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Changes in phenols contents from buckwheat sprouts during growth stage

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Abstract

Germinated buckwheat is buckwheat seeds soaked in water just until it begins to bud. Buckwheat sprouts are seedling plants of buckwheat grown up to 10-15 cm. The purpose of this study was to determine the optimal growth period for accumulating the most abundant functional phenol(s) in germinated buckwheat that had been soaked in darkness and buckwheat sprouts cultivated by hydroponic culture. The rutin contained in germinated buckwheat was analyzed by CE (capillary electrophoresis). Phenols, including isoorientin, orientin, isovitexin, vitexin, and rutin were separated from buckwheat sprouts by HPLC and identified by LC-MS. The highest rutin content in germinated buckwheat was found to be 15.8 mg/100 g DW at 20 hours after germination. Buckwheat sprouts contained five kinds of major phenols. The highest amounts of isoorientin, orientin, isovitexin, and vitexin were measured at day 3, with the exception of rutin, and then a gradual decrease was observed as the sprouts grew. The quantities of isoorientin, orientin, isovitexin, and vitexin at day 3 were 5.8, 11.7, 26.2, and 28.9 mg/100 g FW, respectively. The rutin content rapidly increased to 109.0 mg/100 g FW until day 6. The highest total phenols in buckwheat sprouts were 162.9 mg/100 g FW at day 6. Germinated buckwheat soaked for 20 hours and buckwheat sprouts cultivated for 6 days were rich in dietary phenol(s), which makes these plants a valuable functional food for human consumption.

Introduction

Buckwheat (*Fagopyrum esculentum*), which belongs to the family Polygonaceae, genus *Fagopyrum*, has been a commonly-eaten food in arid and cold regions in the world. For centuries, it has been consumed as groats, flour and noodles, although in modern times, the people of Japan, Italy, and China eat buckwheat mainly in the form of noodles. Buckwheat seeds are richer in protein compared with rice and wheat. Buckwheat protein improves health in various ways, notably reducing serum cholesterol (Kayashita et al. 1995), suppressing gallstones and tumors (Tomotake et al. 2000; Liu et al. 2001), and inhibiting the angiotensin I-converting enzyme (Ma et al. 2006). Moreover, buckwheat is the only cereal that contains rutin, hence it is a beneficial source of this flavonoid (Holasova et al. 2002). Other phenolic compounds and flavones such as hyperin, quercitrin, and quercetin have been isolated from immature buckwheat seeds (Sato and Sakamura 1975).

Sprouts are seedling plants before the unfolding of the true leaves, and are categorized as a vegetable type or a cereal type according to the growth period. Cereal-type sprouts are prepared by soaking the corresponding seeds in water for 12-24 hours. Vegetable-type sprouts are cultivated in the dark until germination, and then under the sun to elongate the stem and leaves. Sprouts are increasing in popularity, and becoming easier to find in grocery stores. Consumers can eat them raw in a salad, or as boiled or steamed vegetables. Germinated brown rice is classified as a cereal-type sprout, and soybean

sprouts, mung bean sprouts, radish sprouts, broccoli sprouts, and buckwheat sprouts are classified as vegetable-type sprouts. Germinated buckwheat has been recently developed as a cereal-type sprout which is commonly prepared in Japan (Tanaka 1997). Buckwheat sprouts are 10-15 cm in length with a pink thin stem and green seed leaf, and are cultivated in Japan, Korea, and China. Large-scale production of sprouts, including buckwheat sprouts, is usually accomplished by hydroponic culture.

Scientists and nutritionists are interested in making use of functional vegetables to increase the health benefits they provide to the body. Cultivation and breeding technology to improve the functional ingredients of vegetables has increased in importance (Amimoto et al. 1996; Dimitrijević-Branković et al. 2002). To prepare cereal-type sprouts, germination time is generally less than 24 hours, as this will achieve a softer texture by cell-wall degradation and improve the taste due to an increase in sugar. It is expected that in the future, cereal-type sprouts will undergo more improvements to their functional constituents than cereals. In germinated barley, it has been reported that the antioxidant activity is higher than that of non-germinated barley (Sharma and Gujral 2010). In germinated brown rice, it has been reported that there is 10 times more GABA (γ-aminobutyric acid), which has an antihypertensive effect, as compared to non-germinated brown rice (Tian et al. 2004). Vegetable-type sprouts usually contain quantities of beneficial vitamins and minerals that are found in higher abundance when compared to mature vegetables or dormant seeds. In soybean sprouts, it has been reported that the content of phenolic

compounds and isoflavones was higher than that of soybeans (Cho et al. 2009; Kim et al. 2006). In lentil sprouts, it has been reported that the vitamin C and total phenols are three times higher than in the seeds (Fernandez-Orozco et al. 2006; Duenas et al. 2009).

