High Performance Liquid Chromatography Method for Determination of Nitrite and Nitrate in Vegetable and Water samples

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Abstract:
A reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous determination of nitrite and nitrate in water and leafy vegetables has been developed. Separation was achieved on a reversed-phase C13 Column, (250 mm × 4.6 mm, i.d. 5 µm) using an isocratic method with aqueous methanol mobile phase. The flow rate was 0.7 ml min⁻¹, temperature of the column was 25°C and the detection was made at 222 nm. The total run time was about 10 min. Recoveries of nitrite and nitrate were in the range 98.3 to 100.3 %. The calibration curves of nitrite and nitrate were extremely linear, where both correlation coefficients were greater than 0.9990 in the range of 0.1 to 100.0 ppm. Detection and quantitative limits were calculated. Excellent accuracy and precision were obtained for nitrite and nitrate. This method is used for quantitative determination of nitrite and nitrate in water and vegetables.

Key words: Nitrite, Nitrate, High performance liquid chromatography (HPLC)

I. Introduction
Nitrite and nitrate in water, agricultural products, and food are of concern due to their adverse effects on human and animal health. In the drinking water standards established by U.S. Environmental Protection Agency (EPA), the maximum permissible contamination levels are 10 mg/L for nitrate and 1 mg/l for nitrite, respectively [1]. The main anthropogenic sources of nitrates in the surroundings are metropolitan and industrial wastes and artificial fertilizers. Nitrogen oxides present in the air and originating from natural and anthropogenic sources
(combustion, transportation) after the reactions with water come back to the earth surface in the form of acid rains [2]. Nitrites appear as intermediates in the nitrogen cycle. They are unstable and, depending on conditions, are transformed into nitrates or ammonia. Their presence in water can be a result of water processing or use of nitrite salts as corrosion inhibitors. Nitrites are commonly used in preservatives. To surface waters they get introduced from the same sources as nitrates, i.e. in municipal wastes, industrial wastes, mining wastes and with water flowing in from artificially fertilized fields. The sources of ammonium ions in surface waters are biochemical decomposition reactions of organic nitrogen compounds, reduction of nitrites and nitrates by hydrogen sulfide and iron (II), humus substances (or other reducing compounds) and, first of all, municipal wastes, industrial wastes and animal farm wastes. Nitrogen compounds enhance eutrophication of surface waters. Organic nitrogen compounds undergo biochemical decomposition into nitrites and later oxidized to nitrates [3, 4].

Various analytical techniques have been developed to determine nitrite and nitrate, oxidation metabolites of nitric oxide (NO) in biological samples [5, 6, 7, and 8]. In previous studies nitrite/nitrate was measured after exposing plasma to Copper-Cadmium-Zinc catalyst to convert nitrate to nitrite and then adding the effluent of the catalyst product to Griess reagent [6]. This procedure carries some major disadvantages. Nitrite only is detected by Griess reagent while nitrate has to be measured indirectly after reduction to nitrite. It is, therefore, not possible to predict how much of the sample's nitrate is converted to nitrite during the reduction. Additionally, the complex formation of nitrite and Griess reagent is a variable process and may influence the measurement of concentrations [6, 9]. Griess reagent reacts with free biogenic amines other than nitrite and may produce false positive results [6]. UV/Vis absorbance [10] and electrochemistry allows simultaneous detection of nitrite and nitrate but is vulnerable to severe interference from chloride present in biological samples [11]. Chemiluminescence [12] and fluorescence detection [13] improve the assay sensitivity and is unaffected by chloride but cannot be applied to simultaneous analysis of nitrite and nitrate. Recently developed fluorometric HPLC method involves the precolumn derivatization of nitrite with 2, 3-diaminonapthalene and enzymatic conversion of nitrite to nitrate [14]. Measurement of nitric oxide itself is complicated by its short half life and would, therefore, require on-line measurement which is suitable for routine use for analysis of biological samples [15].
After an exhaustive investigation, it appears that HPLC, which is a very sensitive, rapid and accurate method with low detection limits for nitrite and nitrate, carries none of these disadvantages and nitrite and nitrate can be measured directly. The chromatographic system developed in this study, after investigating alternatives, readily resolved NO$_2^-$ and NO$_3^-$, with peaks being separated within a minute [15].

