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Determination of nitrate and nitrite in vegetables by capillary electrophoresis with indirect detection

M. Jimidar, C. Hartmann, N. Cousement, D.L. Massart*

Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium

Abstract

Nitrate and nitrite (and some other anions) were determined in vegetables by capillary electrophoresis (CE). The anions were extracted from the vegetables by mixing and diluting the samples with water at moderate temperature. The CE method is divided into two parts: a high-concentration-level method (for nitrate determination) and a low-concentration-level method (for nitrite determination). These CE methods were compared with a reference method (spectrophotometry after Jones reduction: official AOAC reference method for the determination of nitrates in foodstuffs). Parameters such as linearity, detection limit, quantification limit, precision and accuracy of the two techniques were investigated and compared. Both techniques resulted in acceptable linearity within their ranges. The detection limits of the CE methods were sufficiently low for the determination of the anions in vegetable samples. The precision and accuracy of the CE methods were comparable to those of the reference method. The precision was determined by evaluating the repeatability and the time-different intermediate precision, while the accuracy was investigated by comparing the slopes of the standard addition and external calibration lines and by evaluating the agreement between the results obtained with the CE and the reference spectrophotometric methods.

1. Introduction

Nitrate and nitrite are common and natural constituents of many foodstuffs. Their occurrence can also be the result of a deliberate addition during food processing. In the latter case they are considered as food additives. The presence of nitrate in foods is of concern because it can be reduced to nitrite, which is able to induce methaemoglobinaemia. Nitrates can also react with secondary and tertiary amines resulting in the formation of carcinogenic nitrosamines. The acceptable daily intake (ADI) recommended by the World Health Organization is 220 mg nitrate for an adult person of about 60 kg. For nitrite the recommended ADI is 8 mg [1].

The determination of nitrate and nitrite has already been performed by several techniques. Spectrophotometric [2–7], ion-selective electrode [8] and chromatographic [9–12] techniques have been reported. Recently, one can add capillary zone electrophoresis (CZE) to this list. The determination of nitrate and nitrite together with other anions by CZE was first reported by Jones and Jandik [13]. Their method is based on indirect detection of the anions. For this purpose one uses a background electrolyte, which in this

* Corresponding author.

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case is chromate [13–15]. At present CE is used for the determination of a wide variety of ions [13–21]. More recently, a direct UV detection method was described for the determination of nitrate and nitrite in water and urine samples [19].

The method for determining anions by CE has been further optimized by us [20,21]. Instead of using reagents with an unknown composition supplied by Waters (Milford, MA, USA), necessary for the determination of anions, we preferred to work with known substances. Therefore, in the optimization procedure cetyltrimethylammonium bromide (CTAB) was used instead of Anion-BT. The mobility of the anions as a function of the pH and the concentration of CTAB was described with a physical model [20,21]. By applying this model, a selectivity optimization was carried out [21], resulting in a good separation of ten inorganic anions.

The performance of the optimized method with complex sample matrices was studied in this work for the determination of nitrates and nitrites in vegetables. The aim was to compare the results of the CE method with that of a reference method. The AOAC describes several reference methods for the determination of different compounds in various matrices. For ions these are usually photometric methods. A specific reference method for the determination of nitrate and nitrite in vegetables has not been proposed by the AOAC. It does, however, provide a method for the determination of these anions in cheese [4]. Several workers have also applied this method or a slightly modified one to determinations in vegetables [2,3,5,6]. Nitrate is reduced to nitrite over a cadmium column (Jones reduction) and nitrite is then determined after diazotization of an aromatic amine followed by reaction of the diazonium compound with a coupling reagent. The colour intensity of the azo dye formed is proportional to the nitrate concentration [2–4,6].