In this paper, we measured the beneficial functional components in germinated buckwheat and buckwheat sprouts. We determined the rutin content during germination of buckwheat seeds, and tracked changes over time by CE (capillary electrophoresis) analysis. We identified the functional phenols in buckwheat sprouts during the growth period by HPLC (high performance liquid chromatography) and LC-MS (liquid chromatography-mass spectrometry) analysis. Through the two experiments, we determined the optimal growth period during which the most abundant functional phenol(s) in germinated buckwheat and buckwheat sprouts accumulated.

Materials and Methods

Chemicals: All regents used for HPLC and LC-MS were of HPLC grade. Acetone, formic acid, acetonitrile, and methanol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). SDS (sodium dodecyl sulfate) and boric acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). The phenolic acid standards of rutin, isoorientin, orientin, isovitexin, and vitexin were purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Preparation of germinated buckwheat and analytical sample preparation: Buckwheat (*Fagopyrum esculentum*) seeds were provided from SALADCOSMO CO., Ltd. (Gifu, Japan). The buckwheat seeds were surface-sterilized with ozone, and were then immersed in water at 32-33°C for 20 minutes. Treated seeds were germinated on water-soaked urethane foam at 23°C for 0, 4, 8, 12, 16, and 20 hours in the dark. The germinated seeds were frozen in liquid nitrogen, and lyophilized with a freeze-dryer (EYELA FDU-2000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). For each germination time, the dry samples of the germinated buckwheat were ground in an electric mill (300 cc 100 V 900 w MK, Rong Tsong Iron, Taichung, Taiwan) and then extracted with 50% aqueous acetone at room temperature for 2 hours under agitation. The solids were removed by suction filtration, and the filtrates were evaporated under reduced pressure to remove the extraction solvents. The dried residues were used for CE analysis.

Cultivation of buckwheat sprouts and analytical sample preparation: The surface-sterilized buckwheat seeds were immersed in water at 32-33°C for 5.5 hours. After planting on water-soaked urethane foam for 24 hours in the dark at 23°C, the seeds were treated with culture fluid under the appropriate light conditions using a high pressure sodium lamp for about 10 days. Culture fluid was sprayed on the seeds for one minute every 4 hours, and fluid temperature and pH were maintained at 18-20°C and 5.5-6.0, respectively. All parts except for the inedible hull and root were collected from the freshly harvested buckwheat sprouts, and then were lyophilized. The dry samples were ground in the

electric mill and extracted with 50% aqueous methanol at room temperature for 2 hours under agitation. The extracts were centrifuged at 10,000 rpm for 15 minutes and the supernatants were collected. The residual precipitate was extracted again in the same manner for all samples, and the supernatant was combined with the first one that was collected. After evaporation, the resulting residues were dried under reduced pressure and used for HPLC and LC-MS analyses.

CE analysis of rutin: The CE analysis was performed at the CREFAS (Collaborated Research Center for Food Functions, Faculty of Agriculture, Shinshu University). Rutin was analyzed as previously described by Kreft et al. (1999). The rutin standard was dissolved at 0.01 mg/mL in a boric acid buffer (pH 9.0), and the extracts from the germinated buckwheat were dissolved at 2 mg/mL in the buffer. CE analysis was performed through a 75 μm i.d. fused silica column at 25°C with the following electrophoretic solvent: 50 mM boric acid buffer containing 0.1 M SDS (pH 9.0) on a P/ACE™ MDQ capillary electrophoresis analysis system (Beckman Coulter Inc., California, USA). Pressure injection (20 psi × 10 sec) was used to inject the samples. The applied voltage was 25 kV, and compounds in the sample were detected with UV monitoring at 280 nm. Rutin in the sample was identified by relative retention time compared to a standard sample, and mass analysis from the peak. Quantitation was performed by comparing the peak area obtained from the sample with that of the standard.

HPLC analysis: HPLC analysis was also carried out at the CREFAS. The phenolic compounds in

the buckwheat sprouts were identified using a Prominence HPLC system with a LC-Solution workstation (Shimadzu, Co., Kyoto, Japan). Separations were performed using a CHEMCOBOND 5-ODS-W reversed phase column (150 mm × 4.6 mm i.d., Chemco Scientific Co., Ltd., Osaka, Japan). Gradient elution was performed using a mobile phase of acetonitrile with 0.1% formic acid (Solvent B) and 0.1% formic acid in purified water (Solvent A): 0-10 min, 0-5% Solvent B; 10-15 min, 5% Solvent B; 15-20 min, 5-10% Solvent B; 20-40 min, 10-20% Solvent B; and 40-50 min, 20-100% Solvent B. HPLC analysis was performed at 40°C with a flow rate of 0.8 mL/min, and an injection volume of 10 μL. The spectra from all peaks were recorded in the 200-800 nm range, and the chromatograms were acquired at 280 nm. Quantitation was performed by comparing the peak areas obtained from the samples with those of standards.

