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# Simultaneous determination of nitrite and nitrate in meat and meat products using ion-exchange chromatography

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**Abstract**

Nitrite and nitrate salts are widely used as curing agents to preserve meat and meat products. Despite the benefits of using these salts in meat processing, it has some adverse health effects. The high concentration of these salts produces nitric oxide through various metabolic reactions followed by the generation of carcinogenic nitrosamines. In this study, a chromatographic method was developed for the simultaneous determination of nitrite and nitrate level in meat and meat products. Samples were collected from four different locations in Dhaka, Bangladesh and analysed by ion-exchange chromatography. Most relevant validation parameters were evaluated using the standard validation procedure. The linearity was more than 0.995 and recoveries for nitrite and nitrate were more than 96% and 97%, respectively. The Limit of detection (LOD) for nitrite and nitrate was 0.10 and 0.26 mg/L, respectively. Results showed that the highest nitrite and nitrate content was 66.04±0.55 and 55.02±1.4 mg/kg, respectively. However, nitrite and nitrate levels were lower than the prescribed value by international guidelines. Besides, temperature-based recovery revealed that 80°C was the optimum temperature for recovering the nitrite and nitrate from meat and meat products. This developed method applying ion-exchange chromatography can monitor the content of nitrite and nitrate in meat and meat products to ensure food safety.

## 1. Introduction

Traditionally, various chemicals had been used for preserving meat and meat products. These chemicals are known as curing agents. Nitrite and nitrate are mainly used as curing agents to prevent the growth of microorganisms, especially *Clostridium botulinum* (Ma *et al.,* 2018). They also minimize rancidity through the inhibition of lipid oxidation (Pearson and Gillett, 1996).

In acidic conditions, Nitrates spontaneously breakdown into nitrites and nitrogen dioxide. Nitrites supplied with food can additionally react with precursors to produce N-nitroso compounds (such as amines and amides) in the gastrointestinal tract (Ohshima and Bartsch, 1981; IARC, 1987). This reaction of nitrites with secondary amines can form carcinogenic nitrosamines (Archer, 1989). Nitrosamines were known to be related to gastric cancer, oesophagal cancer, colon cancer, and other tumours (Park *et al*., 2015; Bedale *et al*., 2016). Furthermore, irreversible conversion of haemoglobin to methemoglobin occurs in the bloodstream through the interference of nitrite in the oxygen transport system of the body. This incident reduces the ability of haemoglobin to exchange oxygen (Lee 1970; Chan, 1996) which is seriously harmful to infants and pregnant women (Hegesh and Shiloah, 1982; Knobeloch *et al*., 2000).

Nitrite and nitrate were accepted as food preservatives in the European Union under Commission Regulation (EU). The permissible limit of nitrite in processed meat is 150 mg/kg, with an exception of 100 mg/kg for sterilized meat products. However, the maximum limit of sodium nitrate in uncooked meat is 150 mg/kg. The recommendation of the Joint WHO/FAO Expert Committee on Food Additives (JECFA) for maximum daily intake of nitrate and nitrite are 3.7 and 0.07 mg/kg body weight, respectively. The maximum permissible value of nitrate intake in the United States is 400 to 450 mg per week (Hord *et al*., 2011).

The consumption of meat products is increasing day by day in Bangladesh, with the rise in per capita income and urbanization. Dietary patterns have been changing rapidly toward a higher level of consumption of high-value meat products with the economic growth during

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the few years in the urban areas of Bangladesh (World Bank 2019). This demand for processed meat is met by some local industries. However, there is a lack of information available on the contents of nitrite and nitrate concentration in meat products of Bangladesh.

