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Note

Effects of Cooking Conditions on the Relationships Among Oxalate, Nitrate, and Lutein in Spinach

Zheng WANG, Akira ANDO, Atsuko TAKEUCHI and Hiroshi UEDA*

Division of Vegetable Pest Management and Functional Analysis, Institute of Vegetable and Floriculture Science, National Agriculture and Food Research Organization, 360 Kusawa, Anou, Tsu, Mie 514-2392, Japan

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This study sought to determine the optimal cooking conditions under which lutein would be maintained while oxalic acid and nitrate ion would be removed. The effects of cooking temperature and time control on the concentration of oxalic acid, nitrate ion, and lutein in spinach were investigated. The results demonstrated that boiling at 100℃ for 2 min was the optimal cooking condition. This preserved approximately 77% of lutein while 67% of oxalic acid and 30% of nitrate ion were removed. If nitrate ion is disregarded, we also found that submerging in hot water at 60℃ for 30 s was another optimal condition. In this case, 96% of lutein remained, 28% of oxalic acid was removed, whereas the content of nitrate ion was unchanged.

Keywords: spinach, lutein, oxalate, nitrate ion, cooking processing

Introduction

Spinach (*Spinacia oleracea* L.) is an important green leafy vegetable due to its diverse nutritional composition, including vitamins, minerals, and phytochemicals. Spinach is one of the richest plant sources of lutein, which selectively accumulates in the retinal macular area of the eye (Sommerburg *et al.*, 1998; Perry *et al.*, 2009; Abdel-Aal *et al.*, 2013). It is involved in the prevention of age-related macular degeneration and cataracts (Wong *et al.*, 2011). However, oxalate and nitrate are also naturally occurring contents in plants and are found to have relatively high levels in spinach. Oxalate can combine easily with calcium and magnesium to form insoluble oxalate crystals and inhibit calcium or magnesium absorption in humans (Mou, 2008; Genannt Bonsmann *et al.*, 2008; Arias-Carmona1 *et al.*, 2014). High levels of oxalates in the diet can lead to the irritation of the digestive system and the formation of kidney stones (Holmes *et al.*, 2000). Similarly, the amount of nitrate ion in vegetables is an important indicator in food safety evaluation. The intake of vegetables containing a high concentration of nitrate ion can cause infantile methemoglobinemia, cancer, and other diseases (Pennington, 1998; Sanchez-Echaniz *et al.*, 2001). However, there is controversy as to whether nitrate ion in our diets is beneficial or harmful, and it has been reported that nitrate ion is beneficial for health (Bahadoran *et al.*, 2015).

Vegetables are usually consumed in raw and cooked forms. Total and soluble oxalate contents of some raw and/or boiled vegetables have been reported by several groups. For instance, Hönow *et al.* (2002) compared the oxalate content of fruits, vegetables, and other foods such as nuts and cereal crops. Savage *et al.* (2000) reported that soluble oxalate can be removed by boiling. Chai *et al.* (2005) showed that the boiling process markedly reduced the soluble oxalate content more effectively than steaming and baking processes. Abo Bakr *et al.* (1986) reported the lower nitrate content after cooking vegetables. Moreover, Izumi *et al.* (2005) evaluated the influence of different amounts of boiling water on oxalic acid in spinach. The result showed that greater the amount of boiling water, greater was the decrease in the concentration of oxalic acid. Recently, Kojima *et al.* (2017) reviewed the loss of vitamins under various cooking conditions. In spinach, 36%–73% of vitamin C was lost under different boiling conditions.

However, a few studies have focused on the relationships among oxalate, nitrate, and lutein contents during the cooking process. Thus, the purpose of this study was to investigate the

*To whom correspondence should be addressed. E-mail: uedah@affrc.go.jp

effects of different cooking conditions on the changes of oxalate, nitrate, and lutein contents in spinach. The goal was to optimize the cooking conditions that can retain lutein and simultaneously remove oxalate and nitrate contents in spinach.

Materials and Methods

Materials Fresh spinach (*Spinacia oleracea* L., '*Jasuteisu*' cultivar), which was cultivated in the Ibaraki Prefecture, was purchased from a local supermarket on June 27, 2017. Standard lutein, oxalic acid, potassium nitrate, sodium hydroxide, and 2,6-pyridinedicarboxylic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pyrogallol and all organic solvents were of analytical or HPLC grade and purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Hexadecyltrimethylammonium bromide (purity ≧99.0%) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Sample preparation The fresh spinach (n = 6) was boiled with 3 L of tap water at different temperatures (60℃, 80℃, and 100℃) and for a duration of 30 s, 1 min, 2 min, and 3 min, respectively. Water bath was used for conditions at 60℃ or 80℃ and IH cooking heater for 100℃. The temperatures decreased after putting in spinach. The cooking time was counted when the temperatures returned to starting conditions. The boiled spinach was rinsed under running water (<1 min). Squeezing spinach, the excess water was removed using Kimtowels (Nippon Paper Crecia, Tokyo, Japan). All samples were stored at −20℃ until analysis.

