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# A High Performance Liquid Chromatography Method for Determining Nitrate and Nitrite Levels in Vegetables

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## ABSTRACT

In this study, a simple, rapid, precise and sensitive high performance liquid chromatography (HPLC) method using an UV detector was developed for the determination of nitrate and nitrite amounts in vegetables. The optimal conditions were found and applied using 0.01 M octylammonium orthophosphate of aqueous 30% (v/v) methanol of pH 7.0 for the mobile phase at flow rate of 0.8 mL/min. The total time for one sample analysis was within 10 min. Recoveries of nitrate and nitrite were between 96.6% to 105.7%. The calibration curves of nitrate and nitrite were extremely linear, where both correlation coefficients were greater than 0.9990 in the range of 0.1~100.0  $\mu\text{g/mL}$ . Therefore, this HPLC method is applicable for simultaneously determining the nitrate and nitrite levels in vegetables. For application, nitrate and nitrite amounts in 12 marketed vegetables were determined by this HPLC method. The results showed nitrate and nitrite contents varied in a range of 225~4,410 mg/kg and <5~200 mg/kg, respectively.

Key words: vegetables, nitrate, nitrite, high performance liquid chromatography (HPLC)

## INTRODUCTION

Vegetables constitute a major source of human exposure to nitrate and nitrite in human diet. It was estimated that vegetables contribute approximately 80~92%<sup>(1,2)</sup> and 16~43%<sup>(3)</sup> of the average daily dietary intake of nitrate and nitrite, respectively. Nitrate concentrations vary significantly, ranging from 1 to 10,000 mg kg<sup>-1</sup> fresh weight, while nitrite levels in fresh vegetables are extremely low (<2 mg kg<sup>-1</sup>), as published literatures stated<sup>(1,3,4,5)</sup>. Furthermore, nitrate and nitrite have been routinely added during the curing process of certain meat products, serving as a preservative against microorganisms, such as *Clostridium botulinum*, that can cause food poisoning. Both nitrate and nitrite are monitored regularly because of their toxicity. There is an increased awareness of the relationship between nitrate and nitrite content in food and water supplies and methemoglobinemia<sup>(6,7)</sup> found in infants and the formation of carcinogenic nitrosamines<sup>(3)</sup>.

The European Commission (EC) in 1997 established maximum levels of nitrate in lettuce and spinach<sup>(8,9)</sup> and this has been adopted by the UK and other Member states since February 15, 1997. In China, the suggested maximum levels of nitrate in vegetables of 3,100 mg/kg/day have also been established<sup>(10)</sup>. It was even set up by the EC that vegetables producers should gradually modify their farming methods by applying the codes of Good Agricultural Practice (GAP) recommended at national levels, so as to comply with the maximum levels to reduce

nitrate levels and assure concentrations in lettuce and spinach to avoid possible risks to the public health.

A variety of analytical methods for the determination of nitrate and nitrite have been developed and applied to the analysis of food, water, plants and other matrices. These methods include spectrophotometry<sup>(11,12)</sup>, high performance liquid chromatography (HPLC)<sup>(13,14)</sup>, ion chromatography (IC)<sup>(15,16)</sup>, gas chromatography (GC)<sup>(17)</sup>, polarographic method<sup>(18)</sup> and capillary electrophoresis (CE)<sup>(12,19)</sup>. Spectrophotometric methods are traditionally used to determine nitrate and nitrite in food; however, a lack of high sensitivity for the detection of trace levels of the analytes could cause results to be unreliable due to sample matrix interferences. Capillary electrophoresis is a recent developing separation technique with the main advantages of fast simultaneous detection of a wide variety of anions, small sample requirement and low buffer consumption. During the past decade, a number of IC and HPLC methods have been developed, which are generally characterized by faster, more accurate and higher sensitivity than the spectrophotometric methods. Different extraction procedures are dependent on the matrix in samples, which include simple extraction with water followed by deproteinization for biological (food) samples, to ultracentrifugation or ultrafiltration for clinical samples<sup>(12)</sup>.

