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# Optimization and Validation of Reversed Phase Ion-Pair Liquid Chromatographic Method for Accurate Determination of Nitrate in High Saline Seawater

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## Abstract

An accurate method for the determination of low concentrations of nitrate in high-saline seawater is now well optimized and validated. The method is based on using reversed-phase ion-pair liquid chromatography with diode-array detection for accurate determination of nitrate in high-saline seawater without interference from highly dominant chloride and sulfate anions. The method showed satisfactory precision (ranging from 5.8% to 9.5%), linearity, and detection limits, and its trueness was confirmed by participation in the international comparisons of CCQM K-161 with seven national metrology institutes. The key comparison and spiking showed recovery rates between 90% and 110%. The validation results confirm the applicability of the optimized method for detecting very low concentrations of nitrate in high-saline seawater.

***Kewords:*** Nitrate; High Saline Seawater; Method validation; Ion-Pair HPLC

## 1. Introduction

The accurate measurement of nitrate in seawater is fundamental for seawater studies, environmental monitoring, and evaluating a waterbody's quality [1-4]. The measurement of nitrate in seawater at trace and ultra-trace levels has always been a challenge for high and hypersalinity water due to the variations in concentrations, matrix, and interferences, and even requires measurements at concentrations near the method's detection limit. The test methods for nitrate detection in different environmental matrices include flow injection analysis, electrochemical, spectroscopic, electrical, potentiometric, ion-selective electrodes, and ion-selective membranes [5-11]. Each of these analytical methods has its own advantages, but most of them are very sophisticated, costly, and have low sensitivity for detection [12-18]. Therefore, the goal of this study was to optimize liquid chromatographic methods for use in high-saline seawater based on reversed-phase ion-pair liquid chromatography. This technique is currently used for the determination of nitrate and nitrite in food matrices; nevertheless, there is no particular procedure for the use of ion-pair liquid chromatography in the quantification of nitrate in trace and ultra-trace levels in high saline water samples. The method was optimized in regards to pH, concentration of ion-pair reagents, and column stationary phases. The optimum conditions for separation and full baseline resolution were obtained by using gradient elution consisting of 10% methanol, 90% 5 mM tetra-butyl ammonium hydroxide, and a ZORBAX SB-C18 (3.0 x 250, 5µm) column. The detection of nitrate was carried out using a diode-array detector (DAD) operated at 218 nm. These conditions provided a complete separation of nitrate with very good peak resolution in 8 minutes, as well as satisfactory precision (ranging from 5.8% to 9.5%), linearity, and detection limits. The method's trueness was confirmed by participation in the international comparisons of CCQM K-161 [19] with seven national metrology institutes. The key comparison and spike showed recovery rates between 90% and 110%. The validation results confirm the applicability of the optimized method for detecting very low concentrations of nitrate in high-saline seawater.

## 2. Materials and Methods

### *2.1. Chemicals and reagents*

HPLC grade solvents methanol, ethanol, isopropyl alcohol were purchased from Merck (Germany), while trimethylamine, hexylammonium hydroxide, tetra-butyl ammonium hydroxide and orthophosphoric acid were purchased from Sigma-Aldrich. The ultrapure water used for sample and mobile phase preparation was obtained from Millipore, Milli-Q, IQ 7005 system (USA). Primary standard reference material SRM 3185 was purchased from NIST (USA). The stock and calibration solutions were prepared gravimetrically in water.

### *2.2. Liquid chromatograph*

An HPLC Agilent 1100 integrated system with a G1313A automated injector, a G1311A pump, and a G1315B diode-array detector operating at 218 nm was used to perform the chromatographic study. A reversed-phase column, ZORBAX SB-C18

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(3.0 x 250, 5µm) from Agilent (USA), running at constant room temperature (20°C), was used to achieve the chromatographic separation of the nitrate. The eluent employed was a solution of alkyl ammonium hydroxide in water and methanol, and the elution was carried out at a flow rate of 0.4 mL/min with an injection volume of 10 µL. Agilent Chemstation was used for the analysis of chromatographic results.