Several techniques like spectrophotometry, chemiluminescence and fluorimetry are commonly used for the determination of nitrite and nitrate. However, they have some drawbacks like the complex extraction steps, interference with matrix and high detection limit. Therefore these methods are not appropriate for the regular analysis of large numbers of samples (Hardisson*et al*., 1996; Betta *et al*., 2014). On the other hand, High Performance Liquid Chromatography (HPLC) has a fluorescence detection system and Gas Chromatography-Mass Spectrometry (GC–MS) provides improved reliability, repeatability, sensitivity and selectivity. Although these techniques are problematic due to the requirement of a high amount of toxic solvents, time and cost (Gapper *et al*., 2004; Akyüz *et al*., 2009). Considering these drawbacks, interest in analytical techniques that could replace traditional methodology is constantly improving. The ion chromatography technique provides quick separation and high resolution for anionic compounds like nitrite and nitrate analysis. It requires no organic solvent and only small quantities of reagents and samples, resulting in less residue generation (McMullen *et al*., 2005; Cengiz *et al*., 2015).

It is crucial to monitor and control the levels of nitrite and nitrate in meat products, as an excessive amount can cause serious health problems in consumers. Meat processing additives and techniques vary in geographical location depending on the taste and acceptance of local inhabitants. Therefore a rapid and validated ion-exchange chromatography method is required for routine monitoring of the status of nitrite and nitrate by the local authorities. As a result, the goal of this study was to develop an ion chromatography method for analyzing nitrite and nitrate simultaneously in meat and meat products available in Bangladesh.

## 2. Materials and methods

### *2.1 Sample collection*

Frozen meat samples (n = 42) were collected from different grocery shops located in Mohammadpur (n = 12), Dhaka north city corporation and Newmarket (n = 9), Dhanmondi (n = 11), Kalabagan (n = 12), Dhaka south city corporation of Bangladesh. Beef burger patty (n = 12) and beef sausages (n = 15) of different brands were collected from a super shop. All the samples were stored at -20°C until analysis.

### *2.2 Sample extraction*

The sample was extracted according to Hsu *et al.* (2009) with slight modification. Approximately 5g of homogenized meat sample was mixed with 50mL of deionized water in a conical flask and placed in a water bath at 80°C for 10 mins. At ambient temperature, the mixture was centrifuged (Centrifuge machine, Hettich, Universal 320R, Germany). The supernatant was collected and filtrated using a 0.45µm syringe filter prior to analysis in ion-exchange chromatography.

### *2.3 Ion exchange chromatography system*

Chromatographic analysis was carried out using ion-exchange chromatography with a conductivity detector (IC-CD)(Thermo Scientific Dionex ICS-3000 systems, Thermo Fisher Scientific Inc., MA, USA), coupled to a Dionex ICS-3000 SP Single Pump, Dionex AS Autosampler and Dionex ICS-3000 CD Conductivity Detector. Nitrite and nitrate were separated on a Dionex™ IonPac™ AS11-HC, 4 × 250 mm IC Columns (Dionix, USA) which was controlled at 30°C using a temperature-controlled column compartment (Dionex Corporation, 1998). Dionex Chromeleon software (Version 6.80 RS 10) was used for the data acquisition, peak integration, and calibration curve preparation (Table 1).

Table 1. Chromatographic conditions

| | | |
|---|---|---|
| Column | : | AG11-HC 4 mm and AS11-HC 4 mm |
| Eluent | : | 30 mM Hydroxide |
| Flow rate | : | 1.5 mL/min. |
| Detector | : | Dionex ICS-3000 CD Conductivity Detector |
| Injection volume | : | 25 µL |
| Column oven temperature | : | 30°C |
| Suppressor Type | : | ASRS_4mm |
| Current | : | 112 [mA] |

#### *2.3.1 Chemicals and reagents*

Sodium hydroxide, sodium nitrate and sodium nitrite were purchased from Sigma (St. Louis,Mo,USA). Water was purified (18 MΩ $cm^{-1}$ quality) by a Milli-Q system (Millipore, Bedford,MA, USA). Seven anion standard II was supplied by Dionex (California,USA). All glassware was washed with 3.65 g/L HCL (Merck,Germany) solution and deionized water for minimizing contamination.