LC-MS analysis: LC-MS analysis was performed with a Waters 2695 HPLC system (LC) and a Quattro Micro™ API (MS) system (Waters, Milford, MA, USA). The LC conditions were the same as described for the analysis of the phenolic compounds. All mass spectra were acquired in ESI (electrospray ionization) mode using 3500 V capillary voltage, 30 V cone voltage, desolvation gas (N_2) flow of 350 L/h, cone gas (N_2) flow of 50 L/h, source temperature of 100°C, and desolvation temperature of 350°C. Analyses were carried out in positive scan modes from *m/z* 100-1000.

Identification of phenols: Rutin, isoorientin, orientin, isovitexin, and vitexin were identified by

comparing relative retention time and ESI-MS (electrospray ionization-mass spectrometry) spectra with standard compounds.

Statistics: Chromatographic analyses were carried out in triplicate. Data were expressed as the mean ±SD (standard deviation). The quantified values for the rutin in germinated buckwheat were compared with those obtained for non-germinated seeds by a *t* test. The quantified values for the flavonoids in buckwheat sprouts were compared with those values obtained on day 3 after germination by a *t* test. The results were considered to be significant at $P < 0.05$ (*).

Results and discussion

Change in the rutin content of the germinated buckwheat: The rutin content during germination was determined using CE analysis. CE is used to separate compounds by their charge and frictional forces. After voltage is applied, electroosmotic flow of the electrolyte solution occurs in the capillary. In this flow, positively charged compounds are pulled toward the cathode at increasing speed, and negatively charged ones are pulled toward the anode. The bigger the charge on the compound, the more it is affected by these electrical migrations. In addition, every compound interacts with the inner surface of the capillary. By means of these factors, the compounds in the analytical sample are able to be separated in fines by CE. After being identified by a PDA (photodiode array) detector, the separated compounds are displayed as an

electropherogram on the computer. The CE electropherogram of germinated buckwheat that was soaked for 8 hours is shown in Figure 1. The electropherogram pattern of germinated buckwheat corresponded to the rutin standard, with the peak area at 4.2 min; the peak area also increased when mixed injection of the standard occurred. The peak was confirmed by MS analysis.

The change in the rutin content over time of germinated buckwheat extracts and dry powdered germinated buckwheat is shown in Figure 2. The rutin content in the extracts significantly increased to 3.1 mg/g of extracts at 20 hours after germination, which was approximately 1.5 times greater than the rutin content in non-germinated buckwheat seeds (Figure 2A). The rutin content of dry powdered germinated buckwheat also significantly increased with the passage of time, similar to the extracts. The rutin content reached 15.8 mg/100 g DW at 20 hours, and increase from 9.4 mg/100 g DW at pre-germination, approximately 1.5 times greater than the rutin content in non-germinated seeds (Figure 2B). Germinated buckwheat is thus a rich dietary source of rutin in comparison to non-germinated buckwheat seeds.

Soaking increased the amount of rutin in buckwheat seeds. It has been previously reported that rutin is contained in not only the buckwheat kernel, but also the hulls (Watanabe et al. 1997). Therefore, it is thought that the rutin in buckwheat hulls is transferred to the kernel by the soaking treatment. This simple method enabled us to significantly increase the rutin content in germinated buckwheat. Rutin, which possesses anti-inflammatory, vasoactive, antitumor, antibacterial, antiviral and antiprotozoal properties,

has been widely used in treating disease (Calabro et al. 2005). In addition, rutin can ameliorate increased capillary fragility associated with some hemorrhagic diseases or hypertension (Yildizogle-Ari et al. 1991), has a antioxidant effect (Afanas et al. 1989; Lindahl and Tagesson 1997), and is also antispasmodic and anticarcinogenic (Webster et al. 1996).

