kV. The sheath gas flow rate was set at 30 arbitrary units.

4. TLC and HPLC analyses of fagopyrin

We extracted fagopyrin from 100-mg freeze-dried leaves, stems, flowers, hulls, and groats of Rotundatum and Miyazakiootsubu by treatment with 1.25 mL of methanol for 2 d at 25ºC. The samples were centrifuged at 10,700×*g* for 10 min at 25ºC. The concentrations of the supernatants obtained from all samples were 80 mg mL^{-1}.

These solutions (2 μL) were analyzed using TLC on Kieselgel 60 F_{254} (Merck Ltd.) according to the method of Samel et al. (1996), wherein a 50:40:10:20 solution of toluene/ethyl formate/formic acid/pyridine was used; the spots visualized under UV light at 302 nm. For HPLC analysis, the obtained solution (20 μL) was injected into an HPLC system (LaChrom Elite; Hitachi, Japan). Subsequently, HPLC was performed using an Inertsil ODS-SP column (5 μm, 150×4.6 mm id; GL Sciences Inc., Japan) at 25ºC and a flow rate of 1 mL min^{-1} with water/methanol/trifluoroacetic acid (TFA)/tetrahydrofuran (THF) (3:40:1:1). Absorbance was monitored at 590 nm.

5. UV-vis photometric analysis of fagopyrin

The fagopyrin content was determined by the method described by Ožbolt et al. (2008). In brief, 60 mg of freeze-dried leaves, stems, flowers, hulls, and groats of Rotundatum and Miyazakiootsubu in 3 replicates each were extracted using 4 mL of 80% tetrahydrofuran in water at 65ºC for 30 min. The samples were centrifuged at 2,000×*g* for 10 min at 25ºC, and the supernatant was transferred into a glass tube. The sediment was extracted again and then centrifuged. After centrifugation, the supernatant obtained was added to the supernatant collected after the first extraction. The combined extract (0.5 mL) was transferred to a glass tube and evaporated under vacuum. The sediment was dissolved in 1 mL of methanol in an ultrasonic bath and centrifuged at 10,700×*g* for 10 min. The concentrations of the supernatants obtained from all samples were 3.75 mg mL^{-1}.The supernatant (0.5 mL) was transferred to a spectrophotometer cell, and the absorbance was determined at 590 nm (V-560; Jasco). Fagopyrin contents were measured to evaluate hypericin concentrations (Wako, Japan), which ranged from 1.72 μg mL^{-1} to 27.5 μg mL^{-1}–the range over which linearity was indicated by a correlation coefficient of 0.999.

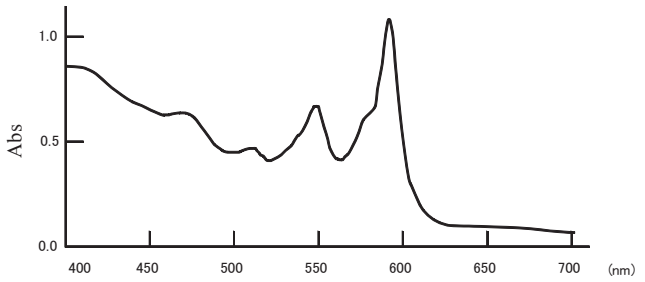

Fig. 2. UV-vis absorption spectra of the red pigment in methanol purified from the extract of Tartary buckwheat 'Rotandatum'.

Results and Discussion

1. Identification of fagopyrin

The TLC analysis of the solution containing the red pigment of Rotundatum yielded a major spot with an Rf value of 0.43 and a minor spot with an Rf value of 0.40 (figure not shown). Samel et al. (1996) obtained 3 distinct spots with Rf values of 0.38, 0.43, and 0.46 by TLC analysis of fagopyrin extracts purified from buckwheat flower. Our result is almost completely consistent with their result.

In the UV-vis absorption spectrum of the red pigment purified with TLC, absorbance maxima (λ_{max}) were observed at 547 and 591 nm. Samel et al. (1996) reported λ_{max} at 546 and 590 nm in the UV-vis absorption spectrum of fagopyrin. Hinneburg et al. (2005) detected λ_{max} at 546.6 and 590.4 nm. Our result is almost completely consistent with these results (Fig. 2).

The negative-ion spectrum of the red pigment purified by TLC showed strong ionization at *m/z* 670.7, which corresponded with the formation of the parent negative ion of fagopyrin (Fig. 3).

These results suggest that the red solution purified from Rotundatum extract contained fagopyrin.