#### *2.3.2 Standard preparation*

Five different concentrations (0.25mg/L, 1 mg/L, 4 mg/L, 16 mg/L and 32 mg/L) of nitrite and nitrate were prepared by using seven anion standard II solution (100 mg/L). The solutions were kept at 4°C for further analysis.

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*2.3.3 Equilibration of column*

The column was equilibrated with 30 mM sodium hydroxide as eluent for 30 mins. A total of 25 µL of the standard solution was injected until the relative standard deviation of areas of the peaks of five consecutive chromatograms was less than 2.0% and then the test solution was injected.

*2.3.4 Peak characterization and quantification*

Nitrite and nitrate were identified by comparing against standard peaks using the retention time and the conductivity spectrum profile. Data were reported as mean ± standard deviations of triplicate independent analyses.

*2.4 Validation procedure*

According to European Analytical Chemistry (Eurachem) guidelines (Magnusson and Örnemark, 2014), the validation process includes the following parameters: linearity, limits of detection (LOD), the limit of quantification (LOQ), precision, repeatability and trueness.

The calibration curves were calculated by analysing five prepared concentrations within the linear range of 0 to 32 mg/L for nitrite and nitrate. At each level concentration, three replication injections (n = 3) were performed in duplicate. Least-squares linear regression was used to prepare calibration curves. Linearity was determined considering 85-115% deviations of the mean calculated levels over three runs for nominal non-zero calibration (Heine *et al.,* 2008). The linearity was measured by the evaluation of regression coefficients and statistical tests. The limit of detection (LOD) and limit of quantification (LOQ) were calculated by regression parameters, respectively as $3.3s_y/x/a$ and $10s_y/x/a$, where the symbol '$s_y/x$' was the residual deviation of the regression and 'a' was the slope of the calibration curve (Magnusson and Örnemark, 2014).

The precision was determined by calculating the percent relative standard deviation (%RSD) of repeatability (intra-day precision) and the intermediate precision (inter-day precision). For evaluating the matrix effect, the analysis of three replicates of each calibration standard in the water matrix was performed on the same day for repeatability evaluation and three non-consecutive days over three weeks for intermediate precision evaluation (Heine *et al*., 2008).

The trueness was assessed from recovery evaluation, by spiking raw meat samples at 0.6 mg/L (10 mg/kg), 1.2 mg/L (20 mg/kg) and 2.4 mg/L (40 mg/kg). After that, the percentage of recovery was calculated as the average value of three independent replicates, by using this equation: $\%R = 100 \times ((M_{sample\ spike} - M_{sample})/M_{spike})$ where $M_{sample\ spike}$ is the concentration of analyte in the spiked sample, $M_{sample}$ is the concentration of analyte in the sample and $M_{spike}$ is the spiked analyte concentration. Moreover, the matrix effect was assessed to measure the impact of potential interferences occurring in real samples on the analytical signal. Due to the absence of method blanks for this analysis, two different calibration curves were created simultaneously and their corresponding slopes were compared. One curve was prepared by analyzing standard solutions in water and the other one was generated by standard solutions in homogenized meat extract (Lopez *et al*., 2016).

*2.5 Conversion of nitrite to nitrate in meat*

This experiment was designed according to Träger (2013)with slight modifications. The minced meat was mixed with sodium nitrite and sodium nitrate (150 mg/kg). The polyethylene-wrapped meats were stored at 4°C for 28 days. The amount of nitrite and nitrate were assessed in ion exchange-chromatography at a certain interval of days.

*2.6 Effect of temperature on recovery*

An experiment was designed to find out the suitable temperature for the extraction of nitrite and nitrate from meat and meat products. The temperature for sample extraction ranged from 65°C to 85°C. The meat was spiked with sodium nitrite and sodium nitrate (100 mg/kg meat). Percentage recovery was calculated by using this equation: $\%R = 100 \times ((T_{sample\ spike} - T_{sample})/T_{spike})$ where $T_{sample\ spike}$ is the concentration of analyte in the spiked sample, $T_{sample}$ is the concentration of analyte in the blank sample and $T_{spike}$ is the spiked analyte concentration.

