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Determination of nitrite and nitrate in sugar using ion chromatography

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Goal

To develop a method for the determination of nitrite and nitrate in sugar using an ion chromatography (IC) system with a Thermo Scientific™ Dionex™ IonPac™ AS11-HC-4µm column

Introduction

Natural sugar products, such as molasses and beet pulp, are material used for food and animal feed. Nitrate and nitrite are widespread in the environment and present naturally in water and plants. However, excessive intake of nitrite and nitrate may increase the risk of cancer or cause food poisoning. Therefore, these compounds have been regulated as undesirable substances in water and sugar products. The United States Environmental Protection Agency (EPA) has set an enforceable standard called a maximum contaminant level (MCL) in drinking water for nitrites and nitrates at 1 and 10 mg/L as nitrogen, respectively.[1] Nitrite and nitrate are measured using an ion chromatography (IC) method described in EPA Methods 300.0 or 300.1.[2] In 2010, the European Union (EU) established Directive 2010/63/EU[3] on undesirable substances in animal feed. The Directive limit for nitrite content in sugar industry feed materials, such as molasses and beet pulp, was set at a maximum of 15 mg/kg (expressed as sodium nitrite, relative to a feeding stuff with a moisture content of 12%). Depending on the operating conditions of individual process steps in sugar production (temperature, pH value, dry substance content), nitrate can be reduced to nitrite by bacteria.[4] Therefore, it is important to determine both anions in sugar products.

Ion chromatography is a well-accepted technique for the determination of anions in water and juice samples.[5,6] An IC method, which used a Dionex IonPac AS11-HC-4µm column, manually prepared eluent, and a steep sodium hydroxide gradient, was published to determine the nitrite and nitrate contamination of sugar by-products.[7] Here, we developed and validated an improved IC method using