In this work, a new reversed-phase HPLC method has been developed for the analysis of nitrite and nitrate in various samples. The method uses RP-HPLC based on isocratic mode analogs to nitrite and nitrate. The nitrate and nitrite anions were well separated by the selected HPLC column under a moderate pH condition. Derivatization was not required for this method. The study was focused on the selection and optimization of reversed-phase HPLC conditions, the demonstration of method performance (sensitivity, accuracy, and precision) and the investigation of interferences from common inorganic anions.

II. Instruments and chemicals

1. High performance liquid chromatography system

The isocratic HPLC system used to perform a chromatographic separation was consisted of LC-2010 CHT (Shimadzu Corporation, Japan). HPLC system contains a reversed-phase C13 Column, (250 mm × 4.6 mm, i.d. 5 µm), solvent delivery pump, auto sampler fitted with 100 µl loop, a column oven and UV detector module. The output signal was monitored and integrated using LC solution software (Shimadzu Corporation, Japan).

2. Chemicals

The reagents were of analytical grade. HPLC grade Methanol was obtained from Merck (Darmstadt, Germany). Water was purified on a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used to prepare all solutions. All chemicals used for the preparation of buffer and other solutions were of analytical grade.

III. Methods

1. Sample preparation for analysis

All vegetable samples including coriander leaves, radish leaves, spinach leaves and cabbage were bought from the local morning market. The vegetable samples were carefully rinsed with tap water, distilled water and deionized water, and were divided into smaller parts. They
were dried at 105 °C for 24 h to constant weight. The dried samples were pulverized and sieved to 60 mesh. The resultant powders were stored in desiccators until analysis.

Dry and powdered samples of vegetables weighing to 0.1000 g were added to a beaker containing approximately 15 ml water. The sample solutions were stirred and heated in boiling water bath for 8 h and transferred to 25 ml volumetric flasks. The sample solutions were shaken for 30 min in supersonic wave, and diluted to a final volume of 25 ml with deionized water. All samples were filtered through membrane filter before use.

2. Preparation of mobile phase and standard solutions

The various aqueous methanol concentrations (20, 25, 30 and 40 %, v/v) and different pH values (2.0, 2.5, 3.0 and 3.5) of mobile phase solution at various flow rates (0.4, 0.7 and 1.0 ml/min) were tested on running HPLC chromatograms. To prepare buffer, 1.08 g of hexane sulphonic acid sodium salt and 1.36 g of potassium dihydrogen phosphate were dissolved in 1000 mL HPLC grade water and 5 ml of triethylamine was added to it and the pH was adjusted to 3.0 with orthophosphoric acid. For the preparation of mobile phase, buffer and methanol were mixed in a ratio of 80:20, 75:25, 70:30, and 60:40 and are filtered through 0.45 µm membrane filters and sonicated for degassing in an ultrasonic bath and then again filtered through 0.45 µm membrane filter before injection. Eventually, the optimal condition of the mobile phase (20% methanol, pH 3.0 and flow rate 0.7 ml/min) was used in the experiment. Standard solution diluted to a series of concentrations containing 0.1, 5, 10, 50, 100 ppm of potassium nitrate and sodium nitrite were prepared and stored at 4 °C for use. The solutions were freshly prepared every seven days. The standard curve and calculated correlation coefficients represents linearity within the tested range of concentrations.

3. HPLC analysis

The mobile phase is filtered through 0.45 µm membrane filter and sonicated for degassing in an ultrasonic bath and then allowed to pass through the HPLC column until a stable baseline signal was observed. The flow rate was 0.7 ml/min and the detecting UV wavelength was 222 nm. The injections of the standard solutions gave reproducible retention times and peak areas with below 2.0 relative standard deviation (RSD). The peaks of the sample were identified by the comparison to the respective 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 suitable concentrations. The injection sample volume was 20 µl.

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 h as intervals) were conducted. The reproducibility precision values were characterized by the relative standard deviation (RSD %).

5. Recovery test
A series of various concentrations of 0.1, 0.5, 1, 5 and 10 ppm standard solutions containing nitrite were spiked into vegetable and water samples. Each concentration spiked was analyzed in triplicate, including a blank test to evaluate average recoveries. Same procedure is applied for nitrate on 1, 5, 10, 50 and 100 ppm concentrations. The average of recovery is determined and recovery precision values were characterized by the relative standard deviation (RSD %).