Saponification and extraction procedure Saponification and extraction methods were developed, which were modified from the methods described by Yasui *et al.* (2015). Frozen spinach was cut into small pieces and immediately crushed using a family use mixer (MJ-M30, National, Tokyo, Japan) after mixing with 3× volume of pyrogallol/ethanol solution (3%, w/v). One milliliter of crushed spinach solution was transferred into a 50-mL centrifuge tube. Ten milliliters of pyrogallol/ethanol solution (3%, w/v) and 1 mL of potassium hydroxide (60%, w/v) were added, and the contents were mixed using a vortex mixer. Furthermore, the tube was incubated for 15 min at 60℃ in a shaking water bath (Personal-11, TAITEC, Saitama, Japan) at 160 strokes/min. After saponification, the tubes were cooled under running water. A solution containing 22.5 mL of sodium chloride (1%, w/v) and 15 mL of ethyl acetate/n-hexane (1:9, v/v) was added into the tube and kept at room temperature with vibration for 5 min. Then the mixture was centrifuged at 6 000 ×g for 5 min and the organic layer containing lutein was collected. This extraction procedure was repeated for 2 times. The collected organic layers were evaporated to dryness by a centrifugal evaporator (CVE-3100, EYELA, Tokyo, Japan). The dry residue containing lutein was dissolved in 10 mL of ethanol for determination by HPLC.

Determination of lutein content The HPLC system consisted of a PU-2080 Plus pump (JASCO, Tokyo, Japan), a MD-2015 plus diode array detector (JASCO, Tokyo, Japan), and a Develosil XG C-30 M column (4.6 × 250 mm i.d., Nomura Chemical Co., Ltd., Aichi, Japan) with a particle size of 5 μm. The column was protected by a guard column (XG-C30, Nomura Chemical Co., Ltd., Aichi, Japan) of the same packing material. The mobile phase was acetonitrile/methanol (65:35, v/v), and the flow rate was 1 mL/min. The column oven temperature was 40℃. The volume injected was 20 μL, and the lutein peak was detected at 450 nm. All samples were filtered by a 0.45-μm pore size hydrophobic PTFE membrane before determination by HPLC.

Extraction and determination of oxalate and nitrate content Frozen spinach was cut into small pieces and mixed with 4× the volume of Milli-Q water in a beaker. The inactivation of enzymes was performed in a microwave oven (NE-M150, National, Tokyo, Japan) until the Milli-Q water was heated to nearly boiling (<5 min). Following this, it was cooled under running water and crushed by a family use mixer (MJ-M30, National, Tokyo, Japan). The mixture was then centrifuged at 15 000 rpm for 5 min, and the supernatant was collected and diluted 4 times with Milli-Q water. All the samples were filtered using a 0.45-μm pore size hydrophobic PTFE membrane before determination by a capillary electrophoresis (CE) system (Model 7100, Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector. An uncoated fused-silica capillary of 100 cm (91.5 cm effective length, 50 μm i.d.) purchased from GL Sciences Inc. (Tokyo, Japan). The CE method used in this study for the determination of oxalate and nitrate contents was a modified form of the methods described by Soga *et al.* (1999) and Horie (2009). The running buffer contained 20 mM 2,6-pyridinedicarboxylic acid, and 0.5 mM hexadecyltrimethylammonium bromide. The pH of running buffer was adjusted to 12.1 using 1 N sodium hydroxide. The standards and samples were injected into the capillary with a pressure of 50 mbar for 5 s. The separation voltage applied was −30 kV, and detection was performed with a diode array detector at wavelengths of 350 nm with a reference at 270 nm. The temperature of the capillary was controlled at 25℃. For successive electrophoretic runs, the capillary was rinsed sequentially with methanol, 0.1 N sodium hydroxide, and the running buffer for 5 min, respectively. The mixture of working standard was prepared by dissolving oxalic acid and potassium nitrate in Milli-Q water with concentrations of 400 and 200 mg/L (nitrate ion), respectively. Oxalate and nitrate were measured as oxalic acid and nitrate ion, respectively.

Statistical analysis Statistical analysis was performed using the EXCEL Statistics version 7.0 (Esumi Co., Ltd., Tokyo, Japan). Data were reported as mean ± SE (n = 6). Two-way repeated measures analysis of variance was performed to analyze the effects of the various cooking temperatures and cooking times on oxalate, nitrate, and lutein contents at a confidence level of 95% ($p < 0.05$). A least significant

difference test with a 95% confidence level was used to compare differences between the treatments.

