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# Determination of Nitrate Ion Concentration in Fresh Vegetable Juices Using Ion-Pair Ultra Performance Liquid Chromatography (IP-UPLC)

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## Abstract

The nitrate ion content of vegetables has become a human health concern. Accurate and fast tissue (root, stem or leaf stalk) testing in crops is a valuable tool to determine fertilizer needs and maximize fertilizer efficiency. Therefore, the nitrate ion concentrations in fresh vegetable juices were measured using ion-pair ultra performance liquid chromatography (IP-UPLC). The separation was performed on a C18 column with a mobile phase containing 0.15 mM tetrabutylammonium chloride as an ion-pair reagent, 0.1% (w/v) ammonium acetate, and 5% (v/v) methanol at pH 6.2. The chromatogram was detected at 220 nm. The IP-UPLC chromatogram was obtained in 8 minutes, which is 39 minutes less than the time required to obtain a complete ion chromatography (IC) chromatogram of the same sample. The linearity of the method for nitrate ions was high, as evidenced by $R^2$ of 0.9996. In nitrate-spiked samples of fresh vegetable juice, the recovery percent consistently approached 100%. Furthermore, the IP-UPLC results obtained without any preliminary sample cleanup were essentially identical to those obtained by IC with a correlation coefficient (R) of 0.999 (n = 10).

**Discipline:** Agricultural chemicals
**Additional key words:** ammonium acetate, ion chromatography, leaf vegetables, root vegetables, tetrabutylammonium chloride

## Introduction

The nitrate ion content of fresh vegetables is important in evaluations of food safety. Ingested nitrate ion has been linked to infantile methemoglobinemia, carcinogenesis, and insulin-dependent diabetes mellitus (Santamaria 2006). Fifty to ninety percent of nitrate ions in the human diet is derived from vegetables (Sohn and Yoneyama 1996). Vegetables are considered an important potential source of the intake of nitrate. However, in view of the well-known benefits of vegetables and the lack of data on the possible effects of vegetable matrices on the bioavailability of nitrate, the 44th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered it inappropriate to directly compare exposure to nitrate from vegetables with the Acceptable Daily Intake (ADI), and hence to derive limits for nitrate in vegetables directly from the ADI (JECFA 1996). The European Community (EC) established a maximum allowable level of nitrate ions in lettuce and spinach (EC 2005), though Japan has not established such a level. The level was revised in 2011, and rucola (*Eruca sativa*) was added (EU 2011). As mentioned above, leafy vegetables have a high concentration of nitrate ions. In this paper, some vegetables including roots and stems or the leaf stalks of leafy vegetables were selected as samples having high concentrations of nitrate ions. The accurate and fast tissue testing in crops is a valuable tool to determine fertilizer needs and maximize fertilizer efficiency.

There are accepted liquid chromatographic methods of measuring nitrate ion levels in vegetables. Ion chromatographic (IC) methods are reliable but typically require relatively long run times (47 minutes) per vegetable sample (Ito *et al*. 2005). However, an IC column has yet to be developed for use in ultra performance liquid chromatography (UPLC). On the other hand, Hiraki *et al*. (2003) developed a method of separating five anions ($NO_2^-$, $NO_3^-$, $I^-$, $S_2O_3^{2-}$, $SCN^-$) using ion-pair (IP) high performance liquid chromatography (HPLC) with a C30 column, though run times of about 18 minutes though a

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C30 column have yet to be achieved for use in UPLC. In contrast, because UPLC methods boast relatively fast analyses (Villiers *et al*. 2006), an IP-UPLC with a C18 column may shorten the run times. Therefore, the current study focused on developing an IP-UPLC-based method of determining nitrate ion levels in fresh vegetable juices.

## Materials and Methods

Vegetable samples were purchased from retail grocers in Japan. Juices from stem and root vegetables are easily obtained by squeezing at the farm or distribution field. On the other hand, the juice yield from squeezing a thin leaf is much lower. Therefore, the stems of cabbage (n = 1), celery (n = 1), and a leaf stalk of Qing ging cai (*Brassica chinensis*) (n = 1) were comminuted with a grater and centrifuged. Leaf stalks of Komatsuna (*Brassica campestris*) (n = 2) and spinach (n = 1) were chopped and squeezed with a garlic press. Carrot (n = 1), radish (n = 1), Japanese radish (n = 1), and the roots of matsusaka-akana (Japanese name, n = 1) were juiced with a juicer. The fresh juice was filtrated using a disposable filter (Advantec DISMIC-25CS020, Toyo Roshi Kaisha, LTD., Japan).