2. Development of an HPLC method for determining the fagopyrin content

There are no reports on the qualitative analysis of fagopyrin by HPLC. Fagopyrin is a derivative of hypericin, which is present in St. John's wort (*H. perforatum* L). We attempted to develop a method for determining the fagopyrin content on the basis of the HPLC method for the detection of hypericin. The HPLC profile of the fagopyrin purified using TLC from Rotundatum extract showed 3 apparent peaks (Fig. 4). The retention times of peaks 1, 2, and 3 were 8.2, 9.5, and 11.4 min, respectively. The UV-vis absorption spectrum of each peak yielded λ_{max} at 547 and 591 nm; this indicated that these peaks corresponded to fagopyrin and unidentified fagopyrin derivatives (Fig. 5). Protofagopyrin is converted to fagopyrin on exposure to light (Brockmann, 1957). Alali et al. (2004) have reported that protohypericin in hypericin-containing HPLC vials is converted to

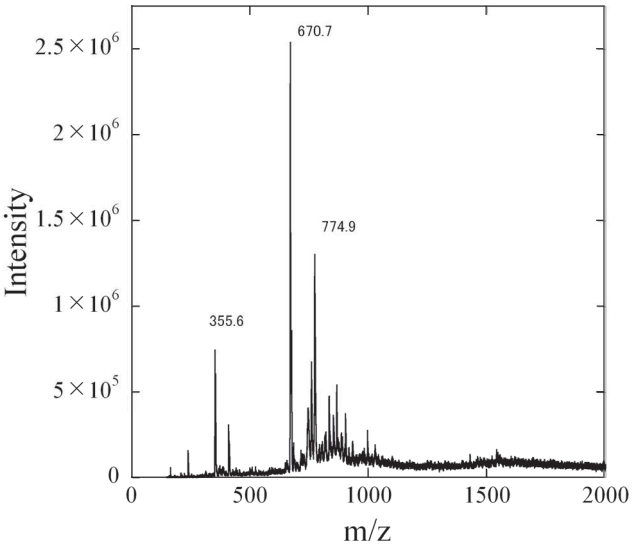

Fig. 3. ESI-MS spectra of the red pigment purified from the extract of Tartary buckwheat 'Rotandatum'.

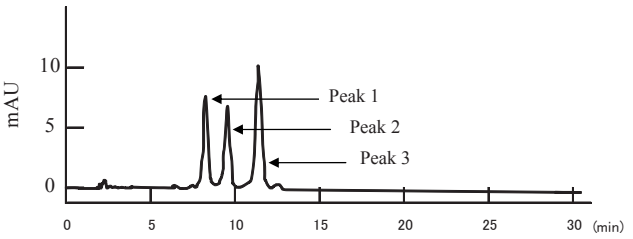

Fig. 4. HPLC profile of fagopyrin and unidentified fagopyrin derivatives purified from the extract of leaves of Tartary buckwheat 'Rotandatum'. Peak 1: 8.2 min, peak 2: 9.5 min, peak 3: 11.4 min.

hypericin on exposure to light immediately before HPLC analysis. We exposed the HPLC vials to a light for 24 h at 4°C to convert protofagopyrin to fagopyrin in our experiment. However, a chromatogram of the purified fagopyrin at 590 nm showed 3 peaks rather than a single peak that could be considered to correspond to fagopyrin. Besides hypericin, St. John's wort contains protopseudohypericin, pseudohypericin, and protohypericin (Xenophontos, 2007). Similarly, it is possible that buckwheat contains unidentified fagopyrin derivatives besides fagopyrin and protofagopyrin. Further research is required to determine the peak that corresponds to fagopyrin and the structures of the unidentified fagopyrin derivatives. In this study, we considered the total content reflected by peaks 1–3 as the fagopyrin content (fagopyrin and unidentified fagopyrin derivatives) of buckwheat. Hinneburg and Neubert (2005) and Ožbolt et al. (2008) determined the fagopyrin content relative to the standard hypericin content, whose structure and UV spectrum are very similar to those of fagopyrin. Thus, we measured the

content reflected by each peak (1, 2, and 3) to evaluate the fagopyrin concentration. The retention time of hypericin dissolved with methanol was 25.0 min. The calibration curve was constructed by plotting the peak area against the concentrations of hypericin which ranged from 1.72 μg mL^{-1} to 27.5 μg mL^{-1}–the range over which linearity was indicated by a correlation coefficient of 0.999.

