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**Abbreviations:** AGPase - ADP-glucose pyrophosphatase; 3-PGA - 3-phosphoglycerate; Pi - inorganic phosphate; TPT - triose

phosphate/phosphate translocator.

### **Abstract**

**Short pulse-chase labeling experiments indicated that there was a considerable difference between soybean and rice in the distribution pattern of  $^{14}\text{C}$  to major metabolites during a 300 sec chase period. The distribution of  $^{14}\text{C}$  to starch at the end of the chase period was largest in soybean but very small in rice. In rice, the distribution of  $^{14}\text{C}$  to sucrose was the largest. Starch formation during the initial stages of photosynthesis implied the existence of a different mechanism of starch synthesis and utilization between soybean and rice. The relative intensity of the flow into starch increased by treatment with 2%  $\text{O}_2$ , suggesting that the decrease in the level of the metabolites involved in the photorespiratory pathway may increase the carbon flow to starch in soybean.**

***Key words:* photorespiratory pathway; primary photosynthate distribution; pulse-chase labeling experiment; rice; soybean.**

## **Introduction**

The use of short pulse-chase experiments enabled to observe the metabolite flow in the Calvin cycle, C<sub>4</sub> photosynthesis, and the photorespiration pathways in plants. Whereas most of these experiments involved soluble metabolites, the insoluble fractions, of which the major component is considered to be starch, have not been the focus of quantitative analysis because the distribution to the insoluble fraction usually accounts for less than 10% (e.g. Nielsen et al. 2002). Thus, detailed analysis of short-term photoassimilated carbon distribution to the insoluble fractions in plants has not been thoroughly performed.

We focused on the several min period after the assimilation to study the difference in the carbon distribution between soybean and rice, using a short pulse-chase experiment. After a 5 min <sup>14</sup>CO<sub>2</sub> assimilation period, a larger amount of <sup>14</sup>C was released from soybean leaves than from rice leaves, especially during the several min period after assimilation, irrespective of either post-assimilation light conditions or the plant nitrogen status (Shinano et al. 1994). In addition, Nakamura et al. (1997) reported that the inhibition of <sup>14</sup>CO<sub>2</sub> release by 2% O<sub>2</sub> treatment was more pronounced in soybean than

in rice, regardless of the growth stage or nitrogen availability, indicating a higher activity of the photorespiratory cycle in soybean leaves. The distribution of  $^{14}\text{C}$  to polysaccharides, organic acids, and amino acids (especially glycine and serine) was also larger in soybean than in rice (Nakamura et al. 1997). However, in these experiments, the duration of the assimilation periods was too long to provide information about the behavior of the  $^{14}\text{C}$  distribution to each compound.

In the present paper, we investigated the differences in the  $^{14}\text{C}$  distribution in rice and soybean after assimilation in a 20 sec pulse – 300 sec chase labeling experiment. This enabled us to analyze the carbon distribution pattern. The 2%  $\text{O}_2$  treatment not only inhibited the photorespiratory cycle but also raised the  $^{14}\text{CO}_2$  assimilation rate. Such a treatment is necessary for acquiring more information about the conditions of carbon distribution to photoassimilates and photorespiratory intermediates.

## **MATERIALS AND METHODS**

**Cultivation and sampling.** Rice (*Oryza sativa* L. var. Michikogane) and soybean (*Glycine max* Merr. var. Kitamusume) plants were cultivated in a

glasshouse at Hokkaido University in pots (5 L pot for rice and 10 L pot for soybean) filled with a brown lowland soil. Rice plants were fertilized with 2.5 g Pot<sup>-1</sup> N (ammonium sulfate), 2.5 g Pot<sup>-1</sup> P<sub>2</sub>O<sub>5</sub> (superphosphate) and 1 g Pot<sup>-1</sup> K<sub>2</sub>O (potassium sulfate). The soybean plants were fertilized with 5 g Pot<sup>-1</sup> N, 5g Pot<sup>-1</sup> P<sub>2</sub>O<sub>5</sub>, and 2 g Pot<sup>-1</sup> K<sub>2</sub>O, 15 g Pot<sup>-1</sup> CaCO<sub>3</sub> and 10 Pot<sup>-1</sup> MgO (magnesium oxide),

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**<sup>14</sup>C assimilation and fractionation.** <sup>14</sup>CO<sub>2</sub> was assimilated under the 21 % O<sub>2</sub> and 2 % O<sub>2</sub> conditions in the 5th leaf of rice and the 4th leaf of soybean, both of which were fully expanded at the vegetative growth stage, respectively.

