A sample stacking-capillary electrophoresis method for simultaneous determination of nitrate and thiocyanate ions of ultra-heat-treated milk samples

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Abstract: Ultra-heat-treated (UHT) cow milk is mostly consumed in Turkey. Heating milk can cause some chemical and biochemical changes that affect different components of milk. However, studies on the constituents of UHT milk are scare. In the present study, nitrate and thiocyanate contents of commercial UHT whole cow milk were determined simultaneously by sample stacking-capillary electrophoresis. The best separation of nitrate and thiocyanate ions was performed by reducing the electroosmotic flow at pH 4.0 using 30 mM formic acid and 30 mM sodium sulfate containing separation electrolyte. Analysis was completed in less than 3 min. The limits of detection obtained at a signal-to-noise ratio of 3 for nitrate and thiocyanate were 0.03 mg/L (0.48 μmol/L) and 0.06 mg/L (1.03 μmol/L), respectively. The nitrate concentration in commercial UHT milk samples ranged between 0.20 and 1.50 mg/L (3.23–24.20 μmol/L) and the thiocyanate ion concentration ranged between 0.41 and 0.67 mg/L (7.06–11.55 μmol/L).

Key words: Capillary electrophoresis, milk, nitrate, sample stacking, thiocyanate

1. Introduction

Small ions, such as nitrate and thiocyanate, are an important part of the human diet. Daily intake of nitrate and thiocyanate varies among cultural groups with respect to geographic regions. While nitrate is commonly used as a preservative in processed meat products, it exists naturally in some vegetables. The source of this ion in cow milk may be the natural content of the vegetable products that the cow has eaten, or the increased amounts of nitrogen-based fertilizers used in plants.[1,2] Although nitrate is not considered dangerous for human health, it can be easily reduced to a nitrite ion with bacterial action. Due to the chemical reactions of nitrite ions with secondary amines, N-nitrosamines are formed, which are known to be carcinogenic.[3] Ingestion of nitrate is a risk factor for infant methemoglobinemia, or blue baby syndrome.[4] Acceptable daily intake for nitrate is 0–3.7 mg/kg body weight per day according to Food and Agriculture Organization (FAO).[5]

Similarly, thiocyanate comes from the feeding regime of cows and occurs naturally in their milk. Thiocyanate activates a natural antibacterial system, the lacto-peroxidase system, in milk.[6] In some regions, thiocyanates are added to milk for this purpose. According to the FAO, treated milk arriving at the dairy plant would contain approximately 10 mg above the natural amount of thiocyanate per liter of milk.[7] However, high levels of thiocyanate can cause hypothyroidism.[8] Therefore, the determination of nitrate and thiocyanate levels in cow milk is important for the health of babies and infants. Recent analysis trends for easy and si-

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multaneous detection of these ions in food or biological samples are either ion chromatography[9,10] or capillary electrophoresis.[11−13]

Cow milk is an important nutrition source for babies and infants. However, from a health point of view, it is important to kill bacteria in milk. Normal pasteurization heat does not kill all bacteria; however, all harmful bacteria in UHT cow milk are killed and this type of milk can be kept at room temperature for months. UHT milk is the most widely consumed milk in Turkey.

Today, a wide variety of food analysis methods are based on modern separation techniques. There are several reports on the nitrate[14,15] and thiocyanate[16,17] contents of milk worldwide by ion chromatography (IC). Despite several applications of capillary electrophoresis (CE) in the determination of nitrate and thiocyanate in meat and vegetable samples,[18] no studies have been conducted on milk samples with this method.

Bjergegaard et al. reported an MEKC method, which involves prepurification for two ions in biological fluids.[13] The method was applied to one blood and one milk sample, but no quantitative results were given. To the best of our knowledge, a simple capillary electrophoresis method for determination of nitrate and thiocyanate in milk samples is not available in the literature. The fused silica capillaries in CE are easily washed between runs and free of irreversible contamination. This is the main advantage of capillaries compared to the packed columns of IC. Moreover, separation time in CE is much faster compared to other chromatographic techniques for small ions.

Studies on the levels of nitrate ions in UHT milk are scarce, and no reports on the thiocyanate levels in UHT milks exist. The heating process of milk causes some chemical and biochemical changes that affect different components, mainly proteins, carbohydrates, and vitamins.[19] Therefore, it might be useful to check the small ion content of UHT milks. This study aims to determine nitrate and thiocyanate levels of UHT milks chosen from the most consumed commercial brands in the Turkish market. Analysis was performed by CE combined with sample stacking. Sample stacking is an online concentration method in CE. This method is based on the principle of creating electric field differences within the capillaries due to buffer and sample conductivity differences. The method was broadly described and reviewed by Quirino and Terabe.[20]

2. Results and discussion

2.1. CE method validation

The electropherogram of standard nitrate and thiocyanate ions is given in Figure 1. The validation of the proposed method was performed according to Eurachem guidelines.[21] Calibration curves for nitrate and thiocyanate ions were obtained for six different concentrations of these analytes in aqueous solution. Every concentration was injected three times and an average was used to plot calibration curves. The limit of detection (LOD) was considered 3 times the average noise taken for three different baseline areas and the limit of quantification (LOQ) was given as ten times the average noise. For the precision of the method, the nitrate and thiocyanate standard solution was injected on three different days, 7 times per day. The method validation values are given in Table 1.