This paper used an Acquity UPLC (Waters, USA) equipped with a photodiode array detector at 220 nm. The eluent was 5% (v/v) methanol (HPLC grade, Wako Pure Chemical Industries, Ltd., Japan), 0.15 mM tetrabutylammonium chloride (ion-paired reagent, Tokyo Chemical Industry Co., Ltd., Japan), and 0.1% (w/v) ammonium acetate (special grade, Wako Pure Chemical Industries, Ltd., Japan) at pH 6.2 (IP-UPLC). The eluent flow rate was 0.21 mL/min. The column was a 100-mm Acquity UPLC™ BEH SHIELD $RP_{18}$ with a particle size of 1.7 μm and I.D. of 2.1 mm (Waters, USA). The temperature of the column was set at 25°C.

IP-UPLC injection was performed with a 10 μL loop in needle overfill mode. Potassium nitrate (special grade, Wako Pure Chemical Industries, Ltd., Japan) was dissolved in distilled water to obtain a 10,000-ppm nitrate ion stock standard solution. To assess the linearity of the standard solution, aliquot volumes of 0.2, 0.4, 0.6, 0.8, and 1.0 μL of 100-ppm standard solution were injected into IP-UPLC. The limits of detection (LOD) were determined with 0.8 μL of 10-ppb standard solution (0.008 ng of nitrate ions, n = 7) using the following equation (Nagata 2013):

$$\mathrm{LOD} = 2 \times t\,(n - 1, 0.05) \times \text{standard deviation}$$

*t*: t-test, *n*: number of injections, 0.05:5% level of significance

Fresh vegetable juice was diluted 100-fold prior to injection into IP-UPLC. Percent recovery was determined by spiking 50 μL of 60-ppm standard solution into 200 μL of each diluted juice prior to injection. The aliquot volume (0.8 μL) was injected into IP-UPLC for the determination of vegetable samples.

For the validation of analytical precision, experiments were repeated using ion chromatography (IC) to determine the nitrate levels in the same vegetable juices. The IC analytical conditions were as follows: the HPLC system consisted of a line degasser (880-50, Japan Spectroscopic Co., Ltd., Japan), a pump (880-PU, Japan Spectroscopic Co., Ltd., Japan), an autosampler (GL-7420, GL Sciences Inc., Japan), a column oven (865-CO, Japan Spectroscopic Co., Ltd., Japan), and an electric conductivity detector (Shodex CD-4, Showa Denko K.K., Japan). The IC column was a Shodex IC I-524A (Showa Denko K.K., Japan, 100-mm length with an I.D of 4.6 mm) with a Shodex IC IA-G pre-column (Showa Denko K.K., Japan, 10-mm length with an I.D of 4.6 mm) (Showa Denko K.K., Japan). The eluent consisted of 1 mM phthalic acid (special grade, Wako Pure Chemical Industries, Ltd., Japan) (pH 4.3) at a flow rate of 1.0 mL/min. The pH of the eluent was adjusted with tris(hydroxymethyl)aminomethane (special grade, Wako Pure Chemical Industries, Ltd., Japan). The column temperature was set at 40°C. Aliquot volumes of 20 μL were injected (Ito *et al*. 2005). Then 100-ppm standard solution was injected into the IC as with IP-UPLC to determine the nitrate ion concentration. A paired t-test was used to assess the significance of any differences observed between the nitrate levels determined by IC and IP-UPLC.