The methods of <sup>14</sup>CO<sub>2</sub> assimilation were as follows: each individual leaf was covered with a clear polyethylene bag (2 L) filled with natural air (control treatment) or 2 % O<sub>2</sub> and 36 Pa CO<sub>2</sub> mixed with N<sub>2</sub> gas (low O<sub>2</sub> pulse treatment) during 60 sec before the chase period. Thereafter, 0.22 Pa of CO<sub>2</sub> and trace <sup>14</sup>CO<sub>2</sub> were liberated by mixing 1 mL of 0.18 mM NaHCO<sub>3</sub>, 0.74 MBq NaH<sup>14</sup>CO<sub>3</sub> and 1 mL of 20% HClO<sub>4</sub> in the bag. The <sup>14</sup>CO<sub>2</sub> released from the mixture was then assimilated for 20 sec under natural light conditions (in excess of 1,000 μmol E m<sup>-2</sup>s<sup>-1</sup>). In the low O<sub>2</sub> pulse treatment,

since a long exposure to 2 % O<sub>2</sub> induced further carbon distribution to other metabolites (Nakamura et al. 1997), the samples were exposed to a 2% O<sub>2</sub> treatment for 60 sec before the chase period. After the 20 sec <sup>14</sup>CO<sub>2</sub> assimilation period, the plants were removed from the bag and placed under ambient gas and natural light conditions and the individual leaves were separated and frozen in liquid nitrogen at 0, 15, 30, 45 and 300 sec after assimilation. They were then lyophilized and stored at -80 °C for subsequent analysis.

Two-step leaf extractions were carried out using methanol, chloroform and water (12/5/3, v/v/v) in the first step and 0.2 mM formic acid in 20% ethanol in the second step. The combined water-soluble supernatant was separated into organic acid, amino acid, sugar and phosphate ester fractions by ion-exchange chromatography using SP Sephadex C-25 (cation resin, Amersham Biosciences, NJ, USA) and QAE Sephadex A-25 (anion resin, Amersham Biosciences), as described by Redgwell (1980). To measure the amount of <sup>14</sup>C-proteins, the insoluble fraction (residue after extraction) was subjected to acid hydrolysis and purified through SP Sephadex C-25, according to the method of Shinano et al. (1994). <sup>14</sup>C in the residual fraction

(total  $^{14}\text{C}$  – soluble  $^{14}\text{C}$  – protein  $^{14}\text{C}$ ) was considered to be the starch fraction because its post-assimilation time was very short. The radioactivity of each fraction was detected with a liquid scintillation counter. To confirm the existence of  $^{14}\text{C}$  in the starch fraction, the residual fractions were solubilized in 1.1 M KOH and incubated at 95 °C for 40 min. After adjustment to pH 5.5, the fractions were incubated for 24 h in the presence of 70-U  $\alpha$ -amylase (Wako Chemicals, Osaka, Japan) and 50-U amyloglucosidase (Roche Diagnostics, Basel, Switzerland) at 30 °C, a process that converts starch to glucose. After removal of the ionic compound by using the anion and cation exchange resin described above and purification for improving the resolution of the paper chromatogram, the distribution of  $^{14}\text{C}$  to glucose was determined by paper chromatography and by using an imaging plate as described in the following section.

**Paper chromatography.** Each soluble fraction was further separated by paper chromatography using Whatman 31 ETCHR paper (Whatman, Kent, UK). Four soluble fractions were separated using different solvents as follows: for organic acids, 1-butanol/methanol/water, 12/3/5 v/v/v (first dimension) and propanol/formic acid (99%)/water, 50/4/9 v/v/v (second

dimension); for amino acids, pyridine/acetone/water, 10/6/4/1 v/v/v/v (first dimension) and propanol/formic acid (99%)/water, 8/1/1 v/v/v (second dimension); for sugars, 1-butanol/methanol/water, 8/2/3 v/v/v; and for phosphate ester, propanol/ammonia/water, 6/3/1 v/v/v (first dimension) and propyl acetate/formic acid (90%)/water, 11/5/3 v/v/v (second dimension).