The recovery experiments were done with one milk sample (E). The milk sample was spiked with standard nitrate and thiocyanate solutions. All spiked concentration and recovery values are given in Table 2. Satisfactory recovery values for nitrate and thiocyanate were between 96.8% and 101%.
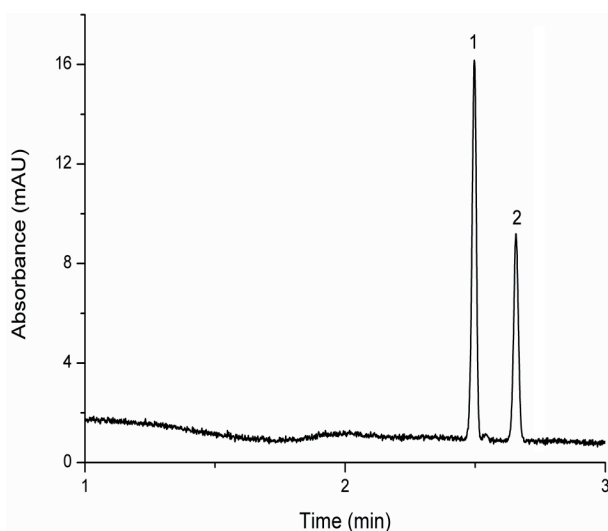
Figure 1. Electropherogram of 0.372 mg/L standard nitrate and 0.696 mg/L thiocyanate ions: 1) nitrate ion; 2) thiocyanate ion. Capillary: 50 μm i.d. and 58 cm (50 cm to detector); injection: 50 mbar, 160 s; running voltage: –25 kV; detection: 210 nm. Separation electrolyte: 30 mM formic acid and 30 mM sodium sulfate at pH 4.00.

Table 1. Method validation values.

	Nitrate	Thiocyanate
Linear concentration range (mg/L)	0.20–1.86	0.29–1.74
Regression coefficients of calibration curves	0.997	0.992
Slopes	0.00921	0.00456
Intercepts	0.00954	0.00383
Standard errors in slopes	0.00877	0.00222
Standard errors in intercepts	0.00043	0.00078
LOD (mg/L)	0.03	0.06
LOQ (mg/L)	0.10	0.20
Intraday precision as corrected area (RSD, %)	3.93	4.32
Interday precision as corrected area (RSD, %)	3.69	3.61
Intraday precision for migration times (RSD, %)	2.09	1.74
Interday precision for migration times (RSD, %)	2.15	2.19

## 2.2. Determination of ions in milk samples

Figure 2 shows the electropherogram of a milk sample (E) and Table 3 gives the nitrate and thiocyanate ion concentrations of eight UHT samples. As seen from Table 3, the nitrate content in UHT milks ranges from 0.20 to 1.50 mg/L. Thiocyanate content of milks is between 0.41 and 0.67 mg/L.

Nitrate values for UHT milks from Portugal obtained via spectrophotometric determination based on Griess reaction were between 0.77 and 4.46 mg/L.[22] No report on thiocyanate levels of UHT milks was found in the literature.

It is not possible to detect a significant difference between the levels of these two ions in fresh milk and UHT milk samples due to insufficient data. Future work on the compositions of UHT milk samples might reveal

Table 2. Recovery values for nitrate and thiocyanate amount in milk sample E.

Analyte	Calculated amount of 1/2 diluted sample E (mg/L)	Amount of spike concentration (mg/L)	Recovery (%)
Nitrate	0.75	0.375	101
		0.75	99.0
Thiocyanate	0.33	0.16	97.5
		0.33	96.8
		0.70	98.6
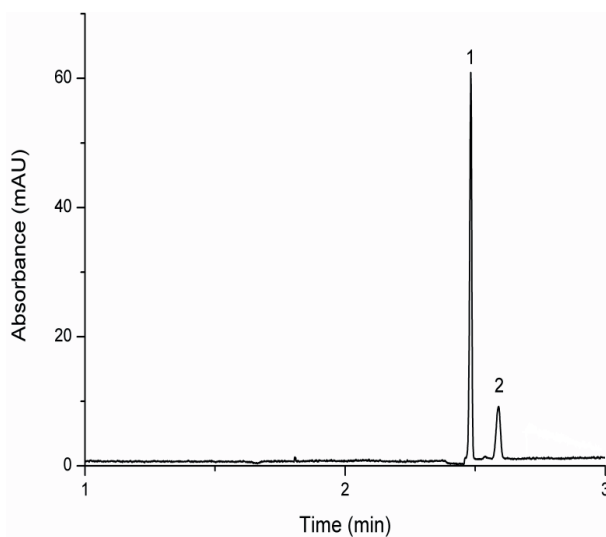
Figure 2. Electropherogram of a milk sample (E): 1) nitrate ion; 2) thiocyanate ion. Separation conditions are the same as in Figure 1.