IV. Results and discussions
1. Optimization of the mobile phase, pH and flow rate
The various aqueous methanol concentrations (10, 20, 25 and 30 %, v/v) and different pH values (2.0, 2.5, 3.0 and 3.5) of mobile phase solutions at various flow rates (0.4, 0.7 and 1.0 ml/min) were tested on running HPLC chromatograms. As shown in Figure 1 the 20 % methanol gives the proper resolution. On 10 % methanol the resolution is achieved but the retention time (RT) is long and 25 % and 30% both gave less resolution. Figure 2 shows the 3.0 is the optimum pH for the best resolution and retention time. This pH is intermediate between the pKa values of both the analytes. In the case of flow rate the Figure 3 shows that the 0.7 ml/min is the preferred flow rate. On 0.4 ml/min flow rate the RT is to long and at 1.0 ml/min flow rate the proper resolution is not achieved. So the 20 % methanol at pH 3.0 and 0.7 ml/min flow rate is considered as optimum conditions. Figure 4 shows the resolved peak of both the analytes on the developed HPLC method.
Figure 1. Effect of Methanol concentration variation on retention times of nitrite and nitrate

Figure 2. Effect of pH variation on retention times of nitrite and nitrate

Figure 3. Effect of mobile phase flow rate variations on retention times of nitrite and nitrate
2. Assay of nitrite and nitrate in vegetables and water samples by HPLC

In this study, efficient and accurate HPLC method is developed 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 20% (v/v) aqueous methanol with the addition of phosphate buffer was applied for real samples. The prepared mobile phase buffer is filtered through 0.45 μm membrane filter and sonicated for degassing in an ultrasonic bath and then filtering (0.45 μm filter membrane) before injection and a flow rate of 0.7 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 5.189 ± 0.01 and 6.645 ± 0.02 min, respectively.

3. The linearity of the standard curve

Figure 5 and 6 provided the standard curves of nitrite and nitrate. Linearity was obtained over the tested concentration range of 0.1, 0.5, 1, 5 and 10 and 1, 5, 10, 50 and 100 ppm of nitrite and nitrate, respectively. The linear regression equations of nitrite and nitrate standard curves were calculated as $y = 1703x + 69.43$ ($R^2 = 0.9990$) and $y = 376.6x + 71.17$ ($R^2 = 0.9990$) respectively. $y$ is the value of peak area and $x$ is the value of various concentrations of standard.
solutions. The correlation coefficients were both greater than 0.999, which showed a very good linearity within the range receptive to nitrite and nitrate.

![Graph of NO₂ concentration vs. Peak Area]

**Figure 5.** Standard curve of nitrite

![Graph of NO₃ concentration vs. Peak Area]

**Figure 6.** Standard curve of nitrate

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 (RSD, %), as
shown in Table 1 and Table 2. Repeated trails all obtained RSD values less than 2.0 %, pointing out high degrees of reproducibility.

**Table 1.** Reproducibility results of inter-day and intra-day analysis for nitrite (n=3)

<table>
<thead>
<tr>
<th>Conc. of Nitrite ppm</th>
<th>% RSD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>0.1</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 2.** Reproducibility results of inter-day and intra-day analysis for nitrate (n=3)

<table>
<thead>
<tr>
<th>Conc. of Nitrate in ppm</th>
<th>% RSD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>1</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>100</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**5. Recovery**

The recoveries of nitrite and nitrate in the study are shown in Table 3 and 4. The recoveries of nitrite for five concentrations (0.1, 0.5, 1, 5 and 10 ppm) into vegetable samples were in the range of 96.4 to 100.3 % and nitrate for five concentrations (1, 5, 10, 50 and 100 ppm) into vegetable samples were in the range of 98.3 to 100.3 %. The average recoveries of nitrite and nitrate were 98.73% and 99.03%, indicating that the method is quite accurate.