The distribution pattern of radioactivity on the paper was observed using an imaging plate (BAS-MP, Fuji, Tokyo, Japan) and a Bio Imaging Analyzer (BAS-1000, Fujifilm, Tokyo Japan). Each spot was identified by GC-MS (GC-Q 4000, Shimadzu, Kyoto, Japan) after derivatization with methoxylamine hydrochloride in pyridine following the addition of *N-tert*-butyldimethylsilyltrifluoroacetamide, according to the method of Fiehn et al. (2000).

## **RESULTS AND DISCUSSION**

The broad scale distribution of assimilated carbon was determined after separation by column ion-exchange chromatography (Table 1). A large proportion of assimilated carbon was incorporated into the phosphate ester fraction immediately after  $^{14}\text{CO}_2$  assimilation in both kinds of plants.

Subsequently, the proportion of the sugar and residual fraction increased. During the 300 sec period after assimilation, almost all (>95%) the residual fraction were considered to consist of  $^{14}\text{C}$ -starch (data not shown). No substantial difference was observed in the distribution of  $^{14}\text{C}$  to phosphate esters between the 21 %  $\text{O}_2$  and 2 %  $\text{O}_2$  treatments in rice leaves. However, in the soybean leaves, the low  $\text{O}_2$  treatment reduced the distribution of  $^{14}\text{C}$  to phosphate esters until 45 sec. The distribution of  $^{14}\text{C}$  to organic acids gradually decreased after assimilation, except for the soybean leaves under low  $\text{O}_2$  conditions. The distribution of  $^{14}\text{C}$  to free amino acids sharply increased during the first 15 sec after assimilation, and then decreased gradually until 300 sec. The maximum relative distribution ratio of  $^{14}\text{C}$  to amino acids during the 300sec chase period was higher in soybean than in rice, and the distribution to amino acids was reduced in both kinds of plants by the low  $\text{O}_2$  treatment. Conversely, only a negligible proportion of  $^{14}\text{C}$  was incorporated into protein, regardless of the kind of plant or  $\text{O}_2$  level under the conditions employed here (data not shown). Thus, there were considerable differences in the quantitative balance of the distribution of assimilated  $^{14}\text{C}$  among the various compounds between soybean and rice, although both

soybean and rice were C<sub>3</sub> photosynthesis plants.

Although research on photosynthate distribution has been carried out over a long period of time, most reports dealt with samples with assimilation for over 10 min (Häusler et al. 1998; Sun et al. 1999; Schneider et al. 2002; Walters et al. 2004). Furthermore, the distribution to starch at a very early assimilation stage had not been well documented. Nakamura et al. (1997) reported a 30 min pulse and 30 min chase experiment on rice and soybean under ambient air conditions. The <sup>14</sup>C distribution to sugars and the insoluble fraction (mainly starch) under ambient air conditions increased during the 30 min chase period by about 0.1 and 0.03 in rice, respectively, and by about 0.1 and 0.05 in soybean, respectively. However, our data (20 sec pulse and 300 sec chase) revealed a more dynamic increase in the <sup>14</sup>C-distribution to soluble sugars and the insoluble fraction during the 300 sec chase period: about 0.22 and 0.31 in soybean, respectively, and about 0.56 and 0.03 in rice, respectively (Table. 1). Thus, it is assumed that the information acquired by a long pulse and chase time experiment (30 min and 30 min, respectively) would not be sufficient for determining the difference in the distribution pattern of primary photosynthates (Häusler et al. 1998;

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Sun et al. 1999; Schneider et al. 2002; Walters et al. 2004). In *Arabidopsis* and tobacco, a number of reports have been published in which longer pulse and chase times were used. Based on these experiments, the distribution pattern to sucrose or starch in each treatment was similar. We assume that if a shorter duration of the pulse and chase periods were used in these experiments, it might be possible to observe different patterns in photosynthate distribution.

