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# Rapid Analytical Method of Nitrite and Nitrate in Fish by Ion Chromatography

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Abstract

**Rapid analytical method was investigated to determine precursors of N-nitrosamine such as nitrite and nitrate in squid(*Illex illecebrosus* and *Sepiell maindroni*), codfish(*Gadus marcrocephalus*) and flatfish (*Paralichthys olivaceus*) by ion chromatography(IC) and colorimetric methods. Recoveries of nitrite and nitrate in fish tissues were 89~98.7% and 94.1~99.8% for IC, and 98.4~103.7% and 67.7~102.2% for colorimetric method, respectively. Using IC, nitrite was not detected and nitrate was 0.89~1.23mg/kg, while using colorimetric method, nitrite and nitrate were ND~0.08mg/kg and 0.3~0.42mg/kg, respectively. Therefore, the IC method showed better recoveries, and was applicable to extract nitrite and nitrate simultaneously, and is simpler compared with colorimetric method in analyzing nitrite and nitrate from fish tissues.**

**Key words**: nitrite, nitrate, ion chromatography, colorimetric method

## INTRODUCTION

Nitrite and nitrate are main precursors for *N*-nitrosamines that can be formed by reacting with secondary and tertiary amines, and also induce methemoglobinemea(1). The use of these compounds should be a serious problem in human health during food processing : where nitrite and nitrate have been used as food additives of maintaining color, texture and growth inhibition of *Clostridium botulinum* in meat products(1,2). Therefore, a highly sensitive and rapid method in the determination of nitrite and nitrate is required. Since most fish contain a lot of amines, especially, it has a danger of forming strong carcinogenic *N*-nitrosamines from nitrite and nitrate in fish. A number of methods for determination of nitrite and nitrate in foods have been based on the colorimetric method. This method needs to be improved due to the limited sensitivity, the use of harmful reagents and the time consuming procedures(3,4).

With the development of analytical apparatus, high performance liquid chromatography(HPLC)(5) and gas chromatography-thermal energy analyzer(GC-TEA)(6) have been proposed for the determination of nitrite and nitrate.

Other methods not only have some apprehensions for accurate analysis of food containning a lot of complex and macromolecules, but the method for the simultaneous determination of nitrite and nitrate has not yet been developed. Ross and Hotchkiss(6) studied the conversion of nitrate to nitrobenzene with measurement by gas chromatography-electron capture detection(GC-ECD) and reported the method suitable for water and air samples. Dull and Hotchkiss(7) analyzed nitrate by GC-TEA, but this method was not able to detect nitrite and nitrate simultaneously. Recently, ion chromatography(IC)(3) using conductivity detector for determination of organic and inorganic substances was introduced. Despite the fact that some improvements have been made with regard to detection sensitivity, resolution of samples, modification of eluents, electrostatic separation and column switching by many researchers, the problem of measuring low amounts of nitrite and nitrate using this method still remains (3,4). Kim and Conca(8) reported that the determination of nitrite in cured meats by ion-exclusion chromatography-electrochemical detection(IEC-ECD) showed good recoveries. However, there are no reports for simultaneous determination of nitrite and nitrate in foods, because of interfering substances such as protein, lipid, chloride ion and phosphate ion(3,7). Nitrite and nitrate are required to be determined simultaneously since these two subatances always exist together and the relative amount of these two change readily during food processing and preservation.

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We set out to investigate the rapid analytical method for determining nitrite and nitrate contents of fish simultanuously using the IC method. These fish species with three different muscle types; squid(*Illex illecebrosus* and *Sepiell maindroni*), codfish(*Gadus marcrocephalus*) and flatfish(*Paralichthys olivaceus*) were selected as experimental materials. The recoveries of nitrite and nitrate measured by IC and colorimetric methods were compared.

## MATERIALS AND METHODS

### Reagents

Stock solution : 1.50g of sodium nitrite and 1.37g of sodium nitrate(Sigma Co., USA) were dissolved in 1L of distilled water, respectively.

Working solution : prepare 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 5, 10, 15 and 20mg/L by serial dilutions of stock solution.

Diazotizing reagent : dissolve 0.1g of sulfamerazine (Sigma Co., USA) in 100ml of 3N hydrochloric acid.

Coupling reagent : dissolve 0.1g N-(1-naphtyl) ethylene diamine dihydrochloride(Wako pure chemical, Japan) in 100ml of 20% acetic acid solution. The solution should be kept in a dark room.