In the present study, the rapid, accurate and sensitive method for determining nitrate and nitrite by HPLC with UV absorbance detection has been further optimized with reference to published procedures of Cheng and Tsang<sup>(14)</sup>. UV absorbance was specific for nitrate and nitrite, eliminating the interference from other ions present at much higher

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concentrations. A simple, frequent and time-saving boiled water method for extraction and filtration for direct analysis was used in this experiment. The main objective of this study was to develop the most optimal conditions for the HPLC method, which can be applied to the determination of nitrate and nitrite in vegetables. This analytical method was then implemented to determine the nitrate and nitrite levels in some of the most commonly consumed vegetables purchased in Taipei supermarkets.

## MATERIALS AND METHODS

### I. Instruments and Chemicals

#### 1. High performance liquid chromatography system

A Shimadzu LC-10AT high performance liquid chromatography system, equipped with a SPD-10A UV-VIS detector and C-R4A chromatopac integrator, was utilized in this study. A Phenomenex Luna C18 HPLC column (5  $\mu\text{m}$ , 250  $\times$  4.6 mm i.d.) was employed for separation. Milton Roy Spectronic Array 3000 spectrophotometer (Waters, New Jersey, USA) was also used. pH measurements were determined using a Metrohm pH meter and a combined glass-calomel electrode. Vegetable samples were homogenized using Nihoneiki Kaisha ACE homogenizer.

#### 2. Chemicals

Octylamine and all other reagents (analytical grade) were purchased from Merck Co. (Darmstadt, Germany). Double deionized water was prepared from a Milli-QSP60 system (Millipore).

### II. Methods

#### 1. Sample preparation for analysis

A total of 12 fresh vegetables were collected from 2 different supermarkets and one organic food retailer in Taipei city. Prior to analysis, non-edible parts of each sample were removed. Each sample was then cut and homogenized with a cutter and a homogenizer, and immediately stored at  $-20^{\circ}\text{C}$  before it is subject to analysis. Fifty mL of deionized water was added to the well-homogenized sample weighed 1g in a 100 mL volumetric flask. The flask was then moved in a boiling water bath for 20 min. at  $80^{\circ}\text{C}$ , shaken up and laid on the table until cooled down, and then diluted to a final volume of 100 mL with deionized water. It was followed by filtering through a 0.45  $\mu\text{m}$  syringe filter. The first filtrate of 3 mL was discarded and the following filtrate of 1 mL was collected for the determination of the nitrate and nitrite. All samples were immediately analyzed within 1 hr after sample preparation.

#### 2. Preparation of mobile phase and standard solutions

The various aqueous methanol concentrations (20, 25, 30 and 40%, v/v) and different pH values (6.5, 7.0 and 7.5) of mobile phase solution at various flow rates (0.5, 0.8 and 1.0 mL/min) were tested on running HPLC chromatograms. Then a series of aqueous methanol (20-40%, v/v) with the addition of a 0.01 M solution of octylammonium orthophosphate reagent was prepared, followed by the adjustment of pH values (6.5~7.5) by adding orthophosphoric acid and then filtering (0.45  $\mu\text{m}$  filter membrane) before injection. Eventually, the optimal condition of the mobile phase (30% methanol, pH 7.0 and flow rate 0.8 mL/min) was used in the experiment.

Standard solution diluted to a series of concentrations containing 0.1, 1, 10, 50, 100  $\mu\text{g}/\text{mL}$  of sodium nitrate and sodium nitrite were prepared and stored at  $4^{\circ}\text{C}$  for use. The solutions were freshly prepared every 7 days. The calculated standard curve and correlation coefficients can be indicative of the linearity within the tested range of concentrations.