## 3. Results and discussion

*3.1 Chromatographic separation*

Figure 1a shows a typical chromatogram of a mixed standard solution ($[NO_2^-]$ and $[NO_3^-]$ 4 mg/L). The repeatability was established by using a standard solution of nitrite and nitrate injected every 6-8 samples and calculating the mean of the observed retention times: 2.6 to 3 mins for nitrite and 3.8 to 4.107 mins for nitrate. The comparison between retention times of nitrite and nitrate in spiked meat (Figure 1A) and for a standard mixture (Figure 1A) showed the absence of interferences in the elution window. The chromatograms of a mixed standard solution, spiked sample (0.2 mg/g of both nitrite and nitrate) and homogenized meat are visible in plots (A) and (B) of Figure 1, respectively. Plot (1B) reports a

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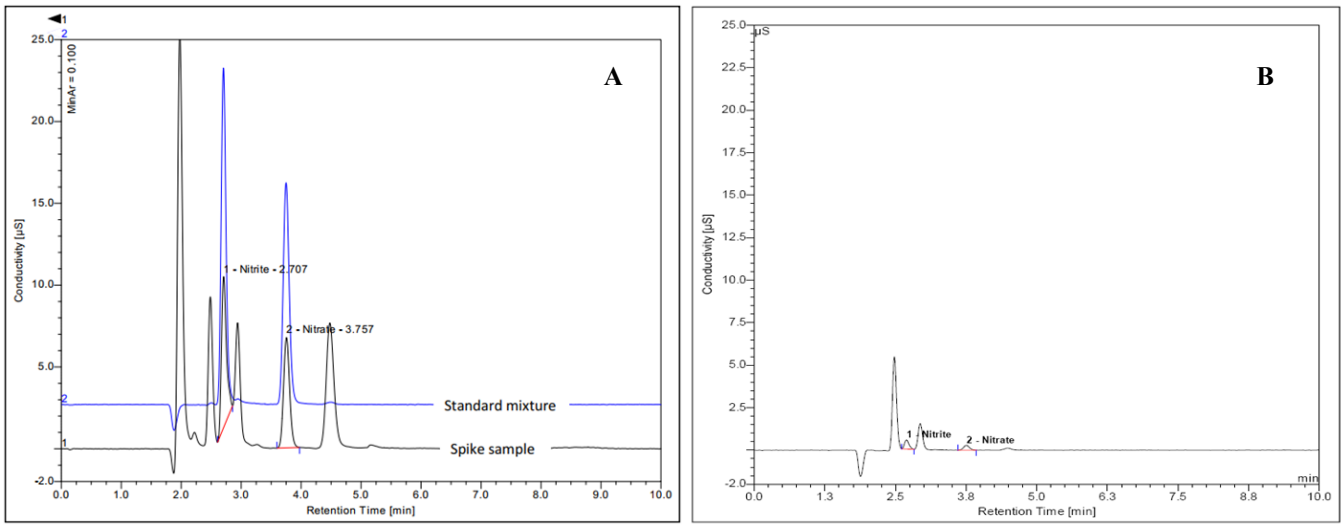


Figure 1. Chromatogram of nitrite and nitrate: (A) standard solution (4 mg/L) and spiked meat sample (0.2 mg/g) and (B) homogenized raw meat

view of the chromatographic profile of a homogenized meat sample.

### *3.2 Method validation*

The validation of the analytical process was achieved by a diagnosis procedure of a method's ability to assess analytes under specific analytical conditions (Magnusson and Örnemark, 2014). Table 2 shows the quality parameters which were obtained during the validation study, such as linearity, precision and trueness.