Eluents : sodium bicarbonate(Fisher Scientific, USA), sodium carbonate(Sigma Co., USA). The eluents and standard solutions were prepared using ultra-pure 18MΩcm water obtained by passing doubly distilled water through a Mill-Q system(Millipore).

### Apparatus

UV spectrophotometer : Shimadzu 1201
Ion chromatography : Dionex - 100

### Preparation of materials

Squid(*Illex illecebrosus* and *Sepiell maindroni*) and codfish(*Gadus marcrocephalus*) were purchased from a commercial market, and flatfish(*Paralichthys olivaceus*) was obtained from a fish farm in Cheju city, and minced using a mixer after removing all viscera and bones. These minced fish tissues were used for analysis of nitrite and nitrate

### Determination of nitrite and nitrate by colorimetric method

Nitrite and nitrate were determinated according to the methods described by Ishibashi and Kawabata(9) and by Kamm et al.(10).

### Determination of nitrite and nitrate by IC

Nitrite and nitrate in fish extracted with distilled water by the method of Kamm et al.(10) and with 200mM sodium borate by the method of Ishibashi and Kawabata(9) and Bosch et al.(3). These extracts were filtered through a 0.22μm membrane filter(Corning Co., USA) and then determinated by IC.

## RESULTS AND DISCUSSION

### Difference between distilled water and sodium borate by extraction solvent

IC chromatograms of the samples extracted with distilled water by the method of Kamm et al.(10) and with 200mM sodium borate by methods of Ishibashi and Kawabata(9) and Bosch et al.(3) are displayed in Fig. 1. As shown in Fig. 1B, the peaks of nitrite(1B-2) and nitrate(1B-3) in the sample extracted with distilled water showed a good resolution. Therefore, when fish samples were extracted with distilled water, the simultaneous separation and the analysis of nitrite and nitrate were possible. However, as shown in Fig. 1C, samples extracted with sodium borate was difficult in determining the nitrite content because the solvent peak was below the baseline and the peak for nitrite (1C-2) appeared nearly approching retention time(RT) with the chloride(1C-1).

From these results, we are confident that the deter-

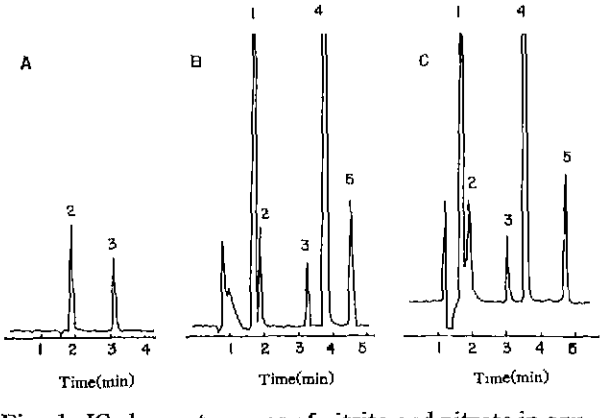


**Fig. 1. IC chromatograms of nitrite and nitrate in squid with different extracting solution.**
A, standard solution(10μg/ml); B, extracted water; C, extracted 200mM borex($Na_2B_4O_7 \cdot 10\,H_2O$). 1, $Cl^-$; 2. $NO_2^-$; 3, $NO_3^-$; 4. $PO_4^-$; 5, $SO_4^-$

mination of nitrite and nitrate by IC is more effective to extract with distilled water than with sodium borate, and we also believe that the interference of the nitrite by $Cl^-$ is a problem to resolve in future.

### Influence of chloride and phosphorous ion

In general, the determinations of nitrite and nitrate by IC in fish samples were difficult because fish contained higher contents of the interfering compounds such as chloride and phosphorus ion. The nitrite peak was merged with that of chloride ion and the nitrate peak was merged with that of phosphorus ion(Fig. 2). Lippsmeyer et al.(4) reported that the determination of nitrite and nitrate in cured meats by AOAC method required the elimination of ascorbic acid and erythorbic acid and a number of chloride contained in the foods. For this reason, we added silver salts as a means of precipitation and elimination of chloride in the foods because the silver salts precipitate bromide, sulfate and phosphate. Posner and Scholffman(11) used silver sulfate for the elimination of chloride and phosphate and obtained good results. Thus, we applied the sample which was treated with silver sulfate to the chromatography for the elimination of chloride and phosphate in fish samples and the IC chromatograms shown in Fig. 2. The addition of silver sulfate made an easier filtering procedure before loading into IC apparatus, and well separated the nitrite(2C-2) and nitrate(2C-3) peaks from that of the interfering substances. The resolutions of the peaks for nitrite and nitrate, therefore, were good.

