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Original scientific paper

DEVELOPMENT OF HPLC METHOD FOR ANALYSIS OF NITRITE AND NITRATE IN VEGETABLE

A. Najdenkoska*

Institute of Public Health, 50 Divisia 6, 1000, Skopje, Republic of Macedonia

*corresponding author: anitanajdenkoska@gmail.com

Abstract
The presence of nitrates and nitrites in food is associated with an increased risk of gastrointestinal cancer and, in infants, methemoglobinemia. Approximately 80% of dietary nitrates are derived from vegetable consumption; sources of nitrites include vegetables, fruit, and processed meats. Nitrate may be reduced into cancerogenic nitrite under various condition. In this work development of a simple HPLC method for simultaneous analysis of nitrite and nitrate was performed. The retention of nitrate and nitrite on the sorbent RP-18 endcapped was studied under the conditions of ion-pair high-performance liquid chromatography, depending on the concentration of organic solvent (methanol) in the mobile phase, pH of the phosphate buffer solution and flow of mobile phase. The optimum conditions were selected for the separation of nitrite and nitrate in the isocratic elution mode within 15 min. A procedure was developed for their determination in vegetable; the detection limit was 50 mg/L for nitrite and 25 mg/L for nitrate. The method is relatively simple, sensitive, selective and can be applied to the monitoring of nitrite and nitrate in vegetable products.
Key words: HPLC, vegetables, nitrate, nitrite

Introduction

The simultaneous determination of nitrite and nitrate in different matrices is of vital significance due to their toxicity to infants and causing methemogloninemia. Highly cancerogenic N-nitrozamines could be formed by the reaction of nitrite with secondary amines and amides, which are generally present in food items, such as meat and fish, where nitrite is used as preservatives (Bilal Butt at al., 2001). Other potential sources of nitrite contamination are wastewater, vegetables and processed food. A variety of analytical methods for determination of nitrite and nitrate have been developed and applied to the analysis of food. These methods include spectrophotometry, high performance liquid chromatography, ion chromatography, polarographic methods and capillary electrophoresis (S-S. Chou, 2003). Considering the physicochemical properties of nitrite and nitrate there is possibility to use liquid chromatography for their accurate and precise determination. In this work Reverse Phase Ion Pair Chromatography and method of suppression of ion is used. Because stationary phase is non-polar it could not be used for separation of charged analytes, so their separation is possible by introducing ion pair reagent to obtain molecular form of analytes, shown on Figure 1.

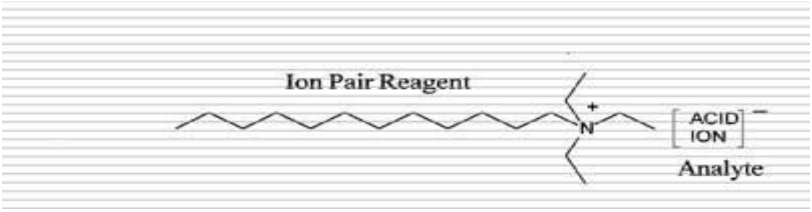

Figure1. Interaction of Ion Pair Reagent with analyte

Materials and methods

Chemicals

All solutions were prepared with distillated deionized water and all chemicals were of analytical reagent grade. Octylamine used as ion-pair reagent was obtained from Supelco. Methanol and standards sodium nitrite and nitrate were supplied from Merck. Standard stock solution was 1000 mg/L for each anion and was prepared together by dissolving appropriate amount of these compounds in deionized water. The working solutions were prepared by serial dilution of stock solution.

Instrumentation

A Perkin-Elmer HPLC system employed for this work was fitted with detector UV–Diode Array 235C, pump, autosampler and column oven Series 200. Analytical balance Sartorius, ultrasonic bath Fisherbrand FB 11021, pH meter WTW 330 Weilheim and ultra-ware KONTES system for filtration of mobile phase were used. Brand transferpettor (1 - 5 mL) and Socorex (10 – 100 μL) were used for precise measurement.

Mobile phase

The mobile phase was consisted from aqueous 10% methanol phase (*V/V*), (aqueous phase is 0.01M octylamine prepared by dissolving of 1.29 g in 900 mL deionized water and adjust pH to 7 with 10 % phosphoric acid and fill up to 1L).