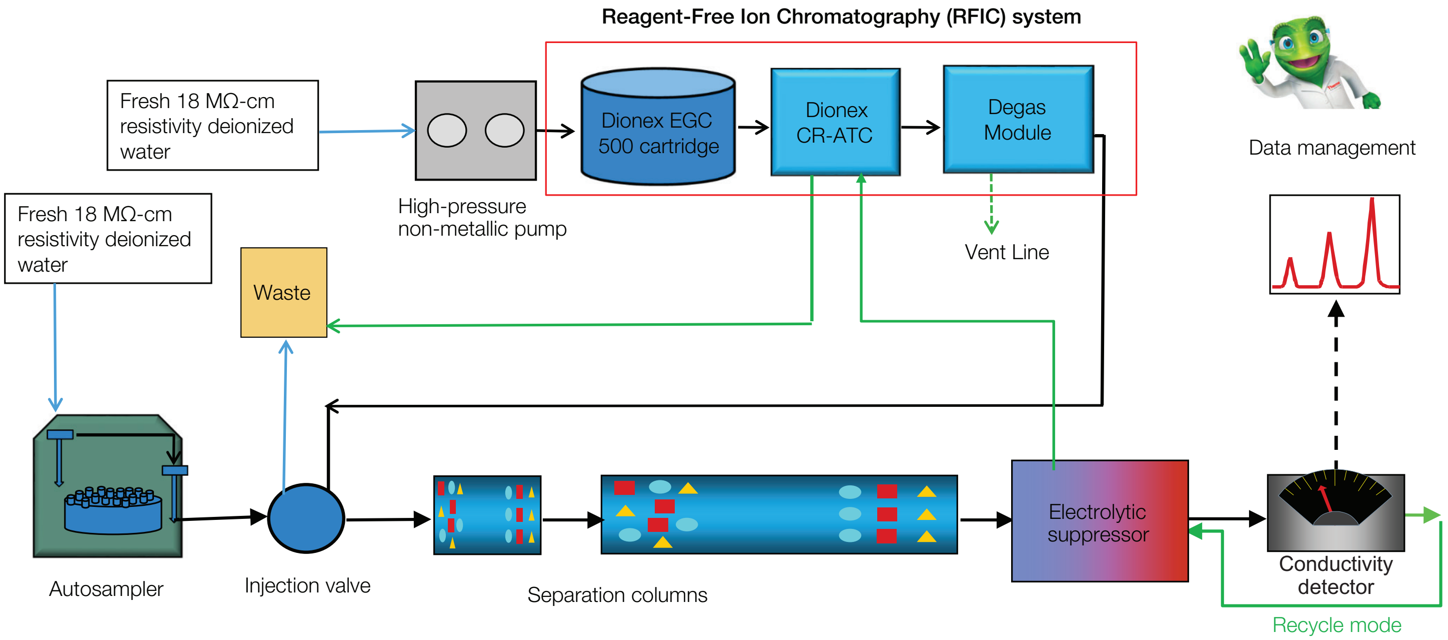


Figure 1. Illustration of the flow diagram of the IC system

a 2 mm version of the Dionex IonPac AS11-HC-4µm column and a Thermo Scientific™ Dionex™ ICS-6000 HPIC™ System with eluent generation and conductivity detection. Figure 1 illustrates the flow diagram of the IC system setup. With this method, nitrite and nitrate are well separated from other anions present in sugar samples. The method is sensitive and can be used to determine nitrite and nitrate in sugar products for regulatory monitoring.

Experimental

Equipment and consumables

- Dionex ICS-6000 HPIC System with RFIC-EG and Conductivity Detection*
- Thermo Scientific™ Dionex™ AS-AP Autosampler with 250 µL syringe and tray temperature control
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.10

*Equivalent results can be achieved using either a Thermo Scientific™ Dionex™ ICS-5000⁺ system or a Thermo Scientific™ Dionex™ Integrion™ HPIC™ system.

Consumables

- Thermo Scientific™ Dionex™ EGC 500 KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 075778)
- Thermo Scientific™ Dionex™ CR-ATC 600 Continuously Regenerated Anion Trap Column (P/N 088662)
- Thermo Scientific™ Dionex™ IonPac™ AS11-HC-4µm Analytical Column, 2 × 250 mm, (P/N 078035)
- Thermo Scientific™ Dionex™ IonPac™ AG11-HC-4µm Guard Column, 2 × 50 mm (P/N 078036)
- Thermo Scientific™ Dionex™ ADRS 600 Anion Dynamically Regenerated Suppressor, 2 mm (P/N 088667)
- Thermo Scientific™ Dionex™ AS-AP Autosampler Vials 10 mL (P/N 074228)

Reagent and standards

- Degassed deionized (DI) water, 18 MΩ·cm resistance or better
- Sodium and potassium salts, A.C.S. reagent grade or better, for preparing anion standards

Samples

- Three sugar samples (sugar beet syrup, white cane sugar, and brown cane sugar) were purchased from a local supermarket.

IC conditions

Table 1. Chromatography conditions

Columns	Dionex IonPac AS11-HC-4µm Analytical column, 2 × 250 mm Dionex IonPac AG11-HC-4µm Guard column, 2 × 50 mm
Eluent	Potassium hydroxide (KOH) gradient: 0–5 min, 5 mM; 5–30 min, 5–20 mM; 30–36 min, 65 mM; 36–40 min, 5 mM
Eluent source	Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 and Dionex high-pressure degasser
Flow rate	0.37 mL/min
Injection volume	2.5 µL (full loop) or 25 µL (full loop)
Column temperature	30 °C
Detection	Suppressed conductivity, Dionex ADRS 600 (2 mm) Suppressor, recycle mode, 60 mA current
Detection temperature	25 °C
Sample tray temperature	4 °C
Run time	40 min

Preparation of solutions and reagents

Anion stock standard solutions (1000 mg/L)

Stock standard solutions are prepared by dissolving the appropriate amounts of the required analytes in 100 mL of DI water according to Table 2. The nitrate stock standard is stable for at least six months at 4 °C. The nitrite stock standard is stable for one month when stored at 4 °C.

Table 2. Masses of compounds used to prepare 100 mL of 1000 mg/L anion standards

Analyte	Compound	Amount* (mg)
Nitrite	Sodium nitrite ($NaNO_2$)	150.0
Nitrate	Sodium nitrate ($NaNO_3$)	137.1

*Compound must be dry.

Working standards

Diluted working standard solutions are prepared from 1000 mg/L stock standards. First, prepare a mixed stock containing 50 mg/mL of nitrite and 100 mg/mL nitrate. Then, dilute the mixed standard stock with DI water to make the six levels of calibration standards (Table 3). The calibration standards were prepared fresh daily. The mixed stock was stored at 4 °C and stable for one month.