Figure 1 clearly shows the difference between rice and soybean in the pattern of  $^{14}\text{C}$  distribution to sucrose and starch. In rice, assimilated carbon was mainly incorporated into sucrose and only a small proportion was distributed to starch, whereas the opposite was observed in soybean. Furthermore, in the low  $\text{O}_2$  treatments in soybean, more carbon was incorporated into starch than under the control conditions just after assimilation, and the amount of carbon increased with time. This indicates that the reduced labeling activity of photorespiration-related metabolites led to an increase in the carbon flow to starch. Although rice and soybean are both  $\text{C}_3$  plants, the quantitative changes in assimilates in soybean were much more conspicuous than in rice under low  $\text{O}_2$  treatments. Moreover, higher

carbon incorporation into starch just after assimilation under low O<sub>2</sub> conditions, rather than under ambient conditions, is a new finding that was not observed in rice. Hendriks et al. (2003) reported that the synthesis of starch is posttranslationally regulated by redox-modification. We suggest that the rapid increase in the distribution of assimilated <sup>14</sup>C to starch which we observed in soybean under low O<sub>2</sub> conditions might be partly attributed to the posttranslational activation of starch synthesis regulated by a rapid change in the redox state. However, there is no evidence as to whether this phenomenon is specific to soybean or brought about by a large accumulation of excess assimilates in soybean. Therefore, further studies on the initial stages of photosynthesis should be conducted in considering enzymatic activities and/or genetic regulation.

Glycine, serine and glycolate are the major intermediates of the photorespiratory pathway. The distribution of <sup>14</sup>C to these metabolites was uniformly larger in soybean than in rice, and was reduced under low O<sub>2</sub> treatments (Fig. 1). The larger distribution of photosynthetically assimilated <sup>14</sup>C to photorespiratory intermediates in soybean could be due to the following three possibilities. First, soybean exhibits a higher ratio of

oxygenation activity to the carboxylation activity of Rubisco than rice, although the enzymatic kinetics of Rubisco in both plants is very similar (Makino et al. 1988). The oxygenation/carboxylation ratio is determined by the  $O_2/CO_2$  concentration in the stroma. Second, soybean displays a lower ability to transport carbon compounds from the intermediate pools derived from the Calvin cycle and the photorespiratory pathway. In soybean, the distribution ratio of  $^{14}C$  to glycolate remained high up to 15 sec, a longer duration than that observed in rice. Thus, labeled carbon distributed to these intermediate pools was stored over a longer period of time inside the soybean chloroplasts than in rice, which may lead to an increased  $^{14}C$  distribution to the photorespiratory intermediates. Third, the existing anaplerotic pathway connecting glycolysis and photorespiration is more active in soybean than in rice. By using this anaplerotic pathway, a larger proportion of  $^{14}C$  can be distributed to the photorespiratory pathway. This hypothesis is partly supported by the observation of synthesis of glycolate from glucose 6-phosphate (Eickenbusch et al. 1975), serine from phosphoglycerate (Larsson and Albertson 1979), and glycolate from pyruvate via isocitrate lyase (Zelitch 1988). Nakamura et al. (1997) reported

that  $^{14}\text{C}$  incorporation into serine under a 2%  $\text{O}_2$  treatment (for 1 h) was higher than under ambient conditions after 30 min of  $^{14}\text{CO}_2$  labeling and a subsequent 30 min chase period. Therefore, since the 2%  $\text{O}_2$  treatment enhanced the distribution of  $^{14}\text{C}$  to serine, despite the inhibition of photorespiration by a low level of  $\text{O}_2$ , there might be a complementary pathway of photorespiratory metabolites.

In soybean under low  $\text{O}_2$  treatments, the  $^{14}\text{C}$  distribution to malate was markedly increased (Fig. 1). Malate production under low  $\text{O}_2$  conditions may be due to two mechanisms. First, a rapid reaction system that stimulated malate synthesis occurred in order to buffer the surplus assimilates. Malate synthesis under low  $\text{O}_2$  conditions may: 1) provide malate ion to correct the electric imbalance or intracellular pH (Sakano 1998) brought about by the rapid metabolic activation, 2) divert surplus assimilates to the carbon skeleton of amino acids. To elucidate this phenomenon, a simultaneous measurement of both photosynthetic rates and the energy status of the leaf should be performed. Second, the contribution of the anaplerotic pathway that supplies carbon molecules to the photorespiratory cycle, which is enhanced by low  $\text{O}_2$  treatment conditions,

especially in soybean, should also be considered. It has been observed that malate was rapidly labeled in the presence of  $^{14}\text{C}$ -oxalate and  $^{14}\text{C}$ -glyoxylate (Havir and Mchale 1988), that the stimulation of malate synthesis could be induced by the inhibition of glutamate synthase (Walker et al. 1984) and that malate accumulation was observed in the mutants lacking aminotransferase or glutamate synthase in leaves (Kendall et al. 1986; Murray et al. 1987).