Phenol content changes in buckwheat sprouts: Buckwheat sprouts used as food are grown for approximately 10 days from germination, shown as "Day 9" in Figure 3. In the germination process, the stem elongates approximately 2 cm in 3 days, and the cotyledon unfolds in 6-7 days, with total growth of 10-15 cm that is achieved approximately 9-10 days after germination. Figure 4 shows the HPLC chromatogram of buckwheat sprout extracts and the UV (ultra violet) spectra of the detected peaks. Five main peaks were detected with retention times of 38.2, 38.7, 40.5, 41.6, and 42.7 minutes, respectively. The UV spectra of peaks 1-5 exhibited characteristics of flavonoids, with bands at 250-280 and 330-350 nm (Jurd 1962). Figure 5 shows the mass spectra of peaks 1-5. LC-MS analyses for the main peaks identified peaks 1 and 2 as isoorientin or orientin at *m/z* 449.0 $[M + H]^+$ (theoretical value: 449.38), peaks 3 and 4 as isovitexin or vitexin at *m/z* 433.0 $[M + H]^+$ (theoretical value: 433.38), and peak 5 as rutin at *m/z* 611.1 $[M + H]^+$ (theoretical value: 611.53). After a mixed injection of standard samples and the buckwheat sample by HPLC, peaks 1-5 were confirmed as isoorientin, orientin, isovitexin, vitexin, and rutin, respectively. The changes in the phenolic content of the extracts are shown in Figures 6A and 6B.

Of these phenols – isoorientin, orientin, isovitexin, vitexin, and rutin – all except rutin have not been previously detected in buckwheat seeds before germination. There were similar levels of isoorientin, orientin, isovitexin, and vitexin in the extracts during the growth stage; the highest amount of these combined phenols was 9.9 mg/g of extracts at day 3 (isoorientin: 0.8 mg/g, orientin: 1.6 mg/g, isovitexin: 3.6 mg/g, and vitexin: 3.9 mg/g of extracts). There were relatively lower levels of isoorientin and orientin as compared to isovitexin and vitexin. Though the rutin content was at the same level as that of the other phenols on day 3, the content rapidly increased after day 3. At day 10, it reached 15.3 mg/g of extracts, which was approximately 5 times more abundant than the amount of rutin at day 3. The changes in the phenolic content of the fresh buckwheat sprouts are shown in Figures 6C and 6D. The changes in phenols in fresh buckwheat sprouts did not correspond to the change that was observed in the extracts. It was estimated that the phenol content of the fresh sprouts decreased relative to the increase in water content. All phenolic compounds, except for rutin, gradually decreased as the sprouts grew from day 6. The amounts of isoorientin, orientin, isovitexin, and vitexin were 5.8, 11.7, 26.2, and 28.9 mg/100 g FW at day 3, and 2.0, 4.2, 8.0, and 8.3 mg/100 g FW at day 10, respectively. The rutin content, which was the same level as the other phenols at day 3 (23.4 mg/100 g FW), rapidly increased to a maximum content of 109.0 mg/100 g FW at day 6. Then the content decreased with plant growth to 43.7 mg/100 g FW at 10 days, which was less than half of that at day 6. The highest total phenols measured were 162.9 mg in fresh

buckwheat sprouts at day 6. Based on our analytical results, buckwheat sprouts cultivated for 6 days from germination possessed the most abundant functional phenolic ingredients. The amounts of rutin, isoorientin, orientin, isovitexin, and vitexin were 109.0, 4.7, 9.6, 19.7, and 20.0 mg/100 g FW at day 6, respectively.

We measured the changes in the phenol content of buckwheat sprouts cultivated hydroponically. The phenol content in buckwheat sprouts decreased with growth from day 6, and the highest total phenol was confirmed at day 6 (Figures 6C, 6D). In other study where buckwheat sprouts were grown by open field cultivation, the content of C-glycosylflavones such as orientin, isoorientin, vitexin, and isovitexin decreased as growth proceeded after germination. In addition, the rutin content increased as the buckwheat grew, with maximum levels measured at 23 days after germination (Watanabe and Ito 2002). These results suggest that we can harvest buckwheat sprouts containing high levels of phenols in a relatively short amount of time with our cultivation method as compared to the open field cultivation. It has been reported that orientin has a vasodepressor effect and antioxidant activity, isoorientin has avasorelaxant effect, and vitexin has antibacterial action (Fu et al. 2005; Budzianowski et al. 1991; Afifi et al. 1999; Afifi et al. 1997). These phenolic ingredients can afford protection and recuperation for those who have lifestyle-related diseases (Iwai et al. 2006; Ojewole 2006).

Conclusion

If buckwheat is to be utilized as a functional food, it is important that it has the maximum amount of beneficial ingredients. It was found by CE analysis that germinated buckwheat soaked for 20 hours contained 1.5 times more rutin than the amount measured before germination. The flour of germinated buckwheat has a higher rutin content, making it an attractive alternative to traditional buckwheat flour. These results indicate that germinated buckwheat soaked for 20 hours is best for use as a cereal or powdered food because it has the highest rutin level.