3. Difference in the fagopyrin content of Tartary and common buckwheat

We measured the fagopyrin content of the leaves, stems, flowers, hulls, and groats of Rotundatum and Miyazakiootsubu by our developed HPLC analysis and UV-vis photometry according to the method of Ožbolt et al. (2008) (Table 1). The correlation coefficient in the relationship between fagopyrin contents UV-vis and those of HPLC analysis was 0.97 (n=24). However, the fagopyrin contents of the leaves and flowers of Rotundatum estimated by UV-vis photometric analysis were 1.8 and 2.6-times higher, respectively, than those estimated by our newly developed HPLC analysis (Table 1). The fagopyrin contents of the leaves and flowers of Miyazakiootsubu as estimated by UV-vis photometric analysis were 3.1 and 2.6-times higher than those in our developed HPLC analysis (Table 1). Fig. 6 shows the UV-vis absorption spectrum of the Rotundatum leaf extract. These extracts contain a considerable amount of chlorophyll and second metabolites other than fagopyrin. Therefore, the fagopyrin content is overestimated by UV-vis photometry at 590nm. Chlorophyll a and b (Wako, Japan) were dissolved in methanol (0.4 mg mL^{-1}) and the authentic

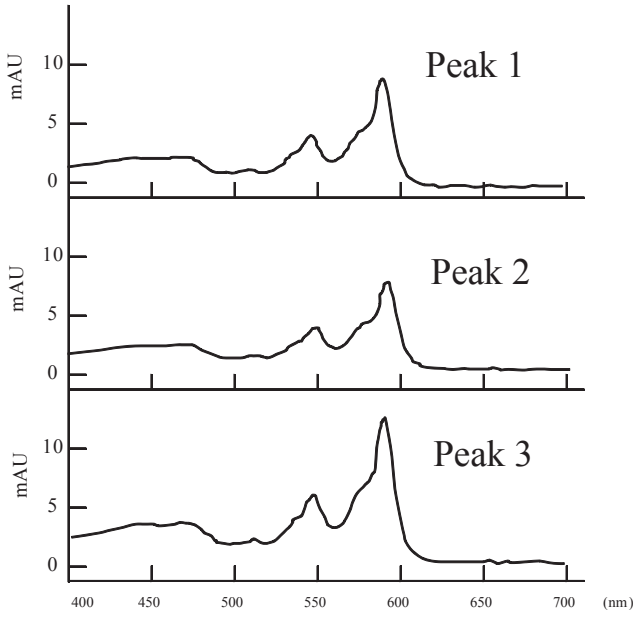

Fig. 5. UV-vis absorption spectra of the peaks 1, 2, and 3 of fig. 4.

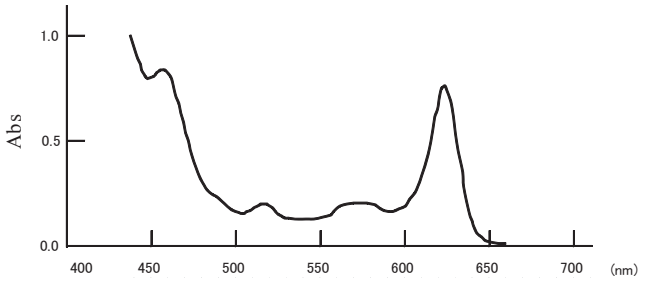

Fig. 6. UV-vis absorption spectra of the leaf extracts of Tartary buckwheat 'Rotandatum'.

Table 1. The content reflected by each peak content was measured to evaluate the hypericin concentration. Each value represents the mean±SD (n=3). N.D., not detected.

Variety	Position	Fagopyrin and unidentified fagopyrin derivative (HPLC)			Fagopyrin (HPLC)	Fagopyrin (UV-vis)
		Peak1 (mg g^{-1})	Peak2 (mg g^{-1})	Peak3 (mg g^{-1})	Total Peak (mg g^{-1})	(mg g^{-1})
Tatary buckwheat 'Rotandatum'	Leaves	0.33 ± 0.00	0.35 ± 0.01	0.37 ± 0.02	1.06 ± 0.03	1.89 ± 0.37
	Stems	0.07 ± 0.03	0.02 ± 0.00	0.02 ± 0.00	0.11 ± 0.03	0.09 ± 0.08
	Flowers	0.88 ± 0.01	0.68 ± 0.02	0.28 ± 0.00	1.84 ± 0.03	4.73 ± 0.33
	Hulls	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.05	0.04 ± 0.05	0.08 ± 0.08
	Groats	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	N.D.
Common buckwheat 'Miyazakiootsubu'	Leaves	0.08 ± 0.01	0.14 ± 0.00	0.17 ± 0.00	0.39 ± 0.00	1.19 ± 0.21
	Stems	0.04 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.03	N.D.
	Flowers	0.31 ± 0.03	0.15 ± 0.01	0.18 ± 0.01	0.64 ± 0.06	1.67 ± 0.19
	Hulls	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.00	0.02 ± 0.03	N.D.
	Groats	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	N.D.	N.D.

The content reflected by each peak content was measured to evaluate the hypericin concentration. Each value represents the mean ± SD (n = 3). N.D., not detected.