コメント [OLE3]: We are unsure what you mean here. GOGAT is the abbreviation for glutamate synthase.

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Recent studies on starch synthesis in leaves have revealed the existence of a considerable heterogeneity, both among cells and among subcellular organs. Tretheway and Smith (2000) first reported the existence of starch-synthesizing enzymes outside the plastids. In addition, it should be noted that, at least in wheat, only approximately half of the leaf cells are mesophylllic (Jellings and Leech 1982). The pattern of starch accumulation is also different in the cells of barley leaves (Williams et al. 1989).

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Micro-scale studies of leaf cells demonstrated a clear differentiation in their primary photosynthate distribution patterns (Koroleva et al. 1998; 2000). Further studies should be carried out to determine whether the rapid distribution of primary photosynthates into starch occurs inside or outside the chloroplasts.

In conclusion, rice and soybean exhibited considerable differences in their patterns of distribution of photosynthetically acquired  $^{14}\text{C}$  to starch. The flexibility of the distribution of  $^{14}\text{C}$  to starch and organic acids points to the existence of a new reaction mechanism to surplus photosynthates in the soybean.

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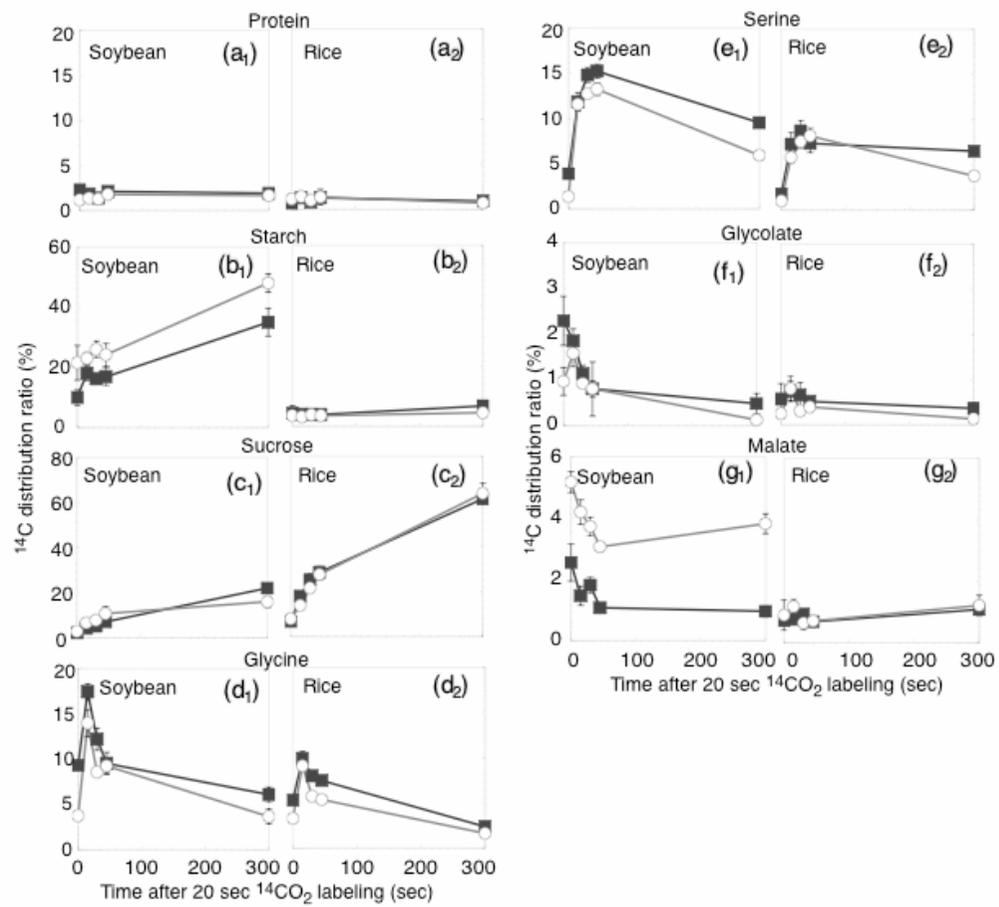
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## Figure legend