Sample preparation

Samples of spinach and lettuce were homogenized in electrical blender. 5g of samples was diluted with 100 mL extraction solution of methanol: water (1 : 1, *V/V*), (J Salomez and G Hofman (2002)). It was ultrasonically homogenized. Extract was passed through a 0.45 μm Milipore syringe and then through a RP-18e column for separation and quantification of nitrite and nitrate.

Results and discussion

Nitrates and nitrites are ionized compounds. As using ion-pair reagent which increase retention time and improve separation of charged analytes isocratic elution mode was adopted. At the beginning of this work different stationary phases were employed (RP-8, RP select B and RP-18). When stationary phase RP-8 is used 22 minutes for determination of nitrates was needed (Fig 2).

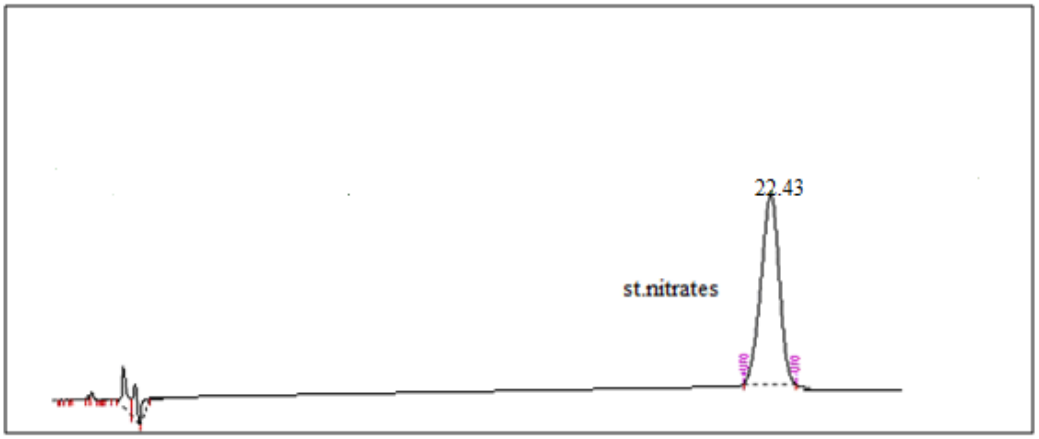

Figure 2. Chromatogram of nitrate standard (50 mg/L) on RP-8 stationary phase.

As shown on Figure 3 under the same condition there is not precise answer for nitrite, two peaks were appeared.

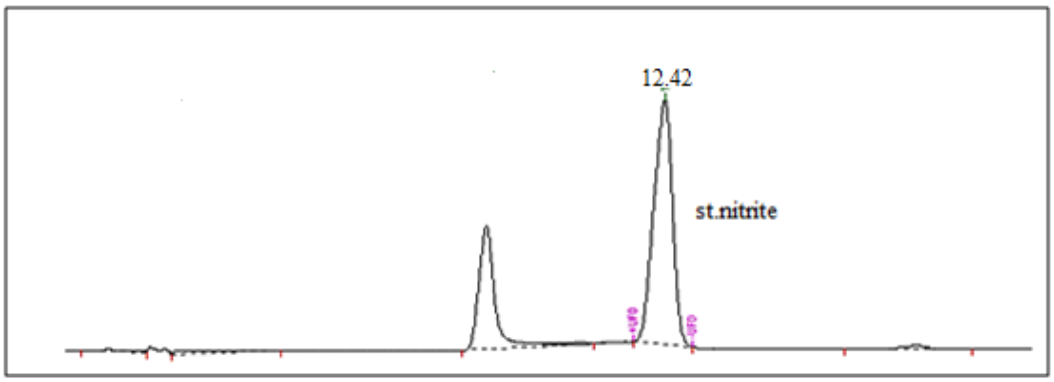

Figure 3. Chromatogram of nitrite standard (50 mg/L) on RP-8 stationary phase.

Further analyses were towards changing of pH of mobile phase in range from 4 to 7. Different retention time for nitrate was achieved by changing of pH. There was not precise peak for nitrite, so octyl phase RP-8 is not suitable for determination of nitrite. It was confirmed by employed of RP select B stationary phase where the shape of the nitrite peak was the same when RP-8 column was used. Retention time for nitrate was shorter on RP select B stationary phase. As a result of decreasing of pH, retention time was increased shown on Figure 4. Considering the retention time neutral pH was selected as optimal.