Results and Discussion

This study was performed to assess the relationships among oxalate, nitrate, and lutein contents in spinach under different cooking conditions. Previous studies have found that oxalate can bind with calcium ions to form calcium oxalate crystals in the mouth (Tanaka *et al.*, 2003; Perera *et al.*, 1990). Additionally, Horie *et al.* (2006) reported that water-insoluble oxalic acid can unlikely affect the taste of spinach. Therefore, this study primarily focused on water-soluble oxalic acid. The concentrations of oxalic acid in raw spinach were 362 ± 18 mg of 100 g of fresh weight (FW) (mean ± SE, n = 6). The variety and environmental factors could affect the biosynthesis and level of oxalate in spinach. For instance, Mou (2008) investigated that there were significant differences in oxalate concentration among the genotypes using 11 commercial cultivars of spinach. After cooking for 30 s, oxalic acid concentrations were significantly reduced at all treated temperatures ($p < 0.05$). More than 30% of oxalic acid was removed at different cooking temperatures (60℃, 80℃, and 100℃) for 30 s, and 40% of oxalic acid was removed at 100℃ after 1 min with 16.7 times the amount of water. This result agrees with that of Izumi *et al.* (2005), wherein they report that 50% of oxalic acid can be removed after boiling at 100℃ for 1 min with 20 times the amount of spinach weight of boiling water. Oxalic acid concentration was significantly decreased after 2 and 3 min (67% and 73%, respectively, $p < 0.05$) at 100℃, whereas there was nearly no change after treating at 60℃ and 80℃ (Figure 1). The results indicate that oxalic acid can easily be removed by hot water treatment (e.g. 60℃ for 30 s). Compared with 60℃ and 80℃, more than twice the amount of oxalic acid was removed at 100℃ for 2 min. It was considered that higher temperature and longer cooking time can break down the tissues of spinach and improve the release of oxalic acid. Other factors, such as the tissue condition of spinach, can also promote the release of oxalic acid. Izumi (2004) reported that the cell wall contained a smaller amount of cellulose and was thinner in summer due to short growing periods, which may result in quick tissue damage and the release of oxalic acid. Izumi *et al*. (2005) also showed that the leaf blade containing oxalic acid was softer than the petiole containing nitrate; therefore, oxalic acid can easily be removed during boiling.

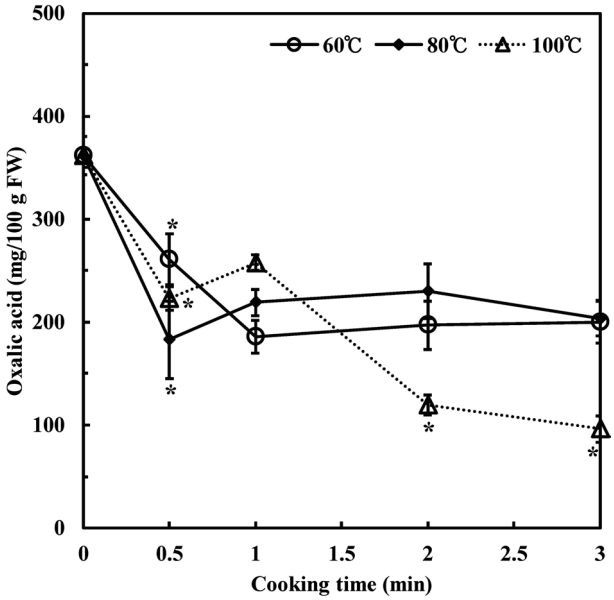

Fig. 1. Effects of cooking times and cooking temperatures on the changes of oxalic acid concentration. Error bars indicate SE (n = 6). Asterisks indicate significant differences (two-way repeated measures analysis of variance, $p < 0.05$).

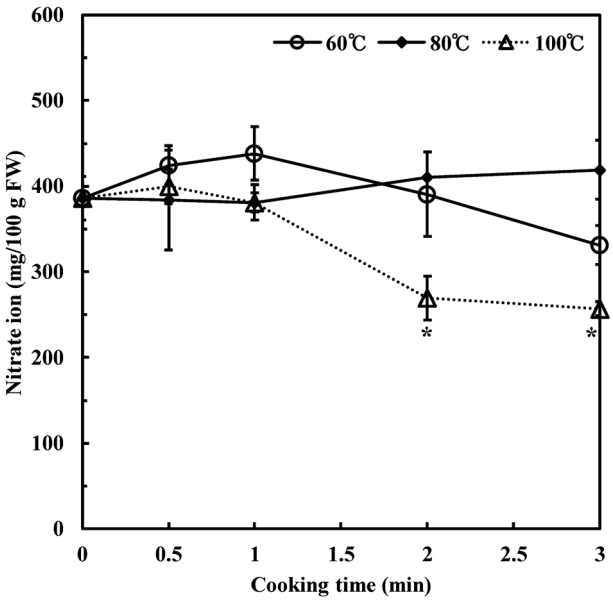

Fig. 2. Effects of cooking times and cooking temperatures on the changes of nitrate ion concentration. Error bars indicate SE (n = 6). Asterisks indicate significant differences (two-way repeated measures analysis of variance, $p < 0.05$).