It was revealed by HPLC and LC-MS analysis that, in addition to rutin, the buckwheat sprouts contained phenols that were produced in the germination process – isoorientin, orientin, isovitexin, and vitexin. These phenols are found in abundance in fresh buckwheat sprouts that have been cultivated for 6 days, and have various positive effects on human health. Buckwheat sprouts cultivated by our method for 6 days are thus good dietary source of phenols. Moreover, hydroponic culture enables the growth of a year-round supply of buckwheat sprouts of consistent quality for consumption. Our CE, HPLC, and LC-MS analysis showed that the germinated buckwheat soaked for 20 hours and the buckwheat sprouts cultivated for 6 days are ideal foods that can help maintain our health on a daily basis.

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Figure Legends

Fig. 1 A typical CE (capillary electrophoresis) electropherogram of germinated buckwheat soaked for 8 hours. The arrow denotes the rutin peak. The detection wavelength was 280 nm.

Fig. 2 Changes in rutin content in (A) germinated buckwheat extracts and in (B) dry powdered germinated buckwheat during germination. Data are the mean ± S.D. (n=3)*; $P < 0.05$; a comparison was made of the quantitation results for each germination time with those obtained before germination by a *t* test.

Fig. 3 Buckwheat sprouts during the growth period.

Fig. 4 HPLC (high performance liquid chromatography) chromatogram of buckwheat sprout extracts and a UV (ultraviolet) spectrum of detected peaks. Peaks **1** and **2** represent isoorientin or orientin; **3** and **4**, isovitexin or isovitexin; **5**, rutin. Detection wavelength was 280 nm.

Fig. 5 MS (mass spectrometry) spectra in positive scan mode for peak 1 (a), peak 2 (b), peak 3 (c), peak 4 (d), and peak 5 (e).

Fig. 6 The changes in phenol content in (A, B) buckwheat sprout extracts and (C, D) fresh buckwheat sprouts during germination. (A, C) Isoorientin, orientin, isovitexin, and vitexin. (B, D) Rutin. Data are the mean ± S.D. (n=3)*; $P < 0.05$; a comparison was made of the quantitation results for each growth period with those obtained on day 3 by a *t* test.