### *2.3. Samples and sample preparation*

The study was based on the received CCQM sample (No. 271 & 274), which was prepared through the National Metrology Institute of China (NIM). The received sample was a mixture of seawater sampling from East China Sea and North Pacific containing high amounts of anions. The sample preparations, dilution, standard preparation were performed gravimetrically. The traceability of measurements to the definition of the SI units was achieved by the use of primary NIST standard solutions directly traceable to the mole and use of calibrated balances traceability to international kilogram.

### *2.4. Method validation*

Experimental design for validating test method and statistical technique for conducting, analyzing, and interpreting data from experiments were conducted in accordance with International Union of Pure and Applied Chemistry (IUPAC) harmonized guidelines for single laboratory validation of analytical methods [20-21]. The method was validated in terms of linearity, precision, trueness, limit of detection (LOD), limit of quantification (LOQ) and ruggedness of the method. The method precision was evaluated by analyzing the CCQM K-161 sample 6 times daily for 10 days. Furthermore, the LOD was estimated from calibration parameters.

## 3. Results and Discussion

### *3.1. Method optimization*

The development of the method for the separation of nitrate from the seawater matrix included the use of different concentrations of individual trimethylamine, hexylammonium hydroxide, and tetra-butyl ammonium hydroxide in water and methanol, as well as the study of the effect of pH to determine the best conditions for the separation of nitrates in seawater. Trimethylamine, whether at low or high concentrations or at different pH values, failed to achieve the desired separation. On the other hand, an appropriate separation was achieved by using hexylammonium hydroxide and tetra-butylammonium hydroxide. Hexylammonium hydroxide and tetra-butyl ammonium hydroxide compositions in the mobile phase were first optimized in the range of 0.5–10 mM and pH from 5 to 7 in a 0.5 pH change to obtain rapid baseline separation of nitrate with acceptable precision. The optimum conditions for separation and full baseline resolution were obtained when using concentrations higher than 3 mM and a pH ranging from 5.5 to 6.5. The effectiveness of separation was equal for both hexylammonium hydroxide and tetra-butyl ammonium hydroxide, and it is clear from Fig. 1 that nitrate was well separated from the matrix in regards to resolution and retention time precision using gradient elution consisting of 10% methanol, 90% 5 mM tetra-butyl ammonium hydroxide, and a ZORBAX SB-C18 (3.0 x 250, 5µm) column. The detection of nitrate was carried out using a diode-array detector (DAD) operated at 218 nm.

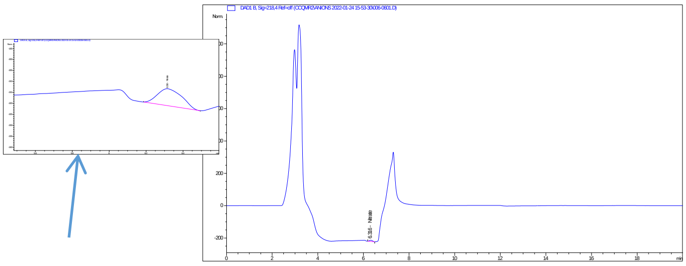

**Fig. 1: Chromatographic separation of nitrate in high saline seawater by reversed phase ion-pair liquid chromatography**

### *3.2. Linearity and detection limits*

The first step of the experimental work involved a screening of nitrate concentration in order to define the experimental design for both the calibration and quantification steps. Preliminarily, the initial screening of nitrate concentration confirmed that the concentration is in the range of 1.7 mg/kg. Based on the detected concentration, four standards and a sample were used for the calibrations. The standard addition calibration approach was applied to minimize the matrix effect. The sample was mixed gravimetrically with ultrapure water in a 1:1 mass ratio, and then the sample was mixed with standards prepared from NIST SRM 3185 in the same ratio to prepare matrix-matched calibration standards. The calibration curve was constructed by injecting a diluted sample and preparing spiked samples. The concentration was calculated by dividing the intercept (0.4812) by the slope (0.6423) multiplied by the dilution factor, 2 [20-21]. Ten calibration curves were constructed for nitrate over ten different days, and the calibration results can be observed in Fig. 2-B. The mean value and reproducibility of the results (1.42±0.12 mg/kg) confirms the absence of any systematic errors. Furthermore, the limit of detection was estimated from the calibration parameters; the LOD was calculated by multiplying the residual standard deviation by 3.3 and dividing the result by the slope of the calibration line. The LOD was found to be 0.11 mg/kg, and the limit of quantification was considered to be three times the limit of detection, 0.33 mg/kg. This LOQ has been considered suitable for the speciation of nitrate at very low levels in high-saline seawater.