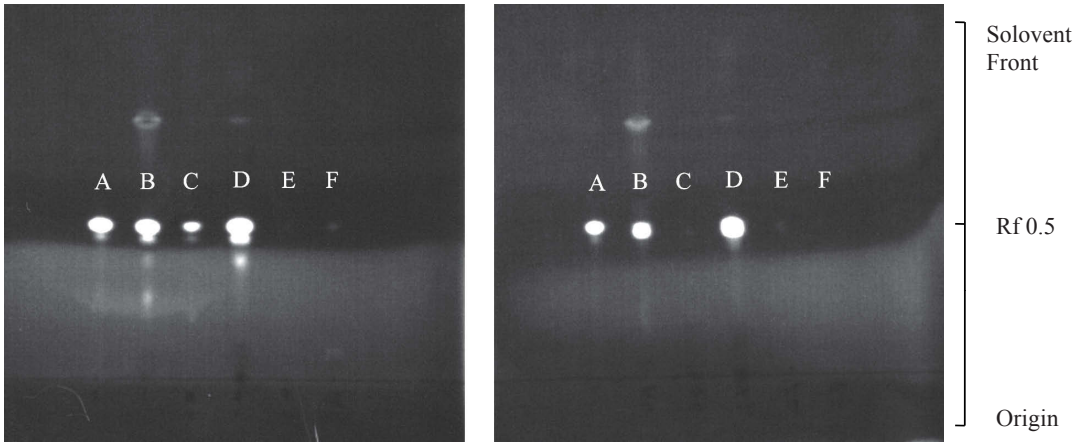

Fig. 7. Fagopyrin detected at different sites in Tartary and common buckwheat. (A) Fagopyrin purified from the extracts from (B) leaves, (C) stems, (D) flowers, (E) hulls, and (F) groats.

samples were analysed by the abovementioned HPLC analysis. Our developed HPLC analysis did not detect chlorophyll a and b (data not shown). Thus, we conclude that this new HPLC analysis method is more efficacious for the measurement of fagopyrin content than UV-vis photometric analysis.

4. Accumulation site of fagopyrin in buckwheat

The size and intensity of the red spots corresponding to fagopyrin changed at certain sites on the TLC plates varied with the cultivar. In Rotundatum, the major red spots were detected in the extracts from the leaves and flowers and the minor red spots in those from the stems, while no spots were detected in the case of the hulls and groats. For Miyazakiootsubu, the major red spots were detected in the case of the leaves and flowers and no spots were detected in the case of the stems, hulls, and groats (Fig. 7).

In this study, HPLC analysis revealed that Rotundatum and Miyazakiootsubu contain a large amount of fagopyrins in their leaves (1.06 and 0.39 mg g^{-1} DW, respectively) and flowers (1.84 and 0.64 mg g^{-1} DW, respectively) (Table 1). Further, small amounts of fagopyrin were found in the stems (0.11 and 0.04 mg g^{-1} DW, respectively), hulls (0.04 and 0.02 mg g^{-1} DW, respectively), and groats (0.01 mg g^{-1} and not detected, respectively) (Table 1). The fagopyrin contents of the leaves and flowers of Rotundatum were approximately 2.7 and 2.9 times higher than those of Miyazakiootsubu, respectively (Table 1).

Brockmann (1957) reported that fagopyrin is present only in small quantities in buckwheat plants and almost exclusively in the flowers. Johnson (1983) reported that the seeds contain very little fagopyrin, and most of it is distributed in the whole plant, especially in the leaves and stems. Joshi et al. (1991) reported that fagopyrin is present only in the flowers and hull and not in the leaves, stems and groats. Our results differed from those of these studies. We revealed that in Rotundatum and Miyazakiootsubu, fagopyrin was mainly present in the leaves and flowers, and to a small extent, in the stems, hulls, and groats by TLC and a newly developed HPLC method. Further, we found that the fagopyrin content of Rotundatum is higher than that of Miyazakiootsubu.

Fagopyrin can induce serious photosensitization problems in livestock that feed on buckwheat. Phototoxicity is an inflammatory reaction that reflects cellular damage caused by a photochemical reaction between a photosensitizer and appropriate radiation incident on the skin. Thus far, buckwheat has not been used as a feed because of the concern of fagopyrism; however, we have revealed that the seeds of Rotundatum and Miyazakiootsubu contained negligible amounts of fagopyrins. Because these seeds contain rutin, they may be efficacious as concentrated feeds with strong antioxidant activity. On the other hand, the leaves and flowers of buckwheat contain fagopyrins at high concentrations, and therefore, buckwheat cannot be used as whole-crop forage as previously reported (Johnson, 1983). Because fagopyrins are more concentrated in the leaves and flowers of Rotundatum than in those of Miyazakiootsubu, the former may cause more severe fagopyrism than the latter when used as whole-crop forage.

In this study, we first developed an HPLC method for measuring the fagopyrin content of buckwheat. This analysis method is easy, quick, and efficacious for screening buckwheat varieties with trace or no fagopyrin content.

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