Fig. 1.  $^{14}\text{C}$  distribution ratio of metabolites related to photorespiratory pathways in the leaves of soybean and rice plants after a 20 sec labeling period with  $^{14}\text{CO}_2$  under 21%  $\text{O}_2$  (■) and 2%  $\text{O}_2$  (○) conditions following a 300 sec chase period. During the chase treatment after labeling, all the plants were exposed to 21%  $\text{O}_2$  air. The amount of  $^{14}\text{C}$  in each fraction was determined with a liquid scintillation counter and an imaging analyzer. Each point represents the mean of 3-5 replicates.

Table 1.  $^{14}\text{C}$  distribution ratio of soluble and insoluble photoassimilates after a 20sec labeling period with  $^{14}\text{CO}_2$  in the leaves of soybean and rice plants under control 21%  $\text{O}_2$  (C) and low 2%  $\text{O}_2$  (L) conditions following a 300sec chase period. During the chase treatment after labeling, all the plants were exposed to 21%  $\text{O}_2$  air. The  $^{14}\text{C}$  in each fraction was determined with a liquid scintillation counter. Each value represents the mean of 4 replicates.



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Table 1.  $^{14}\text{C}$  distribution ratio of soluble and insoluble photoassimilates after a 20sec labeling period with  $^{14}\text{CO}_2$  in the leaves of soybean and rice plants under control 21%  $\text{O}_2$  (C) and low 2%  $\text{O}_2$  (L) conditions following a 300sec chase period. During the chase treatment after labeling, all the plants were exposed to 21%  $\text{O}_2$  air. The  $^{14}\text{C}$  in each fraction was determined with a liquid scintillation counter. Each value represents the mean of 4 replicates.

Sampling time (min)	0		15		30		45		300		
	Treatment	C	L	C	L	C	L	C	L	C	L
<b>Soybean</b>											
Phosphate ester	0.54±0.06	0.51±0.08	0.31±0.03	0.28±0.03	0.38±0.07	0.24±0.03	0.34±0.04	0.17±0.03	0.06±0.01	0.04±0.00	
Soluble sugar	0.05±0.01	0.04±0.00	0.10±0.02	0.09±0.01	0.07±0.01	0.11±0.02	0.09±0.01	0.14±0.01	0.27±0.02	0.21±0.04	
Organic acid	0.15±0.03	0.09±0.01	0.12±0.02	0.11±0.01	0.09±0.02	0.11±0.02	0.09±0.03	0.13±0.02	0.06±0.02	0.11±0.02	
Amino acid	0.16±0.03	0.05±0.02	0.29±0.02	0.30±0.03	0.29±0.03	0.28±0.02	0.25±0.04	0.32±0.02	0.20±0.02	0.15±0.01	
Insoluble	0.10±0.01	0.30±0.08	0.19±0.03	0.21±0.02	0.17±0.04	0.27±0.04	0.22±0.03	0.23±0.03	0.41±0.06	0.49±0.04	
<b>Rice</b>											
Phosphate ester	0.62±0.01	0.69±0.07	0.39±0.03	0.46±0.06	0.34±0.05	0.39±0.02	0.29±0.03	0.31±0.03	0.05±0.00	0.04±0.01	
Soluble sugar	0.08±0.01	0.11±0.02	0.20±0.01	0.18±0.02	0.27±0.03	0.27±0.01	0.34±0.04	0.34±0.03	0.64±0.00	0.74±0.03	
Organic acid	0.15±0.00	0.09±0.03	0.14±0.01	0.12±0.04	0.12±0.01	0.09±0.01	0.10±0.03	0.08±0.01	0.05±0.00	0.04±0.01	
Amino acid	0.10±0.00	0.07±0.01	0.22±0.01	0.20±0.03	0.22±0.00	0.20±0.01	0.21±0.02	0.22±0.01	0.18±0.00	0.13±0.01	
Insoluble	0.05±0.02	0.05±0.01	0.05±0.00	0.05±0.00	0.05±0.01	0.05±0.01	0.05±0.02	0.05±0.01	0.08±0.00	0.05±0.01	

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