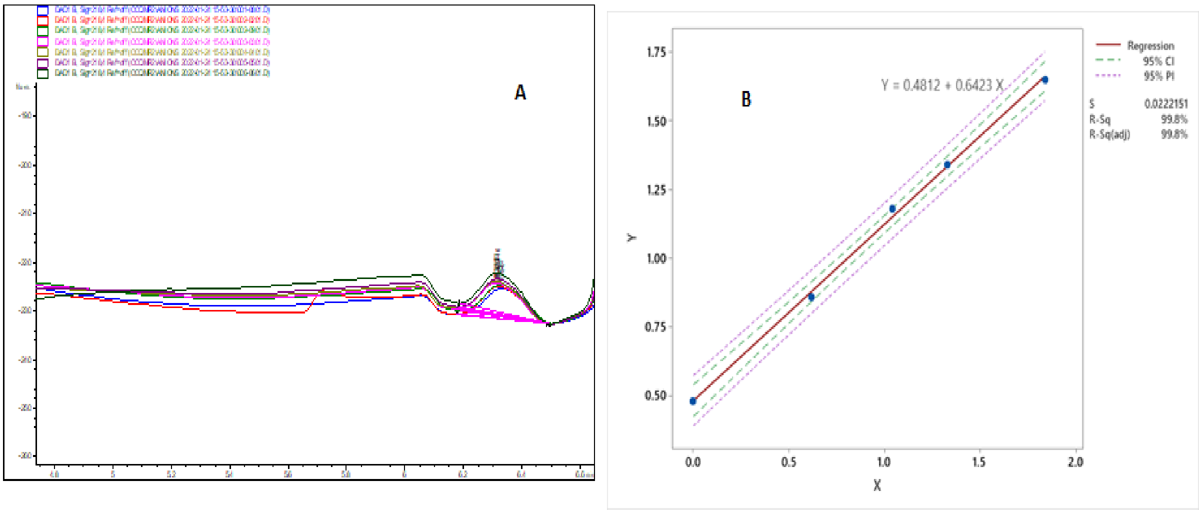

**Fig. 2: Injection repeatability of nitrate in high saline seawater by reversed phase ion-pair liquid chromatography (A), Calibration curve of nitrate by standard addition method (B).**

*3.3. Precision and Trueness*

The precision of measurements, including repeatability and reproducibility, was evaluated by injecting the CCQM K-161 sample six times per day over ten days, as illustrated in Fig. 2-A. The trueness of the measurement results was estimated by the standard addition procedure. Six samples were spiked by the addition of known concentrations, including 0.5, 1, 2, 3, and 5 mg/kg. The mean recovery values were calculated, and the value was 90–110% for all concentration levels [20-21]. The trueness of the measurement results was confirmed by participation in the international comparisons of CCQM K-161 organized by the National Metrology Institute of China [NIM]. The comparison included the participation of national metrology institutes in Thailand, Egypt, Turkey, Japan, China, Canada, Hong Kong, and the Russian Federation. All NIS results, including nitrate, are in agreement with all participating institutes and reference values. The plots of participants' degrees of equivalence (DoEs) were graphically presented in Fig. 3. These results confirm the applicability of the optimized method for detecting very low concentrations of nitrate in high-saline seawater.