#### 3. HPLC analysis

The mobile phase solution was allowed to pass through the HPLC column until a stable baseline signal was equilibrated. The flow rate was 0.8 mL/min and the detecting UV wavelength was 213 nm. When the injections of the standard solution gave reproducible retention times and peak areas, each sample solution was then injected for analysis. The peaks of the sample were identified by comparison to the respectable peaks of the standards. The amounts of nitrate and nitrite in the test solution were calculated from the peak areas by using linear regression equations of nitrate and nitrite standard curves. If the curve of the peak areas was larger than that of the maximum amount from the standard curve, the test solution was diluted to appropriate concentrations. The injection volume was 10  $\mu\text{L}$ . At the end of the analysis, the HPLC column was refreshed by passing a solution of water: methanol (1: 1, v/v) for 4 hr at a flow rate of 0.5 mL/min.

#### 4. Reproducibility test

Intra-day (running 3 times on the same day), and inter-day tests (running 3 times within successive 7 days with at least 24-hr as intervals) were conducted. The reproducibility precision values were characterized by the coefficient of variations (CV, %).

#### 5. Recovery test

A series of various concentrations of 1, 10, 50 and 100  $\mu\text{g}/\text{mL}$  standard solutions containing nitrate and nitrite were spiked into organic broccoli samples. Each concentration spiked was analyzed in triplicate, including a blank test to evaluate the average recoveries.

## 6. The detection limit

The detection limit is defined as the concentration of the standard solution of which the ratio of peak height to noise was 3.

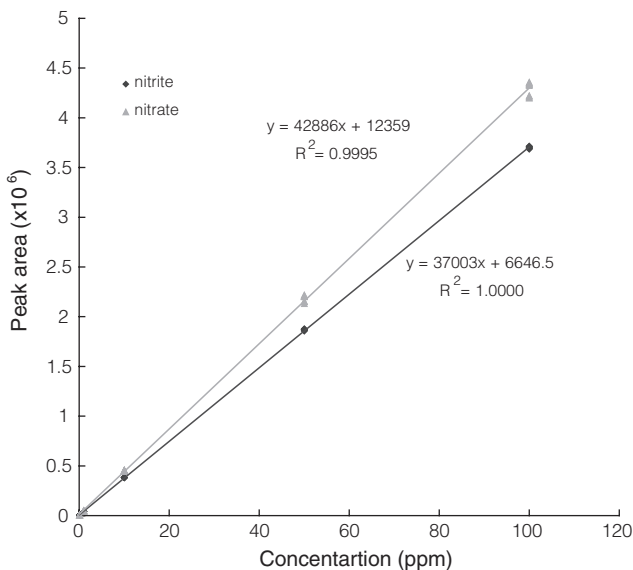
## RESULTS AND DISCUSSIONS

### 1. Assay by HPLC

In this study, a simple, efficient and accurate improved HPLC method mainly derived from the procedures of Cheng and Tsang<sup>(14)</sup> was modified for the determination of nitrate and nitrite in vegetables. Through extensive trials for obtaining the most optimal conditions for the determination, the modified HPLC method using 30% (v/v) aqueous methanol with the addition of 0.01 M octylammonium orthophosphate with adjusted pH value to 7.0 as the mobile phase and a flow rate of 0.8 mL/min was applied. The other related HPLC conditions of the method were as described previously. The total analytical time of the method for one sample analysis was within 10 min. The retention times of nitrite and nitrate were  $7.86 \pm 0.01$  min and  $9.02 \pm 0.02$  min, respectively.

### 2. The linearity of the standard curve

Figure 1 provided the standard curves of nitrate and nitrite. Linearities were obtained over the tested concentration range of 0.1~100  $\mu\text{g/mL}$  of nitrate and nitrite, respectively. The linear regression equations of nitrate and nitrite



**Figure 1.** Standard curves of nitrate and nitrite. The linear regression equations for nitrate and nitrite standard curve were calculated as  $y = 42886x + 12359$  ( $R^2 = 0.9995$ ) and  $y = 37003x + 6646.5$  ( $R^2 = 1.0000$ ), respectively; where  $y$  is the value of peak area and  $x$  is the value of various concentrations of standard solutions using the HPLC method.

standard curves were calculated as  $y = 42886x + 12359$  ( $R^2 = 0.9995$ ) and  $y = 37003x + 6646.5$  ( $R^2 = 1.0000$ ), respectively. The correlation coefficients were both greater than 0.999, which showed a very good linearity within the range of 0.1~100  $\mu\text{g/mL}$ .