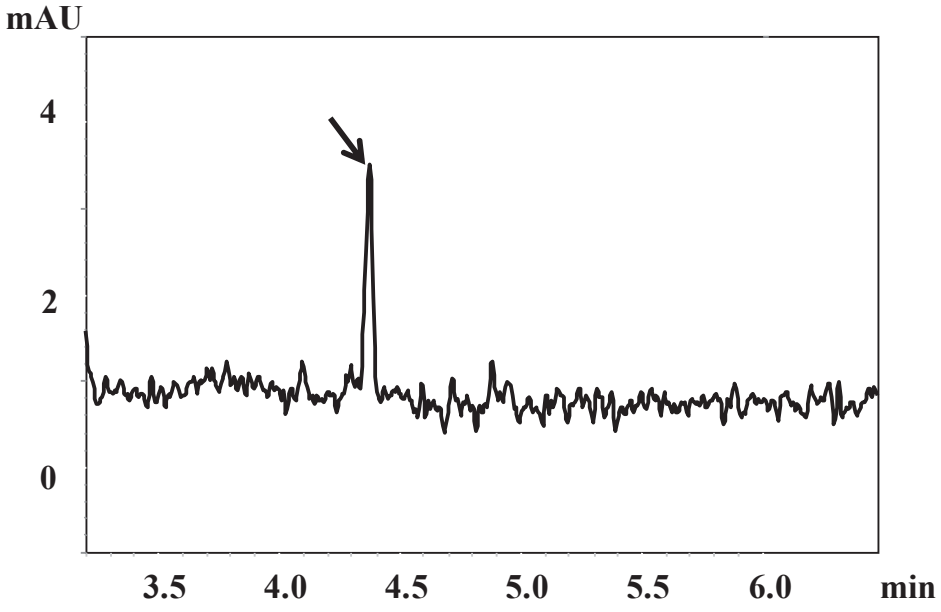


Fig. 1

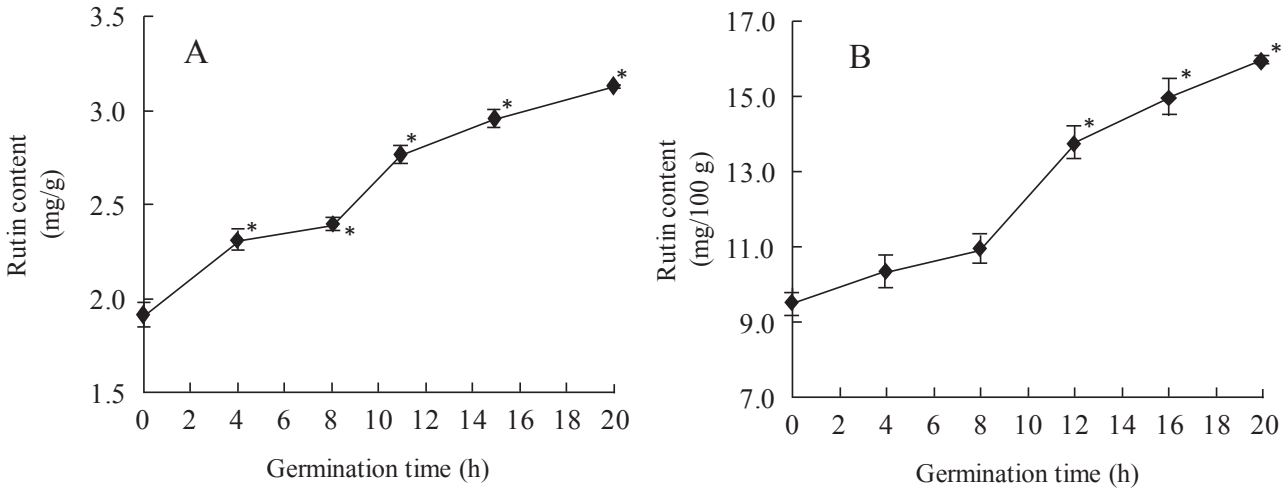
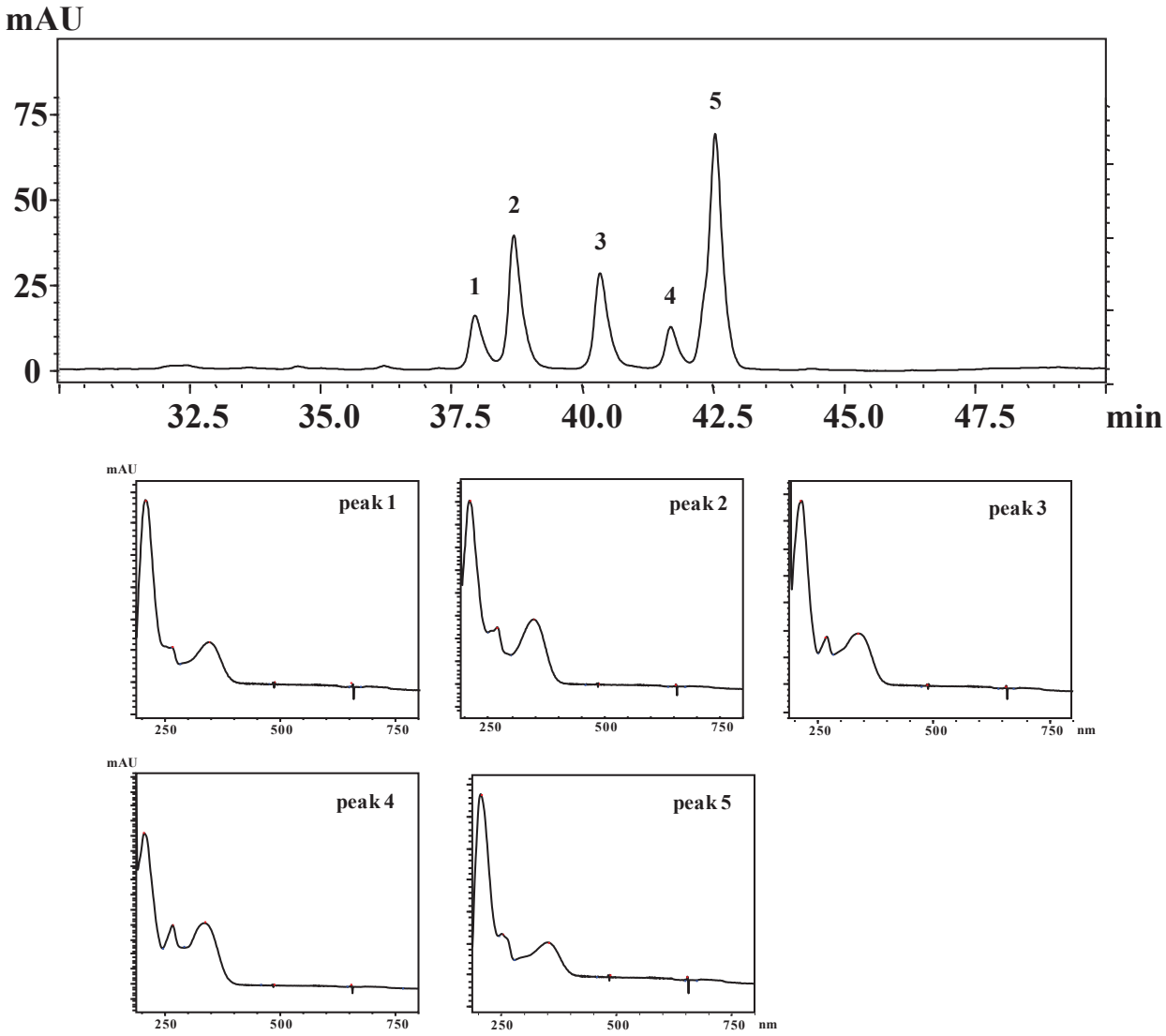