Sample preparation

Weigh 1 g of sugar sample into a 100 mL plastic bottle and add 49 mL (g) of DI water to make 50-fold diluted (50x) samples. Shake the mixture for 5 to 10 min. Filter 10 mL of sample solution through a 0.2 µm PES syringe filter, discarding the first 300 µL of the effluent. Add an equal volume of DI water to make a 100-fold diluted sample for analysis.

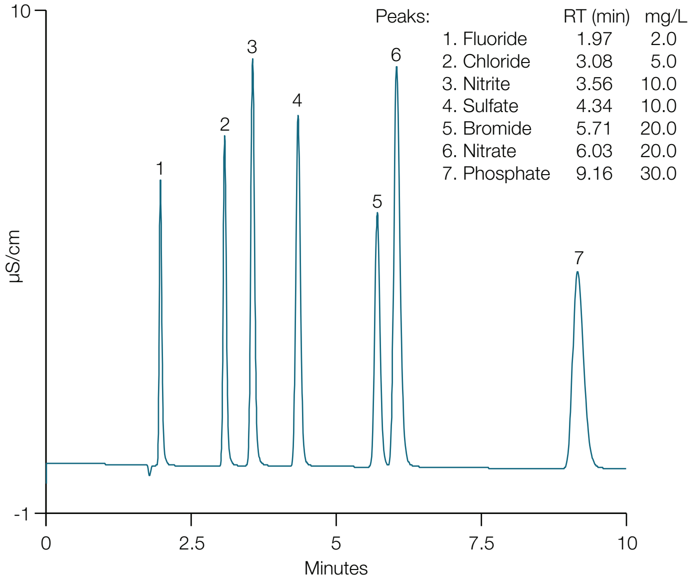
Prepare the spiked sample by adding known amounts of nitrite and nitrate standards into the filtered 50-fold diluted samples. Then, add an appropriate amount of DI water to produce a 100-fold diluted sample.

Results and discussion

Separation

The Dionex IonPac AS11-HC-4µm column is a hydroxide-selective high capacity anion-exchange column developed to determine anions and organic acids in a variety of samples. Figure 2 shows a separation of seven common anions when using isocratic conditions. Nitrate and nitrite are well separated from other common anions in 10 min.

Columns:	Dionex IonPac AG11-HC-4µm, 2 × 50 mm and Dionex IonPac AS11-HC-4µm, 2 × 250 mm
Eluent:	Potassium hydroxide (KOH) 30 mM
Eluent source:	Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 and Dionex high-pressure degasser
Flow rate:	0.37 mL/min
Inj. volume:	2.5 µL (Full loop)
Column temp.:	30 °C
Sampler temp.:	4 °C
Detection:	Suppressed conductivity, Dionex ADRS 600 (2 mm) suppressor, 25 °C, 29 mA, recycle mode
Sample:	A mixed anions standard



Peaks:	RT (min)	mg/L
1. Fluoride	1.97	2.0
2. Chloride	3.08	5.0
3. Nitrite	3.56	10.0
4. Sulfate	4.34	10.0
5. Bromide	5.71	20.0
6. Nitrate	6.03	20.0
7. Phosphate	9.16	30.0

Figure 2. Separation of seven common anions

Table 3. Calibration standards (mg/L)

Analyte	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6*	Level 6
Nitrite (mg/L)	0.01	0.5	2.5	5	10	12.5	25
Nitrate (mg/L)	0.02	1	5	10	20	25	50

*The level 6 calibration standard for large injection volume (25.0 µL) IC method

To quantify nitrite and nitrate in sugar samples correctly, they need to be well separated from common anions and organic acids. We started with the method in reference 7 (Gradient: -1–10 min, 5 mM; 10–15 min, 15 mM; 5–25 min, 30 mM; 25–34 min, 60 mM; 34–37 min, 5 mM) and replaced sodium hydroxide with electrolytically generated potassium hydroxide (KOH). In our lab, the reference method could not separate nitrate from a neighboring peak. To solve the problem, a 40 min gradient method was developed. The simplified IC method includes three steps: first, a group of anions, including fluoride, acetate, formate, etc., were eluted with 5 mM KOH; then nitrite and nitrate were eluted by a shallow gradient of 5 to 20 mM KOH in 25 min with good separation from potentially interfering anionic compounds (chloride, bromide, succinate, carbonate, sulfate, oxalate, and other trace amounts of unidentified organic acids); finally, all other anions were eluted in 6 min with 65 mM KOH. Figure 3 shows an overlay of chromatograms of a mixed seven common anions standard and the sugar beet syrup sample. Nitrite and nitrate are well separated and resolved from common anions and organic acids in this sugar sample. The common anions and organic acids were identified by spiking known compounds into the sample and comparing its retention time to the peaks in the sugar sample.