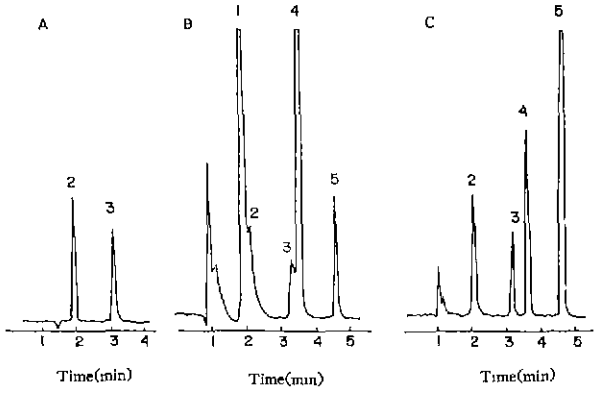


**Fig. 2. IC chromatograms of nitrite and nitrate with and without $Ag_2SO_4$ in fish.**
A, standard solution(10μg/ml); B, without $Ag_2SO_4$; C, with $Ag_2SO_4$.
1, $Cl^-$; 2. $NO_2^-$; 3. $NO_3^-$, 4. $PO_4^-$; 5, $SO_4^-$

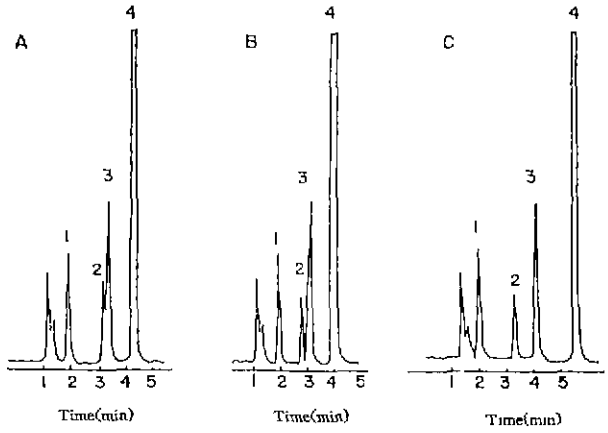


**Fig. 3. IC chromatograms of nitrite and nitrate by eluent composition.**
A, 1.8mM $Na_2CO_3$; 1.7mM $NaHCO_3$; B, 2.5mM $Na_2CO_3$; 1.5mM $NaHCO_3$; C, 1.5mM $Na_2CO_3$; 2.5mM $NaHCO_3$
1, $NO_2^-$; 2 $NO_3^-$; 3, $PO_4^-$; 4, $SO_4^-$

### Effect of eluent composition

The separation of nitrite and nitrate by IC was considerably influenced by the eluent composition. To find the optimum eluent composition for determination of nitrite and nitrate, we prepared the eluents properly mixed with sodium carbonate and sodium bicarbonate. These results are shown in Fig. 3. As shown in Fig. 3, when the eluent of 1.8mM $Na_2CO_3$ and 1.7mM $NaHCO_3$(eluent A) was used, this eluent could not be used to determine the nitrate because the peak of nitrate merged with the peak of phosphate(Fig. 3A). When the eluent of 2.5mM $Na_2CO_3$ and 1.5mM $NaHCO_3$(eluent B) was used, we could obtain the peaks of nitrite and nitrate with a good resolution(Fig. 3B). However, because the peak of nitrate was neighbored with the peak of phosphate, these two peaks were merged. Therefore, we prepared the eluent of 1.5mM $Na_2CO_3$ and 2.5mM $NaHCO_3$(eluent C) in such a way that these peaks are perfectly separated. Using this eluent, we could simultaneously determine the contents of nitrite and nitrate and obtained the peaks with a good resolution for nitrite and nitrate. We could thus obtain the conclusions shown in Table 1; the resolution of the peak for nitrite using C eluent was excellent compared with other eluents tested, while the nitrate peak in A and B eluents were merged with the phosphate peak.

From these results, we are confident that the mixed solution system of 1.5mM $Na_2CO_3$ and 2.5mM $NaHCO_3$

**Table 1. Effect of peak separation by the composition of sodium carbonate and sodium bicarbonate**

| Eluent | Composition | | Effect of peak separation | |
|---|---|---|---|---|
| | Sodium carbonate | Sodium bicarbonate | Nitrite | Nitrate |
| Eluent A | 1.8M | 1.7M | Excellent | Bed, merged between the nitrate and phosphate peaks |
| Eluent B | 2.5M | 1.5M | Excellent | Good, but merged between the nitrate and phosphate peaks |
| Eluent C | 1.5M | 2.5M | Excellent | Excellent |

is the optimum eluent composition for the simultaneous determination of nitrite and nitrate and also established an extraction procedure schematized in Fig. 4. The experimental condition for the analysis is shown in Table 2.