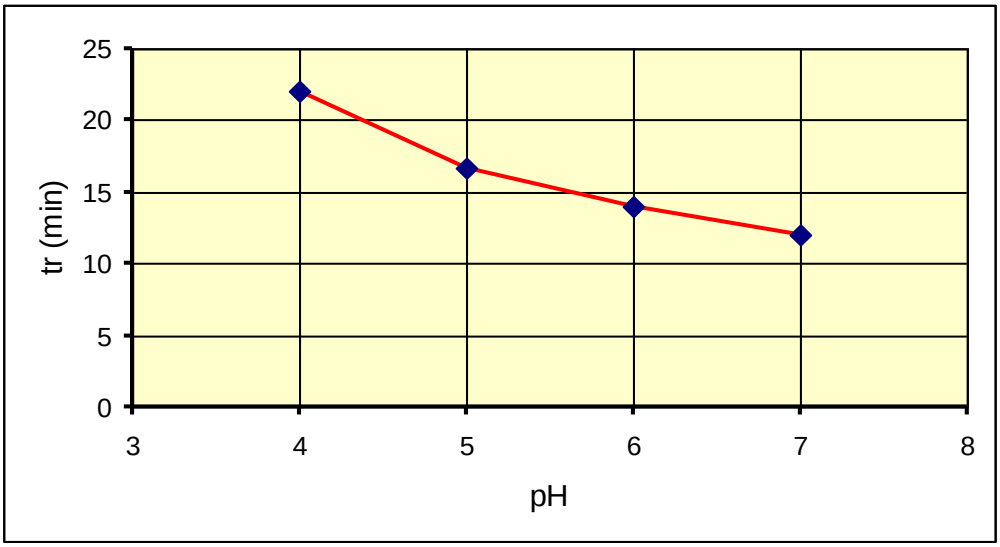

Figure 4. Effects of pH of mobile phase on retention time for nitrate

In the next step of this work octadecil RP-18 column was used. It is more hydrophobic phase compared with RP-8 and RP select B. Applied ratio of aqueous/organic phase was 70:30, *V/V* (S-S. Chou, 2003). Under these conditions chromatogram shown on Figure 5 was obtained, where peaks of nitrite and nitrate were overlapped.

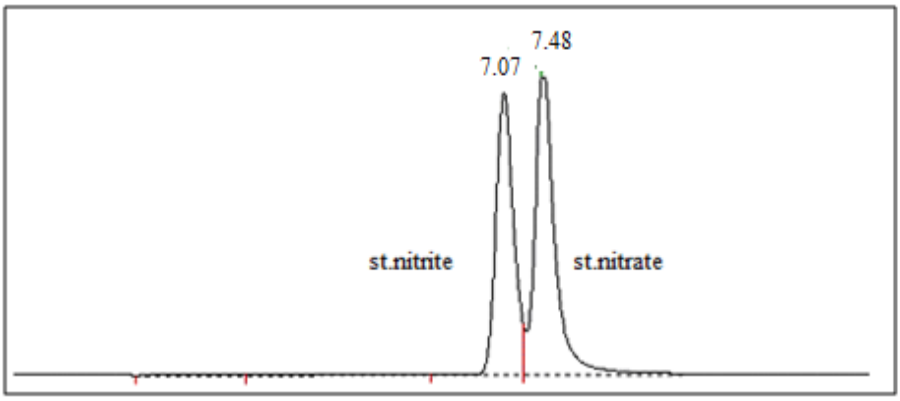

Figure 5. Chromatogram of mixture of nitrite and nitrate standard C = 50 mg/L (aqueous 30 % methanol phase, *V/V*).

Unstable retention time was because of presence of free silanol groups as well as of neutral pH of mobile phase and high percent of water phase in mobile phase. To achieve stable retention time in further examination octadecil RP-18 endcapped column was used. With aim to obtain better separation, ratio of organic and water phase was changed. There is possibility to use high concentration of water in mobile phase when ion-pair chromatography is used and from the other side to obtain stable retention time. Increasing the concentration of water phase lead to better separation, shown on Figure 6.