Columns:	Dionex IonPac AG11-HC-4µm, 2 × 50 mm and Dionex IonPac AS11-HC-4µm, 2 × 250 mm
Eluent:	Potassium hydroxide (KOH) gradient:
Gradient:	0–5 min, 5 mM; 5–30 min, 5–20 mM; 30–36 min, 65 mM; 36–40 min, 5 mM
Eluent source:	Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 and Dionex high-pressure degasser
Flow rate:	0.37 mL/min
Inj. volume:	2.5 µL (Full loop)
Column temp.:	30 °C
Sampler temp.:	4 °C
Detection:	Suppressed conductivity, Dionex ADRS 600 (2 mm) suppressor, 25 °C, 60 mA, recycle mode
Samples:	A (Black): 100 × diluted sugar beet syrup B (Blue): mixed anionic standard containing seven common anions

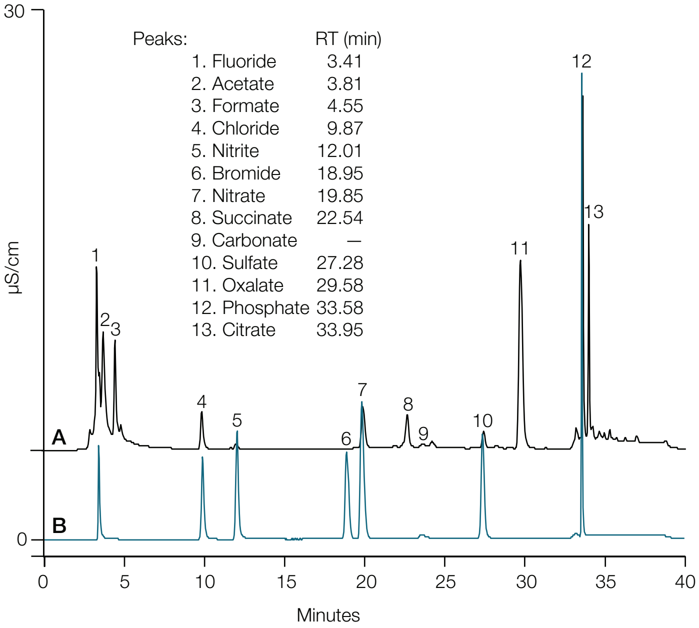


Figure 3. Separation of nitrite and nitrate from common anions and organic acids in a beet sugar sample

Calibration and method detection limits

The method linearity and detection limits were studied using both single component (nitrite or nitrate) and two-component standards (mixed nitrite and nitrate). Because no calibration difference was found for the two standard types, nitrite and nitrate in sugar were determined by an IC method using a mixed standard. The analyst should be aware that if the standard experiences microbial contamination, the amounts of nitrate and nitrite can change. The method detection limits (MDL) were determined by performing seven replicate injections of standards at a concentration of three to five times the estimated detection limits. Here, the standards of 0.01 mg/L nitrite and 0.02 mg/L nitrate were used when determining the MDL for the 2.5 µL injection, and 0.005 mg/L nitrite and 0.01 mg/L nitrate were used when determining the MDL for the 25 µL injection. The results are shown in Table 4. Calibrations and MDLs were determined for two injection volumes, 2.5 and 25 µL.

Table 4. Calibration and method detection limits

Analyte	Injection volume (µL)	Range (mg/L)	Coefficient of determination* (r^2)	Calculated** MDL (mg/L)
Nitrite	2.5	0.01–25	0.9999	0.002
Nitrate	2.5	0.02–50	1.0000	0.006
Nitrite	25	0.01–25	0.9968	0.001
Nitrite	25	0.01–12.5	0.9992	0.001
Nitrate	25	0.02–50	1.0000	0.003

*Calibration type is linear and forced through the origin.