### 3. The sensitivity of the method

Under the previously determined conditions, the detection limit of nitrate and nitrite, defined as a signal-to-noise ratio of 3, was the same 0.05 mg/L. The method showed a very satisfactory sensitivity and can detect trace levels of nitrate and nitrite ( $<5 \text{ mg kg}^{-1}$ ). If the amount of nitrate and nitrite in vegetables is less than  $5 \text{ mg kg}^{-1}$ , this vegetable may be assumed to be safe for consumption. Therefore, extracting 1 g of sample with 100 mL deionied water solution was very reasonable because the detection limit was criterion safe level and the operation was convenient.

### 4. Reproducibility

Reproducibility of the measurements is evaluated by intra-day and inter-day analysis calculated from the results of 3 replicates and illustrated by the coefficient of variations (CV, %), as shown in Table 1. Repeated trails all obtained CV values less than 1.5%, pointing out high degrees of reproducibility.

### 5. Recovery

**Table 1.** Reproducibility of inter-day and intra-day analysis (n=3)<sup>d</sup>

Concentration ( $\mu\text{g/mL}$ )	Coefficient of variation (CV, %) <sup>c</sup>			
	Nitrite		Nitrate	
	Intra-day <sup>a</sup>	Inter-day <sup>b</sup>	Intra-day	Inter-day
0.1	0.80	1.11	0.79	1.41
1	0.38	0.50	0.95	1.32
10	0.28	0.28	0.34	1.24
50	0.14	0.36	0.34	1.47
100	0.18	0.29	0.29	1.49

a: Intra-day: running three times within 24 hr.

b: Inter-day: running three times within successive 7 days with at least 24-hr intervals.

c: Reproducibilities were evaluated by the coefficient of variations (CV, %).

d: The CV of intra-day and inter-day were calculated from results of 3 replicates.

**Table 2.** Recoveries of nitrate and nitrite spiked into organic broccoli<sup>a</sup>

Spike level ( $\mu\text{g/g}$ )	Recovery (%) <sup>b</sup> and coefficient of variation (CV, %)	
	Nitrate	Nitrite
1	96.6 (1.5)	98.9 (0.1)
10	99.5 (0.1)	105.7 (1.1)
50	108.7 (0.3)	99.3 (0.2)
100	101.8 (1.7)	98.9 (0.4)
Average	102.8 (0.9)	100.7 (0.5)