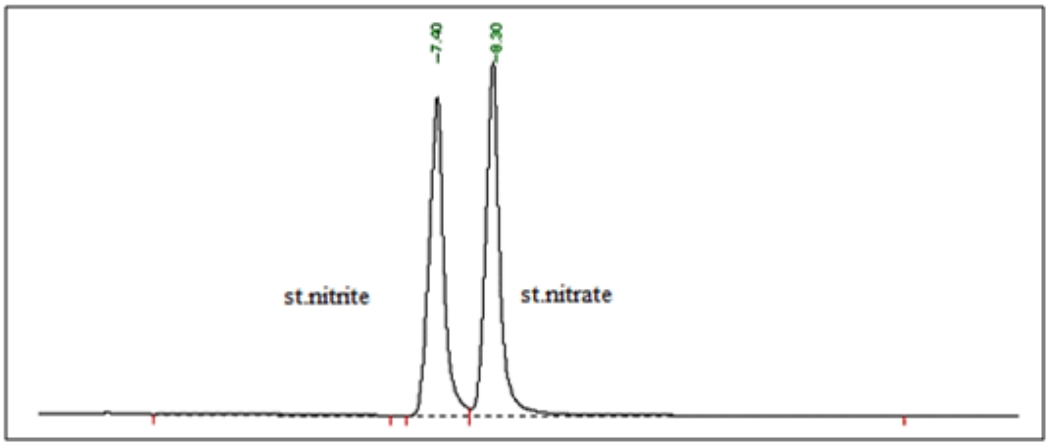

Figure 6. Chromatogram of mixture of nitrite and nitrate standards (50 mg/L) (aqueous 20% methanol phase, *V/V*)

Optimal condition of mobile phase for separation of nitrite and nitrate were selected and they are: aqueous phase 10 % methanol, (*V*:*V*), Flow 0.8 mL/min. Under these conditions chromatogram shown on Figure 7 was obtained.

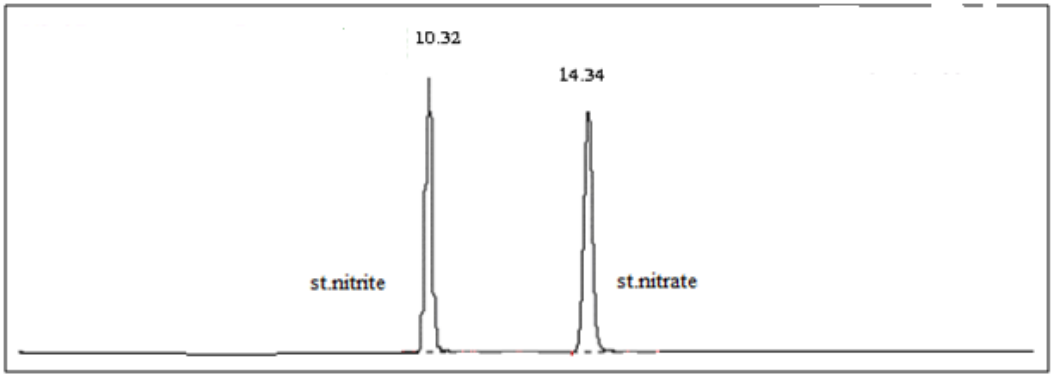

Figure 7. Chromatogram of mixture of standards nitrite and nitrate (50 mg/L) (aqueous 10 % methanol phase, *V/V*).

Total retention time was 14.34 minutes for both compounds. As shown on the next Figure an increase of flow causes decrease of the retention time.