Different extraction procedures have been described for nitrates and nitrites in the literature. Depending on the matrix, these procedures range from simple extraction with water followed by deproteinization for biological (food) samples [2–5,8,12], to ultracentrifugation or ultrafiltration for clinical samples [9,11]. The sample extraction procedure in the AOAC method [4] employs $ZnSO_4$, which would result in a large interfering peak for sulphate in the electropherogram. This makes the determination of anions with mobilities similar to sulphate impossible. An alternative extraction procedure was found in the literature [2,3] which was more compatible with the CE method. The method described by Lox and Okabe [2,3] closely resembles the AOAC procedure, but does not employ $ZnSO_4$ during the sample preparation. The sample preparation procedure consists in extraction of nitrates and nitrites with water at moderate temperature, followed by deproteinization with Carrez solutions. The deproteinization step is not necessary in the sample extraction procedure in the CE determinations, because proteins usually migrate much more slowly than small ions, and can therefore be flushed out of the capillary between the runs. The proteins might lead to fouling of the uncoated silica capillary, but with proper rinsing procedures one can avoid this problem.

2. Experimental

2.1. Reagents

All solutions were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, USA). Sodium chromate, sodium fluoride, sodium bromide, sodium chloride, sodium sulphate, sodium nitrite, sodium nitrate, sodium iodide, sodium thiosulphate, sodium molybdate, sodium tungstate, sodium monohydrogenphosphate, potassium hexacyanoferrate(II), sodium hydrogencarbonate, zinc acetate, sulphanilic acid, hydrochloric acid, (di)-sodium EDTA, ammonia, acetic acid, sodium oxalate, sodium citrate, sodium acetate, sodium propionate, sodium butyrate, sodium hydroxide, potassium hydroxide, cetyltrimethylammonium bromide and acetonitrile were purchased from Merck (Darmstadt, Germany) and sodium formate and 1-naphthylamine from UCB (Belgium).

2.2. Samples

Fifteen fresh vegetables were obtained at a local supermarket (winter season), namely spinach, lettuce, corn salad, celery, leek, watercress, endive, parsley, cauliflower, cucumber, white cabbage, red cabbage, broccoli, onion and tomato.

2.3. Preparation of solutions

Spectrophotometric method

Carrez I solution consisted of 53.00 g of potassium hexacyanoferrate(II) [$K_4Fe_2(CN)_6 \cdot 2H_2O$] in 0.5 l of water and Carrez II of 109.99 g of zinc acetate [$Zn(OAc)_2 \cdot 2H_2O$] diluted to 0.5 l with water. Griess solution A consisted of 1.4997 g of sulphanilic acid + 5 g of sodium chloride and 50 ml of acetic acid diluted to 250 ml with water, and Griess solution B of 0.0593 g of 1-naphthylamine + 50 ml of acetic acid diluted to 250 ml with water. Griess mixture was prepared daily by mixing equal amounts of Griess solutions A and B, and was kept in darkness.

Ammonium buffer consisted of 40 ml of hydrochloric acid + 100 ml of ammonia diluted to 1000 ml with water. This solution should have a pH between 9.6 and 9.7.

Sodium EDTA solution consisted of 20.8 g of Na_2EDTA + 30 ml of 15% NaOH diluted to 500 ml with water.

CE method

Sodium chromate was prepared as a 0.1 *M* stock standard solution. All buffers were prepared in 50-ml aliquots and filtered through a 0.45-mm Millex-HV syringe filter (Millipore, Molsheim, France). The buffers were adjusted to the final pH using 0.01 *M* sodium hydroxide.

The modifier was prepared as a 50 m*M* CTAB solution. The solubility was enhanced by the addition of 5% (v/v) of acetonitrile and stirring on a magnetic stirrer at moderate temperature. After dissolution, it was filtered through the same kind of syringe filter.

The buffer electrolyte was prepared as follows: 5 ml of 0.1 *M* sodium chromate solution and 2.3 ml of 50 m*M* CTAB solution were mixed, the pH was adjusted to 11.50 and the solution was diluted to 50.0 ml. Prior to analysis this solution was filtered through a Millex-HV filter.

Stock standard solutions of 1000 μg/ml of each anion were prepared in Milli-Q-purified water and stored in a refrigerator. Working standard solutions of each anion were prepared daily by dilution. The buffers were adjusted to the required pH using an Orion Model 520 A pH meter.