**MDL = (*t*) x (S)

t = Student's *t* value for a 99% confidence level and a standard deviation with n-1 degrees of freedom (*t* = 3.14 for seven replicates)

S = Standard deviation of the replicate analyses

Figure 4 compares the chromatograms of the beet sugar sample using the two injection volumes. The peaks with the 2.5 µL injection are sharper. Although increasing injection volume can increase the method sensitivity, it also increases the risk of overloading the column and decreasing the linear range for some compounds. Nitric acid is a weak acid, and therefore the calibration response for nitrite does not follow the linear relationship of peak area to concentrations over as wide a concentration range observed for an anion of a strong acid such as chloride. With a 25 µL injection, the nitrite calibration does not follow the linear relationship for the range of 0.01 to 25 mg/L (Table 4 and Figure 5A), with a coefficient of determination (r^2) of 0.9968. A six-level calibration with a range of 0.01 to 12.5 mg/L should be used. If a larger calibration range is desired, quadratic fitting can be explored. It was also found that with a 2.5 µL injection, both nitrite (ranging from 0.01 to 25 mg/L) and nitrate (ranging from 0.02 to 50 mg/L) have linear calibration curves with r^2 of 0.9999 and 1, respectively (Table 4 and Figures 6A and 6B). When using a 2.5 µL injection, the MDLs are 0.002 mg/L for nitrite and 0.006 mg/L for nitrate. When using a 25.0 µL injection, the MDLs are 0.001 mg/L for nitrite and 0.003 mg/L for nitrate. As the regulatory limit for nitrite content in sugar industry feed materials is set at 15 mg/kg, both 2.5 µL and 25.0 µL are sensitive enough for regulatory monitoring, even if the sugar sample was diluted 100-fold. The 2.5 µL injection is a better choice for regulatory monitoring of nitrite content in sugar industry feed materials. It has enough sensitivity and has a larger linear calibration range. If more sensitivity is needed for this sample, a 25 µL injection can be used. When increasing injection volume, the analyst must check analyte recovery to ensure that the column has not been overloaded. Loss of retention time and loss of peak efficiency are signs of column overload. Poor spike recovery confirms column overload.

Columns: Dionex IonPac AG11-HC-4µm, 2 × 50 mm and Dionex IonPac AS11-HC-4µm, 2 × 250 mm
Eluent: Potassium hydroxide (KOH) gradient:
Gradient: 0–5 min, 5 mM; 5–30 min, 5–20 mM; 30–36 min, 65 mM; 36–40 min, 5 mM
Eluent source: Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 and Dionex high-pressure degasser
Flow rate: 0.37 mL/min
Inj. volume: Black trace: 2.5 µL (Full loop)
Pink trace: 25.0 µL (Full loop)
Column temp.: 30 °C
Sampler temp.: 4 °C
Detection: Suppressed conductivity, Dionex ADRS 600 (2 mm) suppressor, 25 °C, 60 mA, recycle mode
Samples: 100 × diluted sugar beet syrup sample