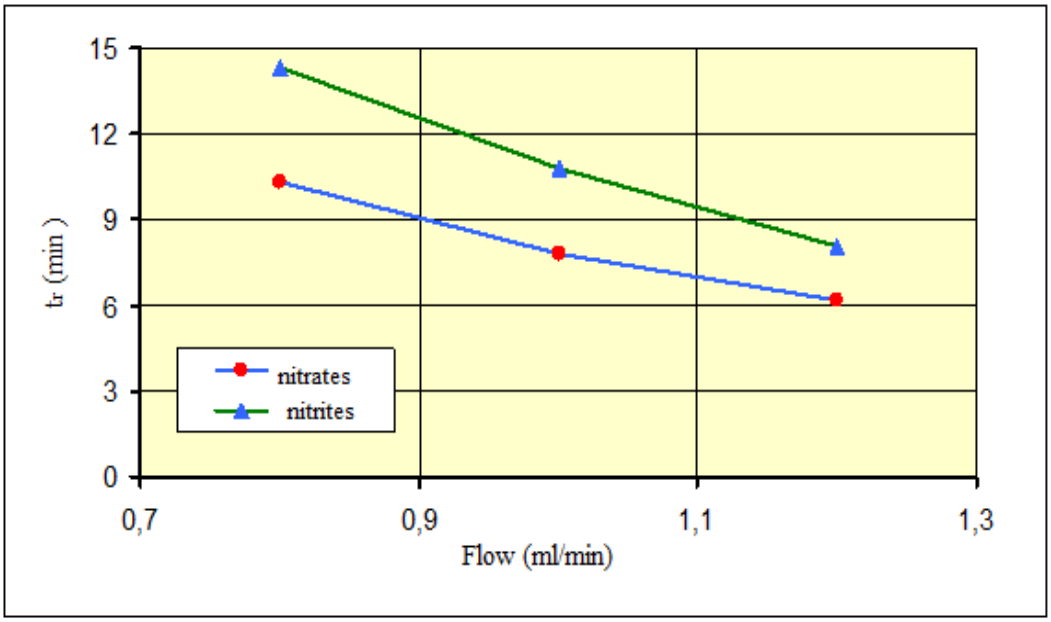

Figure 8. Effects of flow of mobile phase on retention time

With further increasing of water phase up to 95 % better resolution was obtained, but retention time of nitrate was longer for four minutes. Optimal conditions for separation of nitrate and nitrite are selected and presented on Table 1.

Table 1. HPLC conditions for simultaneous separation of nitrite and nitrate

Stacionary Phase	Purospher RP -18 endcapped, (250 x 4.6mm), 5μm
Mobile Phase	methanol (10 %) - 0.01M octylamine (90 %) , isocratic mode
pH	7
Injection *V*	10 μL
Wavelengh	210 nm
Flow	1mL/min
T	30 ^{0}C

UV spectra of nitrite and nitrate shown on Figure 9 were recorded for further identification and confirmation of their presence in vegetables. The detection limit was 50 mg/L for nitrite and 25 mg/L for nitrate.

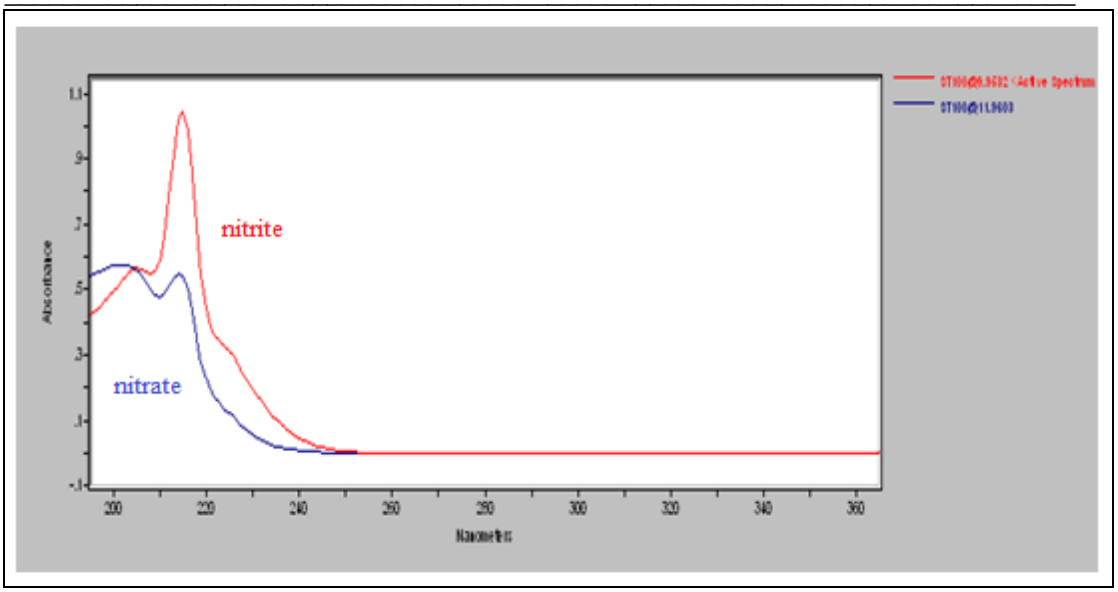

Figure 9. Wavelength scans of standards of nitrite and nitrate

Conclusion

The method reported herein is relatively simple, sensitive, selective, quick and can be applied to the monitoring of nitrite and nitrate in vegetable products. Higher percent of water in mobile phase and more hydrophobic stationary phase were adopted for separation of these components. Considering the retention time neutral pH was selected as optimal.

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