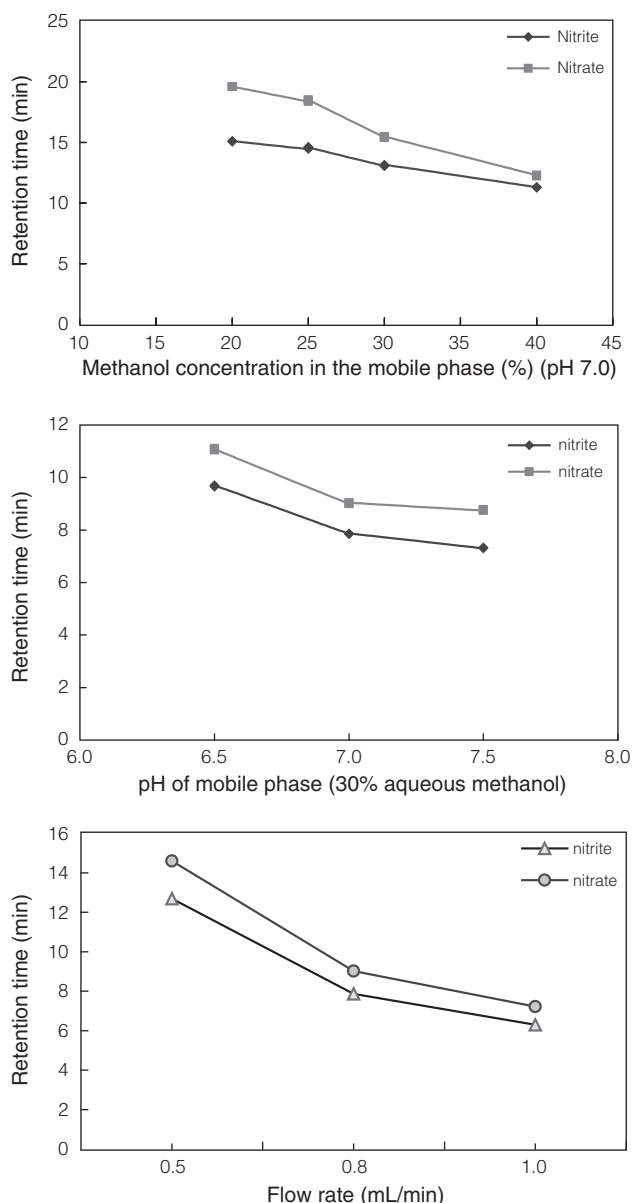
a: The content of nitrate in unspiked organic broccoli was 443.3  $\mu\text{g/g}$ ; nitrite content was not detected.

b: Average of triplicate.

The recoveries of nitrate and nitrite in the study are shown in Table 2. The recoveries of nitrate and nitrite spiked with four amounts (1, 10, 50 and 100  $\mu\text{g/mL}$ ) into vegetable samples were in the range of 96.6~108.7% and 98.9~105.7%, respectively. The average recoveries of nitrate and nitrite were 102.8% and 100.7%, indicating the method is quite accurate.

#### 6. The influence of the mobile phase solution

The various aqueous methanol concentrations (20, 25, 30 and 40%, v/v) and different pH values (6.5, 7.0 and 7.5) of mobile phase solutions at various flow rates (0.5, 0.8 and 1.0 mL/min) were tested on running HPLC chromatograms, as shown in Figure 2. Therefore, although the peaks of



**Figure 2.** The effects of methanol concentration, pH value and flow rate of the mobile phase on the retention time in determining nitrate and nitrite levels using the HPLC method.

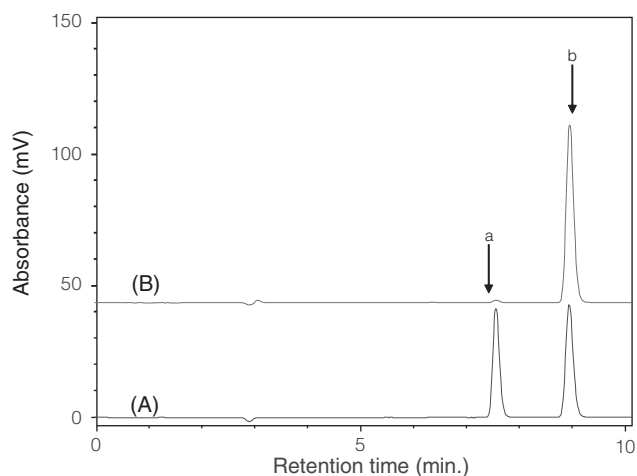
nitrate and nitrite using a flow rate of 1.0 mL/min might appear more rapidly than that of 0.8 mL/min, the latter could differentiate the interfering peaks more clearly. It was concluded that the optimal determined condition for the HPLC method applied in this study was aqueous 30% (v/v) methanol of pH 7.0 for the mobile phase solution at a flow rate of 0.8 mL/min. The results indicated that the running time for one sample was within 10 min.