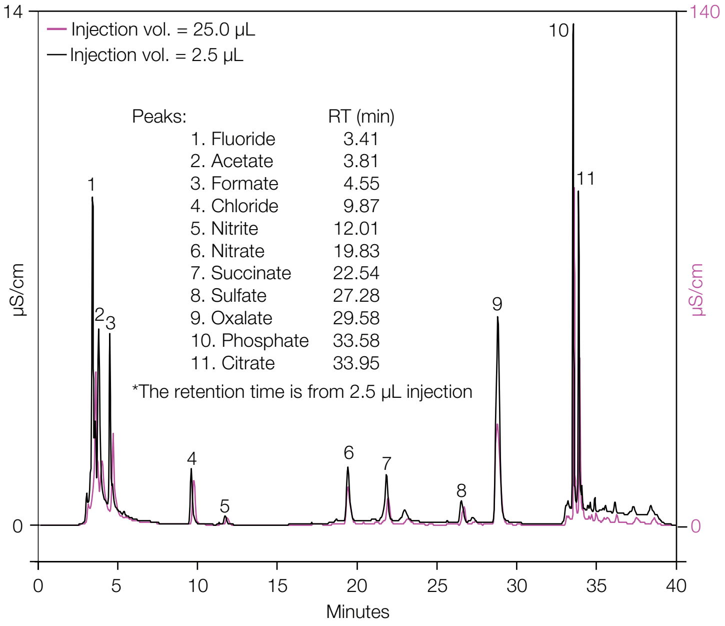


Figure 4. Comparison of the sample analysis with different injection volumes

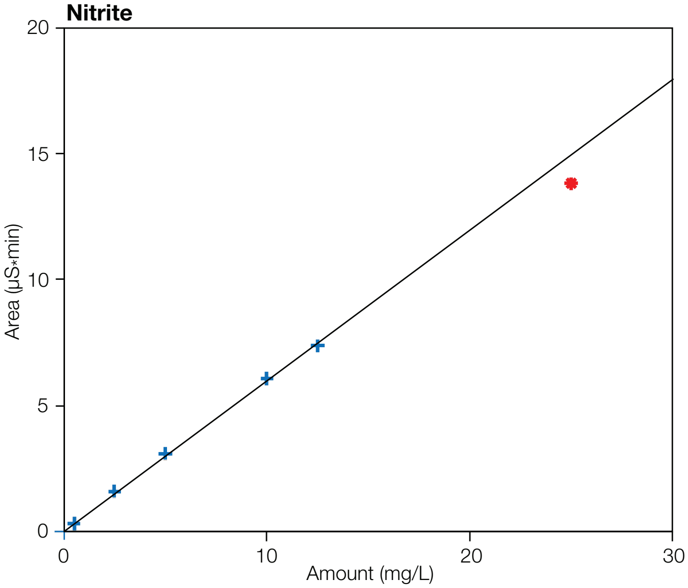


Figure 5A. Calibration plot for nitrite using a 25 µL injection volume

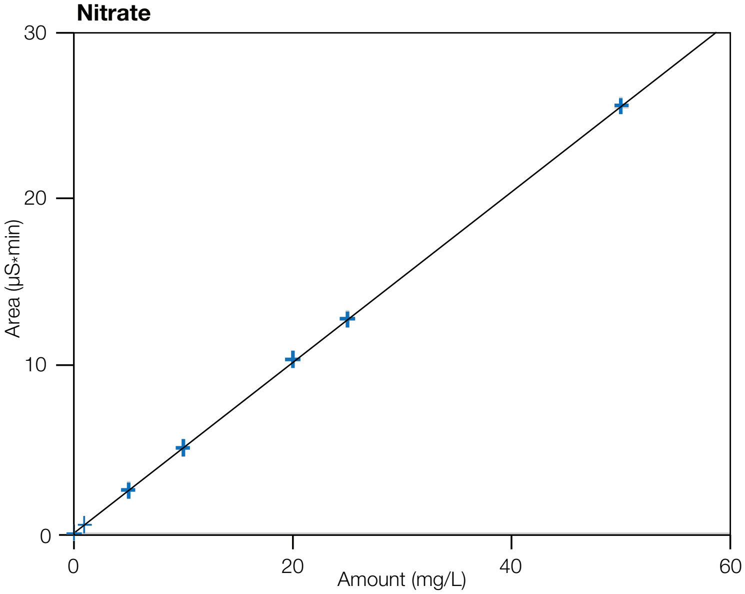


Figure 5B. Calibration plot for nitrate illustrating linearity (using a 25 µL injection volume)

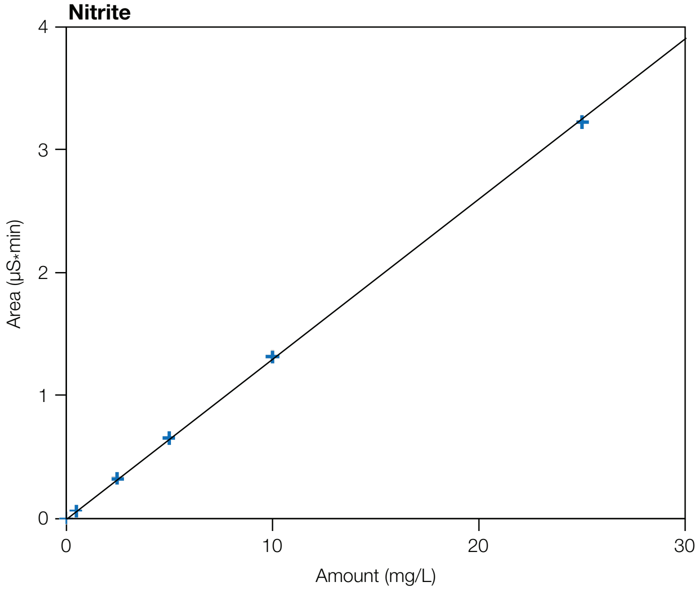


Figure 6A. Calibration plot for nitrite illustrating linearity (using a 2.5 µL injection volume)