## Results and Discussion

Hiraki *et al*. (2003) developed a method of separating five anions ($NO_2^-$, $NO_3^-$, $I^-$, $S_2O_3^{2-}$, $SCN^-$) using IP-HPLC with a C30 column and a UV absorbance detector at 220 nm. A 20-mM phosphate buffer (pH 6.0) containing 2 mM tetrabutylammonium chloride in 15 % (v/v) acetonitrile was used as the mobile phase. At the beginning of this study, a 1.7-mM phosphate buffer (pH 5.4) containing 0.2 mM tetrabutylammonium chloride in 1.5% (v/v) acetonitrile was therefore used as the IP-UPLC eluent. However, this resulted in a split nitrate peak. When the eluent was changed to 0.1% ammonium acetate containing 0.15 mM tetrabutylammonium chloride in 5% (v/v) methanol, nitrate ions eluted stably as a single peak. When the UPLC column split nitrate ion peak into two was used with the eluent, the nitrate ion peak resulted in a split nitrate peak again. The initially used eluent appeared to degrade UPLC column performance, although the specific reasons for the peak split being solved were

unknown. Because a C30 column has yet to be developed for use in UPLC, a UPLC C18 column was used with the aforementioned eluent containing 5% (v/v) methanol. Methanol was required because a weaker eluent is needed with a C18 column relative to a C30 column. Hiraki *et al.* (2003) injected a maximum of 0.4 μg of nitrate ions into eluent containing 2 mM tetrabutylammonium chloride. In the current IP-UPLC method, a maximum of 0.08 μg of nitrate ions was injected with eluent containing 0.15 mM tetrabutylammonium chloride, meaning that the injection of UPLC was reduced to 1/5 of that of IP-HPLC. The retention times of nitrite, nitrate, oxalate, fumarate, and thiocyanate ions were 1.64, 1.79, 2.18, 2.35, and 4.15, respectively. The linearity of the method for nitrate ions was high, as evidenced by $R^2$ of 0.9996 (Fig. 1). The LOD for nitrate ions was 0.044 ng.

Fig. 2 shows an IP-UPLC chromatogram of the leaf stalks of spinach. The main peak on the chromatogram corresponds to nitrate ions, as was also observed in the other samples. The IP-UPLC chromatogram was obtained in 8 minutes. This is 39 minutes less than the time

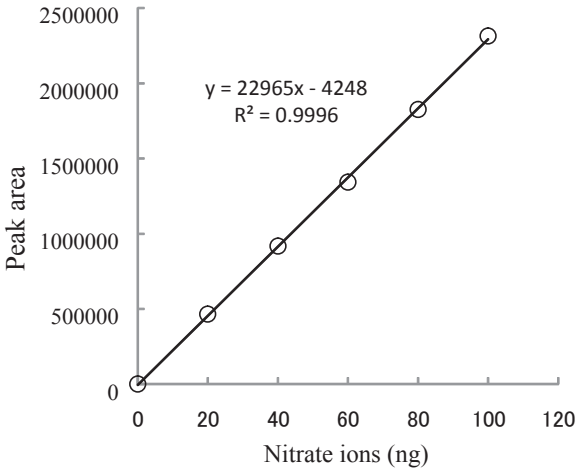


**Fig. 1. Linear IP-UPLC peak area as a function of nitrate ion concentration**

Aliquot volumes of 0.2, 0.4, 0.6, 0.8, and 1.0 μL of 100-ppm standard solution were injected into IP-UPLC using a 10 μL loop in needle overfill mode.

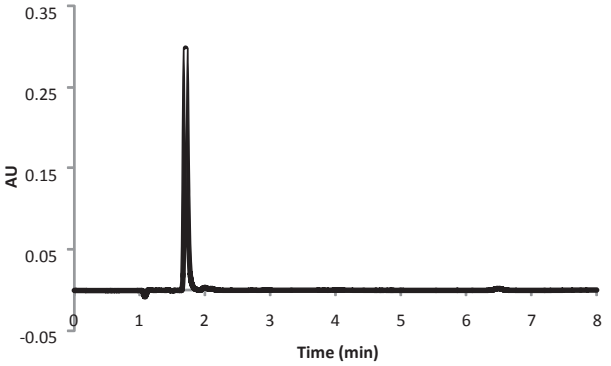


**Fig. 2. IP-UPLC chromatogram of fresh juice from spinach leak stalk**

The main peak of retention times 1.79 on the chromatogram corresponds to nitrate ions (detection at 220 nm). The eluent was 5% (v/v) methanol, 0.15 mM tetrabutylammonium chloride (ion-paired reagent), and 0.1% (w/v) ammonium acetate (pH 6.2). The eluent flow rate was 0.21 mL/min. The column was a 100-mm Acquity UPLC™ BEH SHIELD $RP_{18}$ with a particle size of 1.7 μm and I.D. of 2.1 mm (Waters, USA). The temperature of the column was maintained at 25°C. The aliquot volume (0.8 μL) of 100-fold diluted vegetable juice was injected for determination.