A
Rutin content (mg/g)
3.5
3.0
2.5
2.0
1.5
0 2 4 6 8 10 12 14 16 18 20
Germination time (h)
B
Rutin content (mg/100 g)
17.0
15.0
13.0
11.0
9.0
7.0
0 2 4 6 8 10 12 14 16 18 20
Germination time (h)

Fig. 2

Fig. 3


mAU
75
50
25
0
1
2
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4
5
32.5
35.0
37.5
40.0
42.5
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min
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peak 1
peak 2
peak 3
peak 4
peak 5
250
500
750
nm

Fig. 4

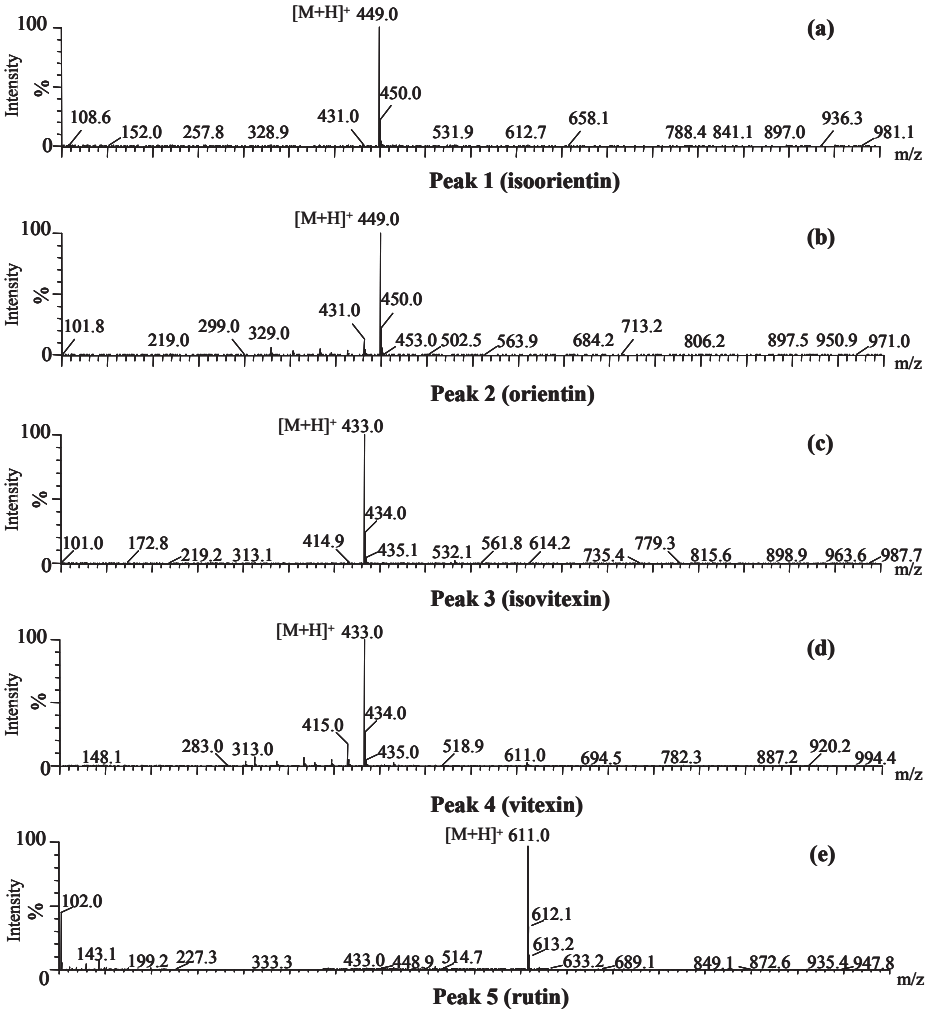
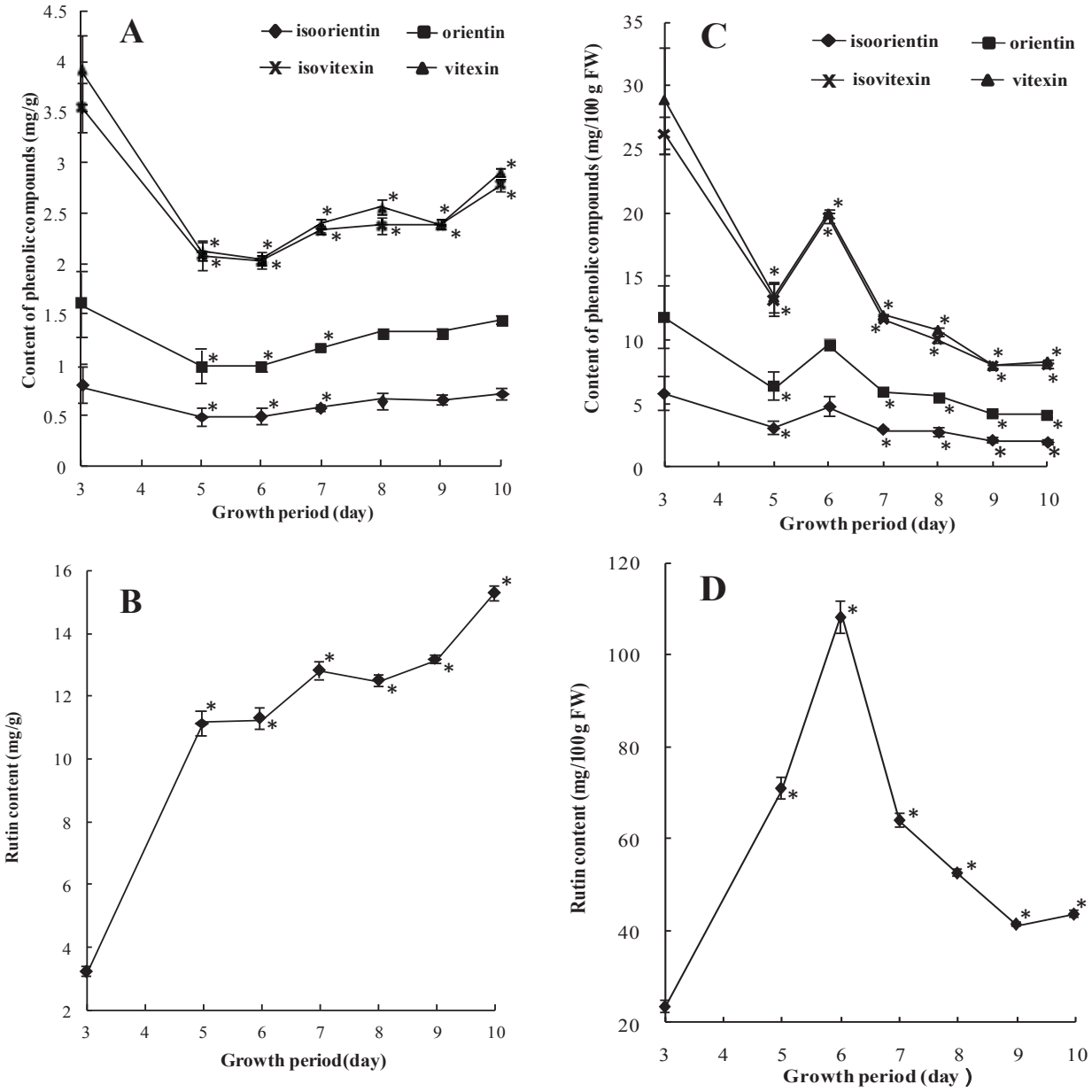