The working ranges were 0.25–32 mg/L for both nitrite and nitrate. The IC-CD working condition displayed a good linearity response (determination coefficient, $R^2$>0.9983). The linearity was supported by a statistical t-test to assess whether the determination coefficient was significant by comparing the calculated value to t tabulated at 99.90% of confidence level. Intra-day and inter-day %RSD variation was less than 1.2% and within the limit of the accepted reference value (15%) (ICH, 2005), thus confirming the suitability of this method for analysing nitrite and nitrate in spiked meat samples. In particular, the %RSD for repeatability ranged between 0.11% and 0.8% for nitrite and 0.02% to 0.15%for nitrate. The percent RSD of intermediate precision for nitrite and nitrate was less than 0.6% and 0.5%, respectively (Table 2). These findings suggest that the IC-CD method has strong repeatability as well as inter-day precision.

The trueness was assessed as the percentage recovery and matrix effect. According to AOAC (2002), the recovery of the analytes was carried out using real samples fortified with three different concentrations of nitrite and nitrate. The nitrite recovery ranged from 97% to 98%, while the nitrate recovery values ranged from

Table 2. Quality parameters of the Ion Chromatography method validated for nitrite and nitrate determination

| Quality Parameters | | Analyte | |
|---|---|---|---|
| | | Nitrite | Nitrate |
| Linearity | Linear range (mg/L) | 0.25-0.32 | 0.25-0.32 |
| | Linearity | | |
| | Regression equation, $y = (m \pm s_m)x + (b \pm s_b)$ | $y = (39 \pm 0.2)10^{-3}x + (2.1 \pm 0.2)10^{-3}$ | $y = (10 \pm 0.1)10^{-3}x + (0.8 \pm 0.3)10^{-3}$ |
| | Determination coefficient ($R^2$) | 0.9993 | 0.9998 |
| Repeatability Precision, %RSD (concentration level, mg/L) | Repeatability | 0.11 (1) | 0.02 (1) |
| | | 0.2(4) | 0.4 (4) |
| | | 0.8(16) | 0.15 (16) |
| | Intermediate precision | 0.2 (1) | 0.3 (1) |
| | | 0.1(4) | 0.5 (4) |
| | | 0.6(16) | 0.18 (16) |
| Trueness | %R (concentration, mg/L) | 96%±2 (0.6) | 97%±2 (0.6) |
| | | 97%±2 (1.2) | 95%±2 (1.2) |
| | | 95%±2 (2.4) | 98%±2 (2.4) |
| | Matrix effect (slope difference %) | 1.85 | 10.3 |
| | LOD (mg /L) | 0.1 | 0.26 |
| | LOQ (mg/L) | 0.31 | 0.8 |

m = slope, $s_m$ = standard error of regression slope, b = intercept and $s_b$ = standard error of regression intercept. precision = percent relative standard deviation (%RSD), trueness = percentage recovery (%R)

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95% to 97% (Table 2). When compared to literature results, there was good agreement with many matrices, implying complete extraction, minimal losses, good alignment between spiking and calibration solution and analytical system accuracy (Siu and Henshall, 1998; Chamandust *et al*., 2016).

The matrix effect has an impact on the trueness due to potential interferences of certain sample components with the analytes of interest. The presence and concentration of these interfering substances were determined by comparing the calibration curve generated by standard solutions in water to that obtained using homogenized meat samples (Figure 2). Without matrix effects, the calibration curves were nearly parallel; the evaluated slope differences for nitrite and nitrate were 1.85% and 10.3%, plots (B) and (A) (Figure 2), respectively, of a homogenized meat sample. Figure 2B shows that the curves of nitrite standard and matrix sample were almost identical. In the case of nitrate, the values of the standards prepared with matrix were 11% lower than that of standards prepared with ultrapure water.