Determination of nitrite and nitrate in sugar

To demonstrate the IC method's application for regulatory monitoring, a 2.5 µL injection was used to determine nitrite and nitrate in the three sugar samples (Table 5 and Figure 7). The two cane sugar samples did not contain nitrite and nitrate, while the beet sugar sample contained 50 mg/L (same as mg/kg) of nitrite and 544 mg/L of nitrate. Therefore, this sample exceeds the European nitrite limit of 15 mg/kg.

Table 5. Determination of nitrite and nitrate in 100-fold diluted sugar samples (mg/L)

	Sugar beet syrup*	White cane sugar**	Brown cane sugar**
Nitrite (mg/L)	0.50 ± 0.01	0	0
Nitrate (mg/L)	5.44 ± 0.09	0	0

*n > 3
**n = 3

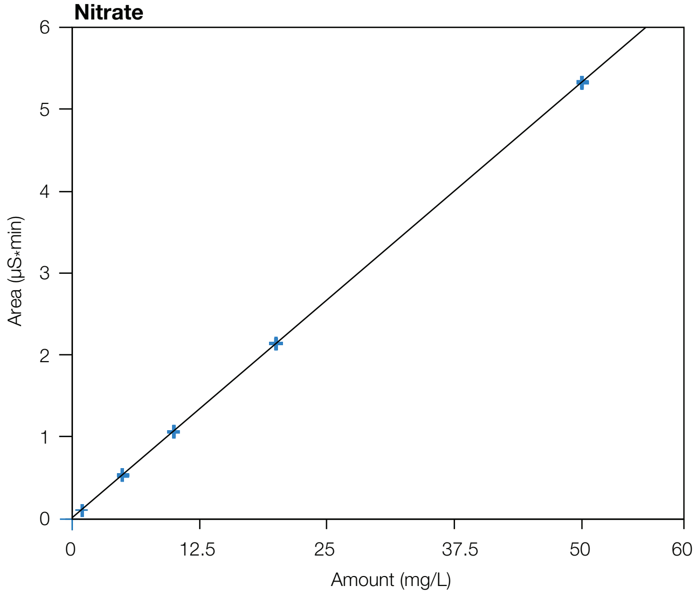
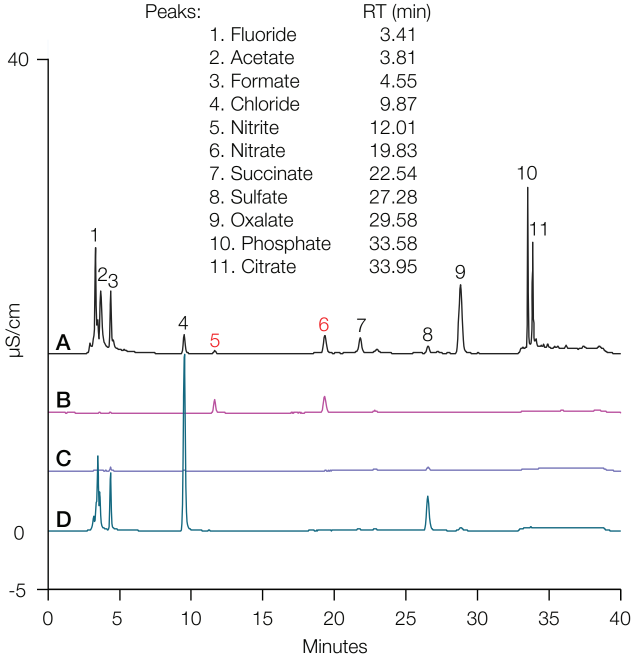


Figure 6B. Calibration plot for nitrate illustrating linearity (using a 2.5 µL injection volume)