**Table 3.** Recovery results of nitrite analysis (n=3)

<table>
<thead>
<tr>
<th>Spiked level</th>
<th>% Average Recovery</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>96.47</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>0.5</td>
<td>99.43</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>97.11</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>100.26</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>100.37</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Average</td>
<td>98.73</td>
<td></td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table 4. Recovery results of nitrate analysis (n=3)

<table>
<thead>
<tr>
<th>Spiked level</th>
<th>% Average Recovery</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.30</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>99.11</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>100.30</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>50</td>
<td>98.90</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>100</td>
<td>98.54</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Average</td>
<td>99.03</td>
<td></td>
<td>0.9</td>
</tr>
</tbody>
</table>

6. Comparison with other analytical techniques

Table 5. HPLC methods for nitrite and nitrate estimation

<table>
<thead>
<tr>
<th>Method</th>
<th>Mode</th>
<th>Detector</th>
<th>Mobile phase</th>
<th>Linear range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsikas et al. [16]</td>
<td>RP</td>
<td>UV (333 nm)</td>
<td>10 mM NaH₂PO₄ in acetonitrile-water (15:85, v/v) (pH 2.0)</td>
<td>0-50 µM for NO₂⁻ 0-100 µM for NO₃⁻</td>
</tr>
<tr>
<td>Yamada and Nabeshim [17]</td>
<td>RP</td>
<td>VIS (540 nm)</td>
<td>10% methanol containing 0.15 M NaCl/NH₄Cl and 0.5 g/L Na₄-EDTA</td>
<td>N.R.</td>
</tr>
<tr>
<td>Rizzo et al. [18]</td>
<td>RP</td>
<td>UV (220 nm) and ECD</td>
<td>10 mM n-octylamine (pH 6.0)</td>
<td>1-1000 µM</td>
</tr>
<tr>
<td>Li et al. [19]</td>
<td>RP</td>
<td>Fluorescence</td>
<td>15 mM sodium phosphate buffer (pH 7.5) containing 50 % methanol (v/v)</td>
<td>12.5-2000 nM</td>
</tr>
<tr>
<td>Present</td>
<td>RP</td>
<td>UV (222 nm)</td>
<td>Phosphate buffer (pH 3.0) containing 20 % methanol</td>
<td>0-10 ppm for NO₂⁻ 0-100 ppm for NO₃⁻</td>
</tr>
</tbody>
</table>

In Table 5, comparison of present method developed for estimation of nitrite and nitrate with previously developed methods is summarized. The detection used in current method is UV which is cheaper and sufficiently sensitive as compared to other detectors. The used phosphate buffer containing potassium dihydrogen phosphate is better than sodium phosphate and the pH of the buffer is in such a way that the pKa values of both the analytes are readily approachable from this pH. Also the common run time for previous developed methods is about 15 min but in current work run time is about 10 min. The method is directly applicable for determination of nitrite and nitrate from the vegetable and water samples.
7. The contamination of nitrite and nitrate contents in vegetables and water

The results for nitrite and nitrate analysis of the eight selected vegetables from India markets and water samples showed that (Table 6) the nitrite and nitrate contents varied significantly in the range of 0-9.4 mg/kg and 58-1540 mg/kg, respectively. The wide ranges and large standard variations in nitrite and nitrate 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. The nitrate amounts in some samples have reached hazardous levels.

Table 6. Average nitrite and nitrate levels in vegetable and water samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nitrite mg/kg</th>
<th>Nitrate Mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coriander</td>
<td>5.2</td>
<td>115</td>
</tr>
<tr>
<td>Radish</td>
<td>7.1</td>
<td>221</td>
</tr>
<tr>
<td>Beet</td>
<td>10</td>
<td>1201</td>
</tr>
<tr>
<td>Spinach</td>
<td>9.4</td>
<td>1540</td>
</tr>
<tr>
<td>Sorrel</td>
<td>2.1</td>
<td>135</td>
</tr>
<tr>
<td>Cabbage</td>
<td>3.9</td>
<td>60</td>
</tr>
<tr>
<td>Tomato</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td>Mint</td>
<td>6</td>
<td>164</td>
</tr>
<tr>
<td>Tap Water</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>Ground water</td>
<td>0.4</td>
<td>5</td>
</tr>
</tbody>
</table>

V. Conclusion

The phosphate buffer of pH 3.0 and 20 % methanol as mobile phase is optimum for nitrite and nitrate analysis using HPLC method. The developed method is rapid, precise and sensitive and successfully applied for determining nitrite and nitrate amounts in vegetable and water samples. Recoveries of nitrite and nitrate were better than 97%. Hence the method may be applied for estimation of nitrite and nitrate in water and other environmental samples.
VI. References