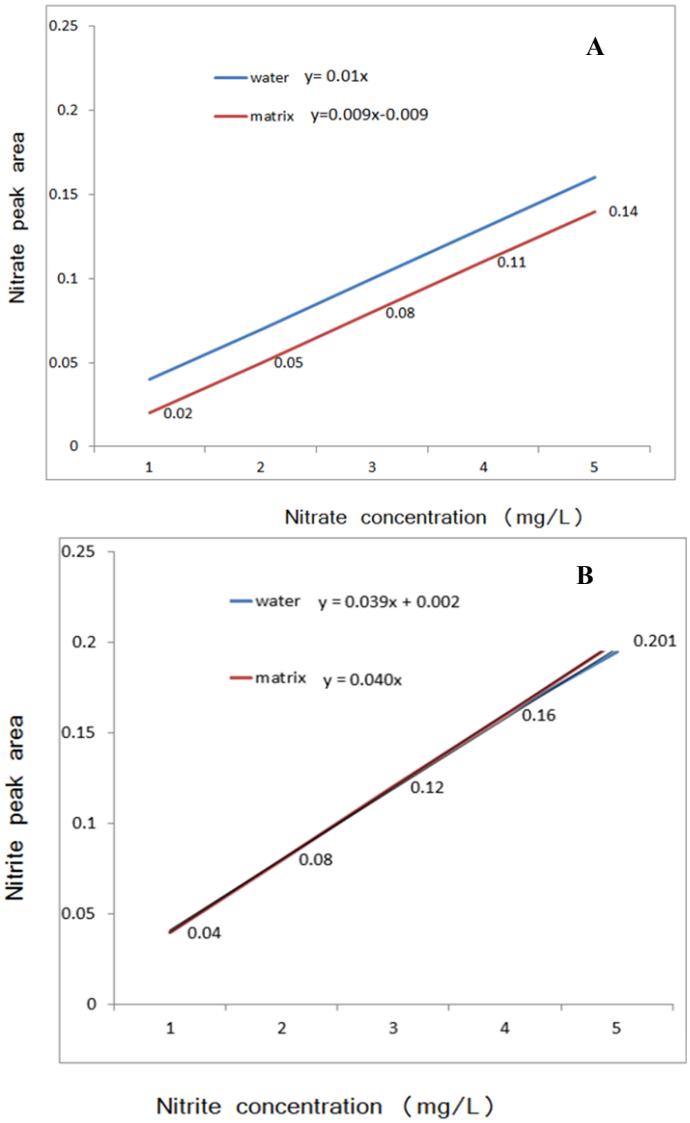


Figure 2. Calibration curves of (A) nitrate and (B) nitrite. Water and Matrix are the standard solution in deionized water and homogenized meat, respectively.

LOD and LOQ were calculated using regression parameters of standard solutions prepared in water as the recovery value and matrix effect were within acceptance criteria (AOAC 2002). The LODs for nitrite and nitrate using a 25uL loop were 0.10 and 0.26 mg/L, respectively and the LOQ values for nitrite and nitrate were 0.31 and 0.80 mg/L, respectively.

### *3.3 Nitrite and nitrate in meat and meat products*

All the meat products contained a higher amount of nitrite compared to raw meat (Table 3). The raw meat from Newmarket contained the highest amount of nitrite (66.04±0.55 mg/kg)though it was within the acceptable limit of the European Union under Commission Regulation (EU). On the other hand, meat from Mohammadpur contained the lowest amount of nitrite (28.06±1.22 mg/kg). According to a survey in China, the nitrite level was 14.3 mg/kg (Yuan *et al*., 2010) in raw meat.

Table 3. Nitrate and nitrite content in meat and meat products

| Meat and meat product | Nitrite (mg/kg) | Nitrate (mg/kg) |
|---|---|---|
| Burger patty (n = 12) | 67.29±1.71 | 23.45±1.32 |
| Sausages (n = 15) | 69.67±1.51 | 41.27±1.46 |
| MB (n = 12) | 28.06±1.22 | 17.70±1.68 |
| MNW (n = 9) | 66.04±0.55 | 55.01±1.42 |
| MD (n = 11) | 53.59±0.99 | 26.53±0.78 |
| KB (n = 12) | 40.23±0.78 | 36.74±1.04 |

MB = Frozen Meat sample collected from Mohammadpur, MNW = Frozen Meat sample collected from Newmarket, MD = Frozen Meat sample collected from Dhanmondi, and KB = Frozen Meat sample collected from Kalabagan.