**Table 1. Analytical precision of nitrate levels and recovery percent of nitrate ions spiked in 100-fold diluted fresh vegetable juices (The number of repeat n = 3)**

| Vegetable samples | Average (ppm) | ±Standard deviation | Recovery (%) | ±Standard deviation |
|---|---|---|---|---|
| Japanese radish | 1052 | 27 | 102.8 | 2.4 |
| Celery | 4468 | 86 | 98.4 | 3.7 |
| Leaf stalk of Komatsuna* | 4790 | 37 | 99.8 | 7.0 |
| Carrot | 251 | 19 | 109.5 | 5.7 |
| Radish | 1381 | 13 | 96.0 | 1.9 |
| Root of Matsusaka-Akana | 2103 | 177 | 100.2 | 5.7 |
| Stem of Cabbage | 2761 | 78 | 101.9 | 7.2 |
| Leaf stalk of Qing ging cai | 4608 | 34 | 100.3 | 1.1 |
| Leaf stalk of Spinach | 6182 | 40 | 102.1 | 2.8 |
| Leaf stalk of Komatsuna** | 9602 | 118 | 100.8 | 0.8 |

*Sampling in Winter.
**Sampling in Autumn.

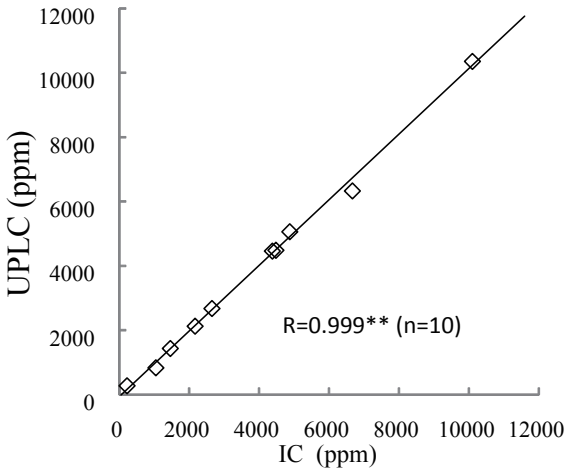


**Fig. 3. Plots of nitrate ion concentration in fresh vegetable juice samples determined by IC and IP-UPLC (**: Significant at 1% level of correlation coefficient)**
The oblique line indicates X:Y=1:1.
Paired t-tests showed no significant differences between the two data sets.

required to obtain a complete IC chromatogram of the same sample (Ito *et al*. 2005). In nitrate-spiked samples of diluted fresh vegetable juice, the recovery percent consistently approached 100% (Table 1). The worst recovery percent (109.5%) was measured with carrot juice, which contained relatively low levels of nitrate ions. When the nitrate ion concentration is low as in carrots, analytical precision may be adversely affected.

Nitrate levels determined by IP-UPLC agreed with those obtained using IC with R of 0.999 (n = 10). Moreover, paired t-tests showed no significant differences between the two data sets (Fig. 3). In a study by Butt *et al*. (2001), water extracts of lettuce and spinach required a preliminary cleanup step with a C18 column prior to measuring nitrate levels by normal-phase IP-HPLC. However, similar samples, analyzed by IP-UPLC in the current study, did not require any additional cleaning steps.

Although the standard analytical conditions of IC call for an eluent containing 2.5 mM phthalic acid at pH 4.0 (http://www.shodex.com/ja/dc/07/02/01.html), these conditions result in an overlap of nitrate and oxalate ion peaks. When 1.0 mM phthalic acid at pH 4.0 was used as the eluent (Yasui *et al*. 1992), the nitrate and citrate ion peaks overlapped. Changing the pH of the IC eluent from 4.0 to 4.3 separated the nitrate and citrate ion peaks (Ito *et al*. 2005). Therefore, IC eluents must be tailored specifically for the vegetable samples being analyzed.

Thus, IP-UPLC is suitable for determining the nitrate ion levels in vegetable juice.

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