[M+H]+ 449.0
(a)
Intensity
%
100
0
108.6
152.0
257.8
328.9
431.0
450.0
531.9
612.7
658.1
788.4
841.1
897.0
936.3
981.1
m/z
Peak 1 (isoorientin)
[M+H]+ 449.0
(b)
101.8
219.0
299.0
329.0
431.0
450.0
453.0
502.5
563.9
684.2
713.2
806.2
897.5
950.9
971.0
Peak 2 (orientin)
[M+H]+ 433.0
(c)
101.0
172.8
219.2
313.1
414.9
434.0
435.1
532.1
561.8
614.2
735.4
779.3
815.6
898.9
963.6
987.7
Peak 3 (isovitexin)
[M+H]+ 433.0
(d)
148.1
283.0
313.0
415.0
434.0
435.0
518.9
611.0
694.5
782.3
887.2
920.2
994.4
Peak 4 (vitexin)
[M+H]+ 611.0
(e)
102.0
143.1
199.2
227.3
333.3
433.0
448.9
514.7
612.1
613.2
633.2
689.1
849.1
872.6
935.4
947.8
Peak 5 (rutin)

Fig. 5


A
isoorientin
orientin
isovitexin
vitexin
Content of phenolic compounds (mg/g)
Growth period (day)
C
isoorientin
orientin
isovitexin
vitexin
Content of phenolic compounds (mg/100 g FW)
Growth period (day)
B
Rutin content (mg/g)
Growth period(day)
D
Rutin content (mg/100 g FW)
Growth period (day)

86

87 **Fig. 6**