Table 3. Nitrate and thiocyanate amount of UHT milk samples.

Sample	Nitrate (mg/L $\pm$ SE)	Thiocyanate (mg/L $\pm$ SE)
A	0.29 $\pm$ 0.00	0.53 $\pm$ 0.05
B	0.91 $\pm$ 0.35	0.57 $\pm$ 0.20
C	0.20 $\pm$ 0.05	0.41 $\pm$ 0.00
D	1.2 $\pm$ 0.5	0.43 $\pm$ 0.20
E	1.5 $\pm$ 0.0	0.67 $\pm$ 0.02
F	0.38 $\pm$ 0.05	0.44 $\pm$ 0.00
G	0.46 $\pm$ 0.17	0.44 $\pm$ 0.22
H	0.94 $\pm$ 0.02	0.47 $\pm$ 0.02

$N = 3$ for all samples; standard error (SE) was calculated at a 95% CL.

more about this issue. When the reported data for nitrate and thiocyanate in fresh milk samples were examined, the levels of both nitrate and thiocyanate were scattered in a large area. The results of this study are inside this range but generally smaller than their mean or median results. For example, the nitrate content of fresh milks

in aseptic packages in Taiwan ranges from 0.3 to 42.3 mg/kg, with 14.3 mg/kg mean.[15] Thiocyanate levels in 1669 raw milk samples from China, 270 samples from New Zealand, and 120 samples from the Netherlands ranged from 0.10 to 16.20 mg/kg and the average concentration of thiocyanate was 2.11 mg/kg.[16]

2.3. Conclusions

The present study provides the nitrate and thiocyanate contents for UHT milk samples from the Turkish domestic market. With the applied CE method, direct injection of the samples is carried out without requiring any preliminary preparation method. Due to their high electrophoretic mobilities, small anions migrate very fast in the capillaries under the separation conditions applied. Separation of both ions occurs at the high resolution value calculated as 4.7. Therefore, it is a very fast method of analysis compared to classical spectroscopic methods. Sensitivity of the method is high for the determination of ions in the milk sample. Since there is a limited number of reports in the literature on nitrate in UHT milk and there is no value for thiocyanate, the values obtained for UHT milk in this study will be a basis in this regard.

3. Experimental

3.1. Materials

Potassium nitrate, formic acid, sodium sulfate, acetic acid, methanol, and trichloroacetic acid (TCA) were from Merck (Darmstadt, Germany); potassium thiocyanate was from Carlo Erba (Sabadell, Spain). All solutions were prepared with water purified with an ElgaPurelab Option 7-15 model system (Elga, UK). Stock standard solutions of nitrate and thiocyanate were prepared in pure water at a 10 mM concentration and used with further dilutions.

3.2. Milk samples

Eight commercial UHT milk samples were purchased from local markets in İstanbul. All milk samples were pure, whole cow milk with 3% milk fat homogenized using ultra-high-temperature technology. All commercial samples were packed in aseptic cartons.

All samples were prepared as milk : 1% TCA : methanol (1:1:1) in centrifuge tubes. After the mixture was centrifuged at 2500 rpm for 15 min with an MRC Scientific Industries centrifuge (London, UK), the samples were filtered through a 0.2-μm microfilter and injected directly to the capillary column.

3.3. CE conditions

Separations were performed with an Agilent CE system (Waldbronn, Germany) equipped with a diode-array detector. The data processing was carried out with Agilent ChemStation software. The wavelength was set at 210 nm. The separation was performed at 25 kV and injections were done from the cathode side, changing the polarity. The temperature was set at 25 °C. Injections were made at 50 mbar for 160 s. The fused-silica capillary used for separation experiments was 50 μm i.d. and was obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 58 cm and the length to the detector was 50 cm. A new fused-silica capillary was conditioned prior to use by rinsing with 1 M NaOH for 30 min and with water for 10 min. The capillary was flushed successively with 0.1 M NaOH for 2 min, water for 2 min, and separation electrolyte for 5 min at the beginning of every working day and between runs.