2.4. Apparatus

The equipment used included a Bamix mixer, a warm water-bath, S&S No. 598$\frac{1}{2}$ filters, Millex-HV 0.45-μm syringe filters (nitrate free) and a Shimadzu UV-2101PC UV–Vis Scanning spectrophotometer.

A Waters Quanta-4000 CE system equipped with a negative power supply was used. The capillaries were ordinary fused-silica capillaries (Waters AccuSep, 60-cm capillaries) of 75 μm I.D. and length 52 cm from the point of sample introduction to the point of detection. The electrophoretic zones were detected with a fixed-wavelength UV detector at 254 nm (mercury lamp). Depending on the concentration level in the samples, the hydrostatic injection mode (10 s) or the electromigration injection mode (10 s, −10 kV) was used for the injection of the samples. The electropherograms were recorded and integrated with a Waters Model 810 data workstation equipped with a W51-watchdog interface.

2.5. Preparation of the capillary

Each time before changing a buffer, the capillary was purged with 0.5 *M* potassium hydroxide solution (KOH) for 5 min, followed by Milli-Q-purified water for 5 min and the buffer electrolyte for 5 min. Between each run, the capillary was flushed with 0.1 *M* KOH for 1 min, followed by flushing with Milli-Q-purified water for 2 min and the running buffer for 2 min. Before shut-down, the capillary was flushed with 0.5 *M* KOH for 5-min and Milli-Q-purified water

for 5 min. The inlet and outlet of the capillary were kept in Milli-Q-purified water.

2.6. Preparation of the modified Jones reductor column

The cadmium column was prepared as described by Sen and Donaldson [5], with the minor difference that instead of generating the cadmium particles starting from cadmium sulphate, we used cadmium particles obtained from Merck. The efficiency of the column was also tested as described by Sen and Donaldson [5].

2.7. Sample preparation

The preparation of the samples consisted of weighing at least 10.0 to up to 50.0 g of fresh vegetable material (cut into small pieces) in a 250-ml beaker, adding 50 ml of Milli-Q-purified water, incubating for 30 min on a warm water-bath at about 50°C and homogenizing with the mixer for 1 min. After cooling, the slurry was transferred quantitatively into a 250-ml volumetric flask. For the spectrophotometric sample extract, 10 ml each of Carrez I and II solutions were added and diluted to volume with Milli-Q-purified water. For the CE sample extract, the addition of Carrez solutions is not needed. Finally, the samples were divided into small parts that were kept in a deep-freezer. Before injection into the CE system, the samples were first filtered through a Millex-HV 0.45-μm syringe filter.

No contamination from glass- and plasticware was observed. The use of Millex-HV filters is preferred as they are free from nitrate. Water (Milli-Q) blank samples, apart from a carbonate peak, did not give any indication of contamination.

2.8. Nitrate and nitrite determination

Spectrophotometry

Nitrate and nitrite were determined according to the method described by the AOAC [4] by direct interpolation of the absorbance of an unknown sample on a calibration line. A volume of each sample filtrate was pipetted into a 25-ml flask and after the addition of 5 ml of Na_2EDTA solution and 5 ml of ammonium buffer it was diluted to volume with water. These solutions were passed through the cadmium column. The eluate and rinsing solutions (ca. 40 ml of a solution prepared by mixing equal amounts of ammonium buffer and Na_2EDTA solutions) were collected in 50-ml volumetric flasks and diluted to volume with water. The calibration graph was prepared by sampling and treating different volumes of a 50 μg/ml standard nitrate solution similarly. Aliquots of 10 ml from each 50-ml flask were mixed with 10 ml of Griess mixture and kept in darkness for 20 min, then the intensity of the developed colour was measured at 526 nm. The procedure for nitrite determination was similar to that for nitrate, except that the samples were not passed through the cadmium column. The concentration of nitrate in the sample extract was obtained by subtracting the amount of nitrite from the original result of the nitrate determination.