Although, raw meat from Mohammadpur contained a higher amount of nitrate (55.02±1.42 mg/kg) compared to sausage(41.27±1.4 mg/kg). Moreover, this sausage had a higher amount of nitrate and nitrite compared to the burger patty (Table 3).

A previous study in Denmark, reported the low content of nitrite and nitrate in cured meat products (Leth *et al*., 2008) and in accordance with the Danish legislation (60 mg/kg for most products and up to 150 mg/kg for certain products) which was similar to the level in cured meat products that were found in Canada in 1996 (Sen and Baddoo, 1997). Likewise in the USA, the levels of residual nitrite were in the range of 0–48 mg/kg (mean values 10 mg/kg) (Cassens *et al*., 1997). Keeton *et al*. (2009) also reported nitrite levels of 7 mg/L in cured meat products (hot dogs, bacon and hams) from five cities in the USA.



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*3.4 Conversion of nitrite to nitrate in meat*

There was a clear view of the conversion of nitrite to nitrate (Table 4). Initially, the concentration of nitrite and nitrate was about 149 mg/kg and 151 mg/kg, respectively. After 24 hrs, the amount of nitrite decreased by 7% and nitrate increased by 12%. Moreover, the amount of nitrite and nitrate decreased to 115 mg/kg and rose to 190 mg/kg, respectively after two weeks. However, the highest increment of nitrate was 11% and depletion of nitrite was 30% after the third week. Finally, in the fourth week, there was a 60% loss of nitrite and a 50% rise in nitrate. According to Trager (2013), nitrate levels can be increased due to the addition of nitrite salt and this increase will continue depending on time and nitrite salt. The results indicate nitrite is converted to nitrate with time. Therefore, it is necessary to measure the content of both nitrite and nitrate in meat products.

Table 4. Conversion of nitrite to nitrate in meat

| Days | Nitrite (mg/kg) | Nitrate (mg/kg) |
|---|---|---|
| Day 0 | 149±0.15 | 151±0.21 |
| Day 01 | 140±1.18 | 169±0.32 |
| Day 03 | 130±1.21 | 181±1.04 |
| Day 07 | 121±1.14 | 187±1.01 |
| Day 14 | 115±1.10 | 190±0.93 |
| Day 21 | 80±0.93 | 211±0.80 |
| Day 28 | 61±1.03 | 226±0.91 |

*3.5 Effect of temperature on recovery*

The temperature had a great effect on the extraction of nitrite and nitrate from meat and meat products (Table 5). At 65°C, the recoveries for nitrite and nitrate were 60% and 55%, respectively. These recoveries were getting higher with the increment of temperature (Leth *et al*., 2008). At 80°C, recoveries were 98% and 99% for nitrite and nitrate, respectively. However, at 85°C, recovery was 102% for nitrite and 95% for nitrate.

Table 5. Effect of temperature on recovery of nitrite and nitrate from meat

| Temperature | Recovery of Nitrite | Recovery of Nitrate |
|---|---|---|
| 65°C | 60% | 55% |
| 70°C | 72% | 70% |
| 75°C | 85% | 87% |
| 80°C | 98% | 99% |
| 85°C | 102% | 95% |

## 4. Conclusion

Investigation of nitrite and nitrate contents in meat and meat products is important for food safety. This was the first systematic approach to develop and validate an ion chromatographic method to evaluate the nitrite and nitrate content in meat and meat products of Bangladesh. The highest value for nitrite and nitrate was 70 mg/kg and 55 mg/kg respectively, which were lower than the guideline. The results demonstrated that the method is well suited for the simultaneous accurate analysis of nitrite and nitrate in meat and meat products with a simple and fast extraction technique and rapid analytical procedure, which has importance in routine check analysis to ensure food safety.

## Conflict of interest

The authors declare no conflict of interest.

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