#### 7. The interference of sampling substances

In this study, due to the constitution of vegetable samples in a simple medium, there were no problems such as protein interference as in the analysis of meat samples for chromatographic analysis, which could result in deteriorating column performance. The vegetable samples were filtered through a 0.45  $\mu\text{m}$  syringe that filtered and isolated possible foreign substances. Moreover, Figure 3 lists the analytical results for the HPLC chromatograms of the nitrate and nitrite standard solutions as well as a representative vegetable sample—organic non-heading Chinese cabbage under the optimal chosen conditions. The peaks of nitrate and nitrite were indicative from a graph of very good resolution and suggested that most of the ions or foreign substances did not bring about any interference in the chosen analytical condition.

#### 8. The contamination of nitrate and nitrite contents in vegetables

The results for nitrate and nitrite analysis of the 12



**Figure 3.** HPLC chromatograms of organic non-heading Chinese cabbage sample (B) and standard solution (A) containing 10  $\mu\text{g/mL}$  of nitrate and nitrite using the HPLC analytical method under the condition for a mobile phase solution of 0.010 M octylammonium orthophosphate of 30% aqueous methanol and adjusted pH value 7.0  $\pm$  0.02. The injection volume of 10  $\mu\text{L}$  and a flow rate of 0.8 mL/min was applied. The individual nitrite and nitrate peaks of the sample and standard solution were shown as arrows a and b as indicated, respectively.

selected vegetables from Taipei supermarkets showed that the nitrate and nitrite contents varied significantly in the range of 225~4,410 mg/kg and <5~200 mg/kg, respectively (Table 3). The wide ranges and large standard variations in nitrate and nitrite levels for the same vegetables purchased from different places and periods were not surprising because nitrate levels in vegetable plants are highly sensitive to inherent and environmental variables such as species, maturity, fertilizer application and storage temperature. In this investigation, green bean sprout, pakchoy, musu bean sprout, lettuce, swamp cabbage and celery had significantly higher nitrate contents than other types of vegetables and were analyzed to have mean concentrations of 4,410 mg/kg, 4,160 mg/kg, 3,910 mg/kg, 3,440 mg/kg, 3,180 mg/kg and 3,020 mg/kg, respectively. The extremely high nitrate amounts have reached hazardous levels. In contrast, broccoli contained the lowest nitrate concentrations with a mean value of 225 mg/kg, while the nitrite level was quite high at 200 mg/kg. Spinach also contributed a higher amount of nitrite at 122 mg/kg.

### CONCLUSIONS

Using 0.01 M octylammonium orthophosphate of aqueous 30% methanol of pH 7.0 for the mobile phase, a simple, rapid, precise and sensitive HPLC method was developed for determining nitrate and nitrite amounts in vegetables. Recoveries of nitrate and nitrite were better than 96%. The method was applied to determine the levels of nitrate and nitrite in 12 selected vegetables. The results showed the method was fast, reliable and sensitive.

Therefore, in the future, the investigation of nitrate and nitrite in vegetables should be continuously implemented to obtain the needed relevant data to ensure the safety of vegetables for public consumption.

**Table 3.** Average contents of nitrate and nitrite in various vegetables determined by the HPLC method

Vegetables	Nitrate* (mg/kg)	Nitrite* (mg/kg)
Organic swamp cabbage	3180 ± 260	58.2 ± 4.2
Organic green bean sprout	4410 ± 310	85.1 ± 5.8
Organic musu bean sprout	3910 ± 220	22.2 ± 2.0
Organic lettuce	3440 ± 290	< 5.0
Organic non-heading Chinese cabbage	2860 ± 80	41.9 ± 3.6
Organic Chinese spinach	1810 ± 160	< 5.0
Chinese cabbage	1220 ± 90	< 5.0
Broccoli	225 ± 20	200.0 ± 16.7
Spinach	2270 ± 210	122.0 ± 8.5
Swamp cabbage	1630 ± 160	10.1 ± 1.1
Pak choy	4160 ± 80	< 5.0
Chinese kale	2340 ± 220	83.8 ± 5.3
Crown daisy	2640 ± 150	< 5.0
Celery	3020 ± 290	< 5.0
Malabar spinach	641 ± 50	28.1 ± 2.2

\* mean ± S.D. (n = 3).

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