Capillary electrophoresis

In CE of high-concentration-level samples, the extracts are injected for 10 s hydrostatically and run at 20 kV (negative potential). No further sample preparation, except sometimes dilution, is required. This procedure was applied to the determination of nitrate in vegetables, as it occurs in large amounts.

For the analysis of low concentration level samples, the injection was carried out by electromigration for 10 s at −10 kV. To reduce the baseline noise, the runs were also carried out at a lower voltage, namely −15 kV. This procedure was applied mainly to the determination of nitrite in the samples.

3. Results and discussion

With the CE method it is possible to separate several anions simultaneously within a short analysis time. In Fig. 1, an example of an electropherogram for a standard mixture of eighteen anions obtained with the low concen-

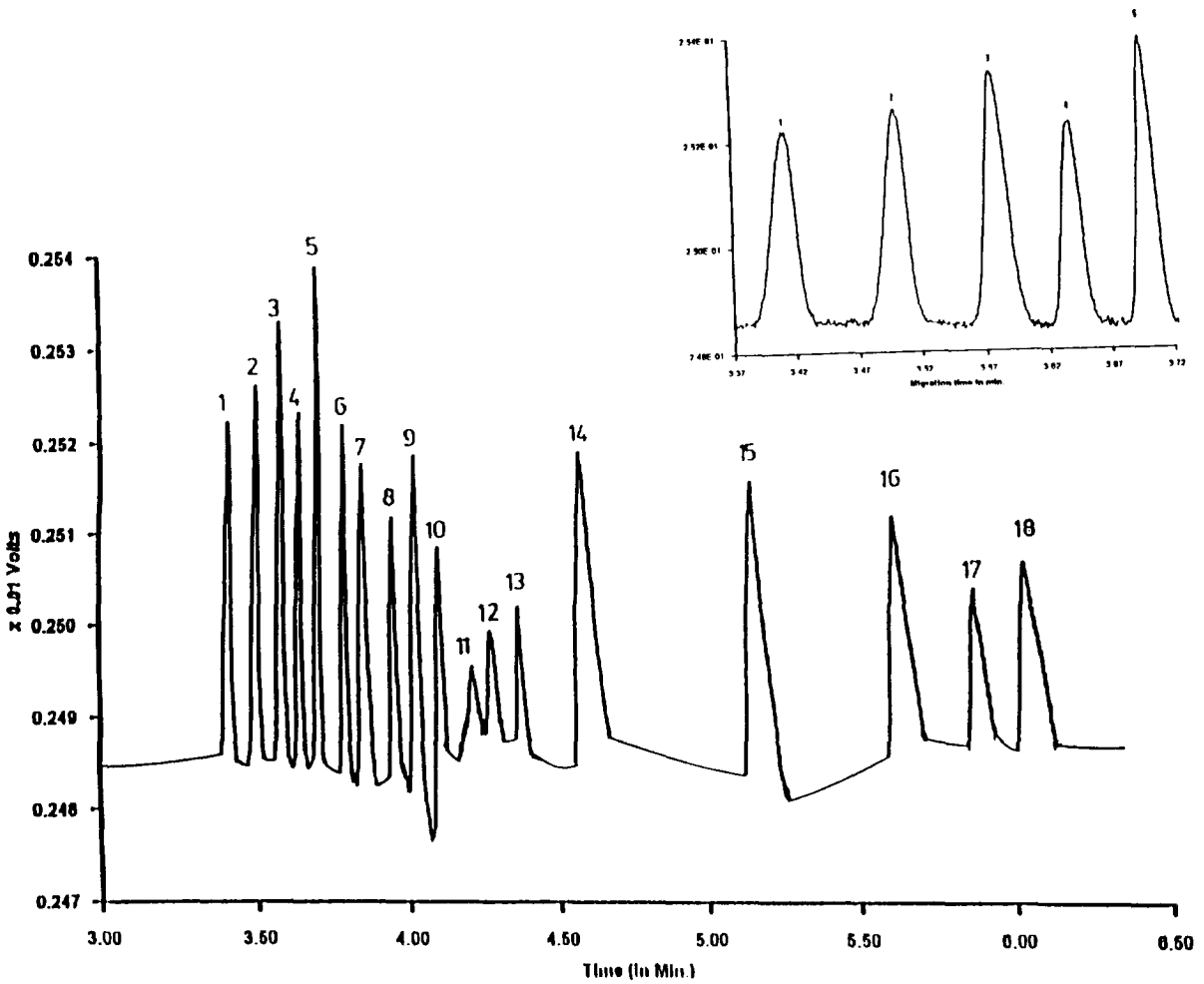

Fig. 1. Electropherogram of a standard mixture. Conditions: $[CrO_4^{2-}] = 10$ mM; pH = 11.50; [CTAB] = 2.30 mM. A mixture of the anions was injected for 10 s at −10 kV by electromigrative injection; the running voltage was −15 kV (with a current of ±30 μA). Peaks: 1 = Cl^-; 2 = Br^-; 3 = NO_2^-; 4 = SO_4^{2-}, 5 = $S_2O_3^{2-}$; 6 = oxalate; 7 = NO_3^-; 8 = MoO_4^{2-}; 9 = HCO_3^-; 10 = WO_4^{2-}; 11 = F^-; 12 = formate; 13 = HPO_4^{2-}; 14 = citrate; 15 = acetate; 16 = propionate; 17 = BO_3^{3-}; 18 = butyrate. The concentration levels are given in the text.

tration level CE method is presented. As can be observed, all the anions, chloride (Cl^- 0.10 μg/ml), bromide (Br^- 0.50 μg/ml), nitrite (NO_2^- 0.10 μg/ml), sulphate (SO_4^{2-} 0.10 μg/ml), thiosulphate ($S_2O_3^{2-}$ 0.10 μg/ml), oxalate (0.10 μg/ml), nitrate (NO_3^- 0.10 μg/ml), molybdate (MoO_4^{2-} 0.10 μg/ml), hydrogencarbonate (HCO_3^- 0.10 μg/ml), tungstate (WO_4^{2-} 0.10 μg/ml), fluoride (F^- 0.05 μg/ml), formate (0.10 μg/ml), hydrogenphosphate (HPO_4^{2-} 0.10 μg/ml), citrate (1.0 μg/ml), acetate (1.0 μg/ml), propionate (1.0 μg/ml), borate (BO_3^{3-} 1.0 μg/ml) and butyrate (1.0 μg/ml), are separated