## Recovery study

To confirm which of the nitrite and nitrate is precisely extracted, or which of these had a loss, we performed a recovery test. After adding 10 and 20ppm of the standard solutions for nitrite and nitrate in the samples, the contents of nitrite and nitrate for the unspiked samples and spiked samples were determined by IC and colorimetric method.

Sample(5g)
| ← 60ml, distilled water
Homogenizing in a mechanical blender
|
Heating for 15 min in water bath(80°C)
|
Making up to 100ml with water
| ← 15mM $Ag_2SO_4$ 10ml
Filtering(5A) or centrifuging(5000rpm, 15min)
|
Filtering(0.22μm microfilter)
|
I.C. analyzer

**Fig. 4. Schematic procedures for determination of nitrite and nitrate by ion chromatography.**

**Table 2. Condition of ion chromatography for nitrite and nitrate in fishes**

| | |
|---|---|
| Ion chromatograph | DIONEX-100 |
| Column | IonPac AS4A-SC 4mm |
| Detector | Conductivity detector |
| Eluent | 1.5mM $Na_2CO_3$/2.5mM $NaHCO_3$ |
| Supressor regenerant | 25mN $H_2SO_4$ |
| Flow rate | 1.7ml/min |
| Sample injection | 25μl |

The recoveries of nitrite and nitrate in fish by the IC and colorimetric method are shown in Table 3. The contents of nitrite and nitrate measured by the IC method showed recovery ranges of 89~98.7 and 94.1~99.8%, respectively. Colorimetric method showed the ranges of 98.4~103.7 and 67.7~102.2%, respectively. The nitrite showed a good recovery by both methods, while the nitrate showed a poor recovery by the colorimetric method.

Based on these results, the IC method has advantages to determine the nitrite and nitrate in fish simultaneously. The procedures for extraction and determination are simple, while the colorimetric method requires time consuming analysis and is not able to simultaneously determine these two substances. The limit of determination of nitrite and nitrate by IC were less than 10μg/kg.

**Table 3. Recoveries of nitrite and nitrate in fishes by IC and colorimetric methods[1)]** (%)

| Samples | IC[2)] | | Colorimetery[3)] | |
|---|---|---|---|---|
| | Nitrite | Nitrate | Nitrite | Nitrate |
| Squid(cheju) | 98.7 | 94.1 | 103.7 | 67.7 |
| Squid(atlantic) | 97.2 | 94.6 | 98.4 | 102.2 |
| Codfish | 98.6 | 99.8 | 99.8 | 69.5 |
| Flatfish | 89.0 | 94.8 | 98.5 | 73.7 |

[1)]Mean of triplicate experiments
[2)]10mg/L of std. soln.
[3)]0.5mg/L of std. soln.

**Table 4. The contents of nitrite and nitrate in fishes by ion chromatographic and colorimetric methods** (mg/kg)

| Sample | Contents | | | |
|---|---|---|---|---|
| | IC | | Colorimetry | |
| | Nitrite | Nitrate | Nitrite | Nitrate |
| Squid(cheju) | ND | 0.89 | 0.02 | 0.37 |
| Squid(atlantic) | ND | 0.93 | 0.08 | 0.42 |
| Codfish | ND | 1.08 | ND | 0.33 |
| Flatfish | ND | 1.23 | ND | 0.30 |

ND: not detected

### The Contents of nitrite and nitrate in fish

The contents of nitrite and nitrate in fish measured by IC are shown in Table 4. As shown in Table 4. the nitrite in fish was not detected by IC, but ranged between ND to 0.08mg/kg by the colorimetric method. The contents of nitrate by IC and colorimetric method ranged between 0.89 to 1.23mg/kg and 0.12 to 0.54mg/kg, respectively.

From these results, we believe that the method for the determination of nitrite and nitrate by the IC method can be widely used in other food products as well as in fish.

The previous research on fishery foods have been focued on the contents of nitrite and nitrate from salted anchovy(12), commercial salted fish(13) and dried fish products(14). The contents of nitrite and nitrate in this investigation ranged between ND and 21.13mg/kg, respectively.

## ACKNOWLEDGEMENTS

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