Columns:	Dionex IonPac AG11-HC-4µm, 2 × 50 mm and Dionex IonPac AS11-HC-4µm, 2 × 250 mm
Eluent:	Potassium hydroxide (KOH) gradient:
Gradient:	0–5 min, 5 mM; 5–30 min, 5–20 mM; 30–36 min, 65 mM; 36–40 min, 5 mM
Eluent source:	Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 and Dionex high-pressure degasser
Flow rate:	0.37 mL/min
Inj. volume:	2.5 mL (Full loop)
Column temp.:	30 °C
Sampler temp.:	4 °C
Detection:	Suppressed conductivity, Dionex ADRS 600 (2 mm) suppressor, 25 °C, 60 mA, recycle mode
Samples:	A - 100 × diluted sugar beet syrup B - Nitrite (2.5 mg/L) and nitrate (5 mg/L) C - 100 × diluted white cane sugar D - 100 × diluted brown cane sugar



Peaks:	RT (min)
1. Fluoride	3.41
2. Acetate	3.81
3. Formate	4.55
4. Chloride	9.87
5. Nitrite	12.01
6. Nitrate	19.83
7. Succinate	22.54
8. Sulfate	27.28
9. Oxalate	29.58
10. Phosphate	33.58
11. Citrate	33.95

Figure 7. Determination of nitrite and nitrate in sugar samples

The method precision was evaluated by the determination of nitrite and nitrate in the sugar beet syrup over five separate days and expressed as the relative standard deviation (RSD) of the results (Table 6). Each day three samples were prepared and analyzed by the IC method with triplicate injections. The method is precise with intraday precision from 0.2% to 0.9% for nitrite and from 0.3% to 1% for nitrate, and interday precision of 1.3% for nitrite and 1.6% for nitrate.

Table 6. Determination of nitrite and nitrate in a sugar sample using the IC method with a 2.5 µL injection

	100-fold diluted sample (mg/L)	Intraday precision range* (%)	Interday** precision (%)
Nitrite	0.50 ± 0.01	0.2–0.9	1.3
Nitrate	5.44 ± 0.09	0.3–1.0	1.6

*n = 3 for each day
**over 5 days

The method accuracy was validated by spiked recovery experiments. Spiked beet sugar samples were analyzed together with the non-spiked sample. The recovery percentages were calculated using the formula shown below:

% Recovery = (c in spiked sample - c in non-spiked sample)/c added

c = concentration of nitrite or nitrate

Table 7 summarizes the recovery results. The method was accurate with both 2.5 and 25 µL injections: nitrite recovery ranged from 89% to 90% and nitrate recovery ranged from 97% to 101% when a 2.5 µL injection was used; nitrite recovery ranged from 94% to 96% and nitrate recovery ranged from 98% to 100% when a 25 µL injection was used.

Conclusion

This application note demonstrates the development and validation of an IC method for the determination of nitrite and nitrate in sugar products. The IC method uses a 2 mm version of the high capacity Dionex IonPac AS11-HC-4µm column and a three-step KOH gradient. With this method, nitrite and nitrate are well separated and resolved from all common anions and organic acids found in the sugar samples. The method is linear for both nitrite (r^2 = 0.9999 for the established range of 0.01 to 25 mg/L) and nitrate (r^2 = 1 for the established range of 0.02 to 50 mg/L) with an injection volume of 2.5 µL. It is sensitive (MDL of nitrite = 0.002 mg/L, MDL of nitrate = 0.006 mg/L), precise (RSDs ≤ 1.6%) and accurate (recovery 89% to 101%) for the determination of nitrite and nitrate in sugar. The sugar beet syrup sample tested contained 50 mg/kg of nitrite and 544 mg/kg of nitrate, which exceeds the EU Directive nitrite limit of 15 mg/kg for animal feed. These data show this method can be used for the regulatory monitoring to determine nitrite and nitrate in sugar products.

Table 7. Spiked recovery of nitrite and nitrate in sugar beet syrup using different injection volumes

Injection volume	Analyte	Spiked-1		Spiked-2	
µL		Added (mg/L)	Recovery (%)	Added (mg/L)	Recovery (%)
2.5	Nitrite	1	90	2	89
	Nitrate	2	101	4	97
25	Nitrite	1	96	2.5	94
	Nitrate	2	98	5	100

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