completely in a separation window of about 3 min and with a run time of only 6.5 min. Most of the organic anions and borate are slow-moving anions compared with chromate. For this reason, they show significant peak tailing. Therefore, the concentration of these anions in Fig. 1 is ten times higher. For the high concentration level CE method these times are shorter as the runs are performed at higher voltage. This is the major advantage of the CE method over the spectrophotometric method. As can be observed

in the enlarged part of Fig. 1, the separation of the peak pairs chloride–nitrite and nitrite–sulphate shows high resolution. This is one of the advantages of the optimized method proposed by us [20,21] compared with the method of Jones and Jandik [13,15]. For this reason, the injection (e.g., by electromigration) of large amounts of chloride and sulphate ions does not interfere in the determination of nitrite.

As nitrate occurs in large amounts in most of the samples, the high concentration level CE method was applied to determine this anion in vegetables. The injection for this method was performed hydrostatically and resulted in an acceptable precision. Therefore, there was no need for an internal standard. In the low concentration level CE method the injection was performed by means of electromigration. As was expected, this injection procedure resulted in a poor precision. An internal standard was required to obtain low R.S.D. values. As can be observed in Fig. 1, thiosulphate, tungstate, molybdate and borate can be employed as possible internal standards. However, sodium thiosulphate was selected as it does not occur in the vegetable samples and it migrates just after sulphate and before oxalate (between the nitrite and the nitrate peak). If necessary one can always use one of the other possible internal standards. The low concentration level method was applied mainly for the determination of nitrite in the samples. Nitrites can occur in very low concentration levels in the samples. Owing to its toxicity, it is necessary to detect nitrite below the 0.1 μg/ml level [1].

Examples of the electropherograms obtained for real samples are shown in Fig. 2a, b, c and d for samples of spinach, spinach diluted 50-fold, endive and tomato, respectively. Some of the samples, e.g., spinach had to be diluted in order to obtain a signal in the linear range and to observe the separation between the oxalate and the nitrate peaks. As was stated before, several anions can be determined simultaneously with the CE method. However, this study was focused mainly on the determination of nitrate and nitrite in the vegetables. Since the method was not fully validated for the determination of the other anions, the concentrations obtained for these anions are shown only for indicative purposes (Table 1). As can be observed in the electropherograms in Fig. 2, several peaks are not identified. In general, peak identification in CE remains a difficult task until better detection systems become available for this purpose, e.g., CE–mass spectrometry. The unknown peaks are probably organic in nature. However, we were not able to identify them. In Fig. 2b, the height of the carbonate peak is not consistent with what is expected from the undiluted sample (Fig. 2a). Carbonate is known to show a strange quantitative behaviour in CE. Especially at low concentrations the reproducibility of the peak height is very poor. The determination of carbonate requires specific precautions in order to avoid contamination from the environment (CO_2) [15]. This anion is therefore usually not determined. The results for the determination of nitrate by the reference spectrophotometric (AOAC) and the high concentration level CE methods are shown in Table 2. Nitrite was not detected in the samples investigated in this study by either the spectrophotometric method or the low-concentration-level CE method. This means that the probable occurrence of nitrite in the vegetables lies below the limit of detection of these methods.

3.1. Linearity

High-concentration-level CE method

The linearity of the high-concentration-level method was judged from the residual plots. These plots indicated that the residual patterns were almost homogeneous, i.e., that there was no need for weighted regression. The quality coefficients [22] obtained for the regression lines were within the acceptable range. The linearity of the methods was also determined by an analysis of variance (ANOVA) test for lack of fit. At each concentration level (at least five), four runs were carried out for this purpose. In spectrophotometry no significant lack of fit was detected for either nitrite ($p > 0.1$) or nitrate ($p > 0.9$). In CE a significant lack of fit was detected for nitrite ($p < 0.025$), but not for nitrate ($p > 0.9$). In the regression procedure for nitrite in the CE and AOAC methods, a signifi-

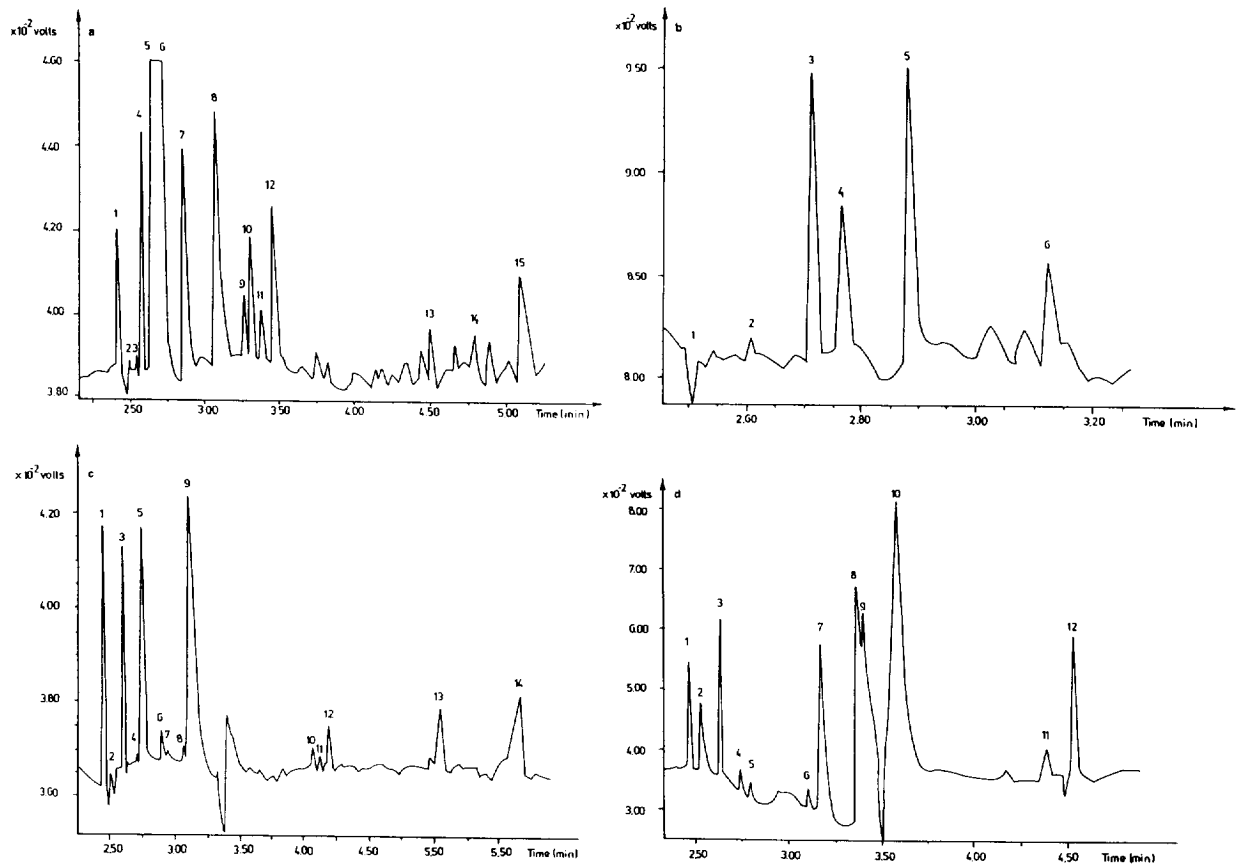

Fig. 2. Electropherograms of different samples. Conditions: $[CrO_4^{2-}] = 10$ mM; pH = 11.50; [CTAB] = 2.30 mM. The samples were injected hydrostatically for 10 s and the runs were performed at −20 kV (with a current of ±48 μA). (a) Spinach. Peaks: 1 = Cl^-; 2 = Br^-; 3 = unknown; 4 = SO_4^{2-}; 5 = oxalate; 6 = NO_3^-; 7 = CO_3^{2-}; 8 = HPO_4^{2-}; 9–11 = unknown; 12 = citrate; 13–15 = unknown. (b) Spinach diluted 50-fold. Peaks: 1 = Cl^-; 2 = SO_4^{2-}; 3 = oxalate; 4 = NO_3^-; 5 = CO_3^{2-}; 6 = HPO_4^{2-}. (c) Endive. Peaks: 1 = Cl^-; 2 = Br^-; 3 = SO_4^{2-}; 4 = oxalate; 5 = NO_3^-; 6 = CO_3^{2-}; 7 = unknown; 8 = formate; 9 = HPO_4^{2-}; 10 and 11 = unknown; 12 = propionate; 13 and 14 = unknown. (d) Tomato. Peaks: 1 = Cl^-; 2 = Br^-; 3 = SO_4^{2-}; 4 = oxalate; 5 = NO_3^-; 6 = formate; 7 = HPO_4^{2-}; 8, 9 = unknown; 10 = citrate; 11, 12 = unknown.

cant quadratic term in the linear model was detected. In the residual plots the lack of fit of the linear model for nitrite in the CE method was also observed. After reducing the range (up to 8 μg/ml), the lack of fit was found not to be significant. However, as nitrite occurs at very low levels in the samples, usually lower than the detection limit of the high concentration level CE method, one should apply the low concentration level CE method.

For nitrate both the high concentration level CE method and the spectrophotometric method gave fair linearity, only the ranges were different: The spectrophotometric method was found to be linear up to 2.5 μg/ml of nitrate, whereas the CE method was linear up to 10 μg/ml. Some statistics for the calibration lines are given in Table 3.

Low-concentration-level CE method

The low concentration level method was linear from 0.1 μg/ml up to 2.5 and 1.6 μg/ml for nitrite and nitrate, respectively. However, from the residual plots it was observed that the data were heteroscedastic. Therefore, weighted regression (weights: $1/s^2$) had to be applied which resulted in homogeneous residual patterns. These weighted calibration lines were used for quantitative purposes. Some statistics of these lines are also shown in Table 3.

Table 1
Results for the determination of anions in vegetables by CE

Sample	Concentration (mg/g)						
	Cl	Br	SO_4^{2-}	HPO_4^{2-}	Formate	Citrate	Oxalate
Spinach	0.239	0.001	0.457	1.815		1.088	9.015
Lettuce	0.446	0.001	0.242	6.75			0.047
Corn salad	2.241	0.031	0.363	3.583			
Celery	1.797	0.080	1.274	3.249			
Leek	0.431	0.110	0.103	3.185	0.099		
Watercress	0.364	0.097	0.942	3.408			
Endive	0.414	0.041	0.436	2.298			
Parsley	1.966	0.396	0.656	14.018	0.271		
Cauliflower	0.139	0.020	0.240	1.058			
Cucumber	0.230	0.130	0.246	2.329			0.009
White cabbage	0.187	0.001	1.025	1.454		1.777	
Red cabbage	0.142	0.089	0.688	0.642			0.002
Broccoli	0.108	0.105	0.827	1.109			0.009
Onion	0.217	0.638	0.157	1.284	0.179	6.865	
Tomato	0.238	0.761	0.379	0.637		25.083	0.054

3.2. Limits of detection (LOD) and quantification (LOQ)

The LOD was estimated as the concentration of nitrate or nitrite that would result in a signal three times higher than the background noise (blank). For the high-concentration-level CE method this resulted in a detection limit of 0.32 μg/ml for nitrate and 0.31 μg/ml for nitrite. The low-concentration-level CE method had an LOD

Table 2
Results for the determination of nitrate in vegetables

Sample	Points in Fig. 3	Concentration (mg/g)					
		Reference method			CE method		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean
Spinach	1 and 12	3.34	3.33	3.335	3.395	3.405	3.4
Lettuce	2 and 13	2.33	2.46	2.395	2.422	2.563	2.493
Corn salad	3 and 14	3.04	2.93	2.985	2.905	2.887	2.896
Celery	4 and 15	3.818	4.029	3.924	3.431	3.717	3.574
Leek	5 and 16	0.14	0.11	0.125	0.113	0.112	0.113
Watercress	6 and 17	3.64	3.73	3.685	3.962	3.758	3.86
Endive	7 and 18	1.54	1.56	1.55	1.328	1.313	1.321
Parsley	8 and 19	0.299	0.252	0.276	0.293	0.274	0.284
Cauliflower[a]		0.009	0.009	0.009	0.009	0.011	0.010
Cucumber[a]		0.028	0.02	0.024	0.026	0.021	0.0235
White cabbage	9 and 20	0.046	0.039	0.043	0.043	0.046	0.045
Red cabbage	10 and 21	0.026	0.03	0.028	0.025	0.028	0.0265
Broccoli	11 and 22	0.171	0.175	0.173	0.243	0.261	0.252
Onion[a]		0.009	0.012	0.010	0.015	0.012	0.014
Tomato[a]		0.058	0.058	0.058	0.055	0.054	0.055

[a] Concentration of nitrate in the sample is below the limit of quantification.

Table 3
Regression statistics for the calibration and the standard addition lines

Parameter[a]	Nitrate		Nitrite	
	External	Addition	External	Addition
Spectrophotometric method				
Slope	0.331	0.332	0.505	0.540
CI slope	0.300–0.362	0.274–0.390	0.479–0.530	0.481–0.599
Intercept	−0.0045	0.2610	−0.0018	−0.0088
CI intercept	−0.055 to 0.046	0.166 to 0.356	−0.031 to 0.027	−0.076 to 0.059
Standard error	0.0286	0.0490	0.0131	0.0303
r	0.99662	0.99217	0.99962	0.99823
High-concentration-level CE method				
Slope	139.84	145.43	197.50