A recently published CE procedure for the simultaneous determination of nitrite and nitrate ions in processed fish products was adapted for the separation of nitrate and thiocyanate ions in milk samples.[23]

Electroosmotic flow inside the capillary was reduced using a separation electrolyte at a low pH. Samples were injected from the negative electrode side and the polarity was changed. In this way, small anions migrate to the detector, which is placed on the positive electrode side. Though they migrate counter to the electroosmotic flow direction, due to the high electrophoretic mobilities of nitrate and thiocyanate ions compared to the low electroosmotic mobility inside the capillary the migration of nitrate and thiocyanate ions to the detector side was very fast. Since the preliminary experiments showed that the levels of ions in UHT milk are comparatively low, sample stacking was applied by increasing both separation electrolyte concentration and injection volume. In sample stacking, ions migrate faster in low-conductivity sample zones due to the high electrical field of that zone, and when they reach the boundary of the sample zone and high-conductivity separation electrolyte zone, the electrical field suddenly decreases on the boundary and ions stack and are concentrated. Thereby, with this application, low detection limits for small inorganic ions are reached. Here, a salt, sodium sulfate, was added to the formic acid/formate buffer to increase the conductivity of the separation medium. Injection time increased up to 160 s. The optimized separation electrolyte was a mixture of 30 mM formic acid and 30 mM sodium sulfate at pH 4.00.

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References

1. Stagnari, F.; Di Bitetto, V.; Pisante, M. *Sci. Hortic.* **2007**, *114*, 225-233.

2. Indyk, H. E.; Woollard, D. C. In *Encyclopedia of Dairy Sciences*; Academic Press: San Diego, CA, USA, 2011, pp. 906-991.

3. Mitacek, E. J.; Brunnemann, K. D.; Suttajit, M.; Caplan, L. S.; Gagna, C. E.; Bhothisuwan, K.; Siriamornpun, S.; Hummel, C. F.; Ohshima, H.; Roy, R. et al. *Nutr. Cancer* **2008**, *60*, 196-203.

4. Greer, F. R.; Shannon, M. *Pediatrics* **2005**, *116*, 784-786.

5. WHO. *Health Criteria and Other Supporting Information. Vol. 2*; WHO: Geneva, Switzerland, 1998.

6. Fonteh, F.; Grandison, A. A. S.; Lewis, M. J. *J. Dairy Res.* **2002**, *69*, 401-409.

7. Food and Agriculture Organization for the United Nations. *Guidelines for the Preservation of Raw Milk by Use of the Lactoperoxidase System*; FAO: Rome, Italy, 1991.

8. Green, W. L. In: *Mechanisms of Action of Antithyroid Compounds*; Werner, S. C.; Ingbar, S. H., Eds. Harper & Row: New York, NY, USA, 1978.

9. Blount, B. C.; Valentin-Blasini, L. *Anal. Chim. Acta* **2006**, *567*, 87-93.

10. Connolly, D.; Barron, L.; Paull, B. *J. Chromatogr. B* **2002**, *767*, 175-180.

11. Vitali, L.; Favere, V. T.; Micke, G. A. *J. Chromatogr. A* **2011**, *1218*, 2327-2333.

12. Nutku, M. S.; Erim, F. B. *J. High Resolut. Chromatogr.* **1998**, *21*, 505-508.

13. Bjergegaard, C.; Moller, P.; Sorensen, H. *J. Chromatogr. A* **1995**, *717*, 409-414.

14. Chanmandust, S.; Mehrasebi, M. R.; Kamali, K.; Solgi, R.; Taran, J.; Nazari, F.; Hosseini, M. J. *Int. J. Food Prop.* **2016**, *19*, 1983-1993.

15. Yeh, T. S.; Liao, S. F.; Kuo, C. Y.; Hwang, W. *J. Food Drug Anal.* **2013**, *21*, 73-79.

16. Yong, L.; Wang, Y.; Yang, D.; Liu, Z.; Abernethy, G. L. *J. Food Chem.* **2017**, *215*, 61-66.

17. Kirk, A. B.; Dyke, J. V.; Martin, C. F.; Dasgupta, P. K. *Environ. Health Persp.* **2007**, *115*, 182-186.

18. Öztekin, N.; Nutku, M. S.; Erim, F. B. *Food Chem.* **2002**, *76*, 103-106.

19. Ferrer, E.; Alegria, A.; Courtoisi, G.; Farre, R. *J. Chromatogr. A* **2000**, *881*, 599-606.

20. Quirino, J. P.; Terabe, S. *J. Chromatogr. A* **2000**, *902*, 119-135.

21. Eurachem. *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*; Eurachem: Torino, Italy, 1998.

22. Reis Lima, M. J.; Fernandes, S. M.V.; Ranges, A. O. S. *Int. Dairy J.* **2006**, *16*, 1442-1447.

23. Kalaycioglu, Z.; Erim, F. B. *Food Anal. Method.* **2016**, *9*, 706-711.