The concentrations of nitrate ion in raw spinach were 386 ± 25 mg of 100 g FW (mean ± SE, n = 6). The environmental conditions, nitrogen fertilization regime, temperature, and use of herbicides can also increase the plant nitrate content (Iammarino *et al.*, 2014). Figure 2 shows the changes of nitrate ion concentration in spinach after different cooking conditions. The remains of nitrate ion depended on the cooking temperature and time. Compared with 60℃ and 80℃, the concentrations of nitrate ion in boiled spinach at 100℃ were reduced to 70% and 67% at 2 and 3 min, respectively ($p < 0.05$). These results indicated that oxalic acid contents can be removed more easily than nitrate ion contents at the same cooking conditions. For nitrate ion removal, it was required to boil the spinach at 100℃ only for 2–3 min.

The cultivar and environmental conditions can affect the lutein content in spinach (Oowashi *et al.*, 2014). The

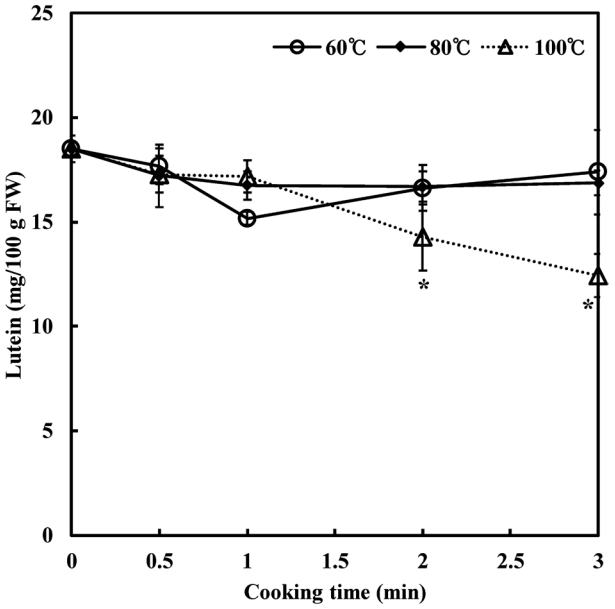

Fig. 3. Effects of cooking times and cooking temperatures on the changes of lutein concentration. Error bars indicate SE (n = 6). Asterisks indicate significant differences (two-way repeated measures analysis of variance, $p < 0.05$).

concentrations of lutein in raw spinach were 18.5 ± 0.6 mg of 100 g FW (mean ± SE, n = 6). The remaining lutein concentration in spinach after cooking is shown in Figure 3. The lutein concentration was significantly decreased at 100℃ for 2 min and approximately 77% of lutein remained ($p < 0.05$). In contrast, it was slightly decreased when the spinach was cooked at 60℃ or 80℃ for 2 min (<10%). This result suggested that the cooking temperature and cooking time can affect the stability of lutein in spinach. Approximately 50% decrease of lutein was reported in spinach with different storage methods or boiling time (Bunea *et al.*, 2008).

Oxalate, nitrate, and lutein can be synthesized by plants with different precursors and pathways in leaves, stems, and storage tissues (Betsche *et al.*, 2005). Each component can be released with different cooking conditions. The release behavior of each component depends on the chemical properties and/or the chemical forms of each component in plants. In future, sensory evaluation is also required for spinach processed with each condition. In this study, our results showed that oxalic acid and nitrate ion of vegetables can be removed by the process of cooking in boiling water. However, many beneficial compounds such as lutein and vitamins may be damaged. The results of this study optimized the best condition (100℃ for 2 min) to remove oxalate and nitrate ion in spinach while keeping as much the lutein content as possible. Moreover, previous studies have shown that dietary consumption of nitrate ion has numerous health benefits such as anti-inflammation, regulation of glucose homeostasis and insulin signaling pathway, and also improvement of cardiovascular function (Bahadoran, *et al.*, 2015; Bedale *et al.*, 2016; Bondonno *et al.*, 2016; Delmastro-Greenwood *et al.*, 2015). From this perspective, another optimized condition can be suggested as following. 28% of oxalic acid can be removed while 96% of lutein can be remained at 60℃ for 30 s. However, although the beneficial properties related to nitrate ion have been investigated in different experiments, it is necessary to confirm the effects by long-term clinical studies with various doses of inorganic nitrate supplementation.

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