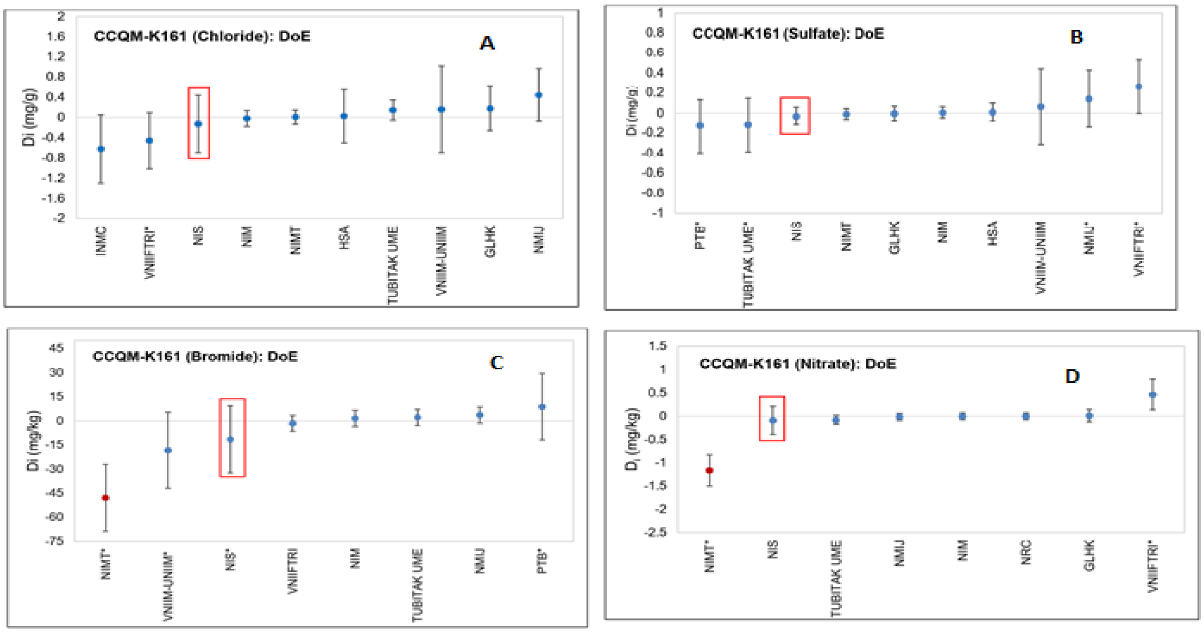

**Fig. 3: The NIS results for anions in seawater are part of the international comparison of CCQM K-161, (A) chloride, (B) sulfate, (C) bromide and (D) nitrate.**

### *3.4. Measurements Uncertainty*

The main sources of uncertainty in the chromatographic determination of nitrate are the uncertainty of the primary calibrator (SRM), the amount of sample used for a determination, errors from the calibration function (Ordinary Least Squares Regression, OLSR), and the precision of HPLC measurements. The sources and uncertainty value of each source are presented in Table 1. The combined uncertainty includes the four sources mentioned [22-23]. Therefore, the relative combined standard uncertainty of nitrate measurements is calculated by using equation No. 1.

$$u_C = \sqrt{\left(\frac{u_{SRM}}{SRM}\right)^2 + \left(\frac{u_d}{mass}\right)^2 + \left(\frac{u_{LSR}}{X_{Cal}}\right)^2 + \left(\frac{u_P}{X_{Nitrate}}\right)^2} \qquad \text{Eq.1}$$

**Table (1): Sources and corresponding uncertainty values**

| Parameter | Source of uncertainty | Typical value | Standard uncertainty | Unit | Type |
|---|---|---|---|---|---|
| $u_{SRM}$ | Uncertainty of SRM | 999.8 | 1.15 | mg/kg | B |
| $u_d$ | Gravimetric preparation of samples | 500.0 | 2.0 | mg | A |
| $u_{LSR}$ | Uncertainty from calibration curve (ordinary least squares regression) | 0.71 | 0.06 | mg/kg | A |
| $u_P$ | Precision of HPLC measurements | 1.42 | 0.12 | mg/kg | A |

## 4. Conclusion

The current study presents an optimized reversed-phase ion-pair liquid chromatographic method for accurate determination of nitrate in high-saline seawater. While the use of reversed-phase ion-pair liquid chromatographic in separation and quantification of nitrate and nitrite is not new, its usage in determination of very low level of nitrate in seawater complicated matrix is a novelty. The method was validated internally in terms of linearity, precision, trueness, and detection limits. Additionally, the applicability of the method and the trueness of the results were confirmed by participation in the international comparisons of CCQM K-161 organized by the National Metrology Institute of China [NIM]. The optimized method was found to be efficient for the separation and accurate determination of nitrate at very low concentrations in high-saline water samples (TDS > 20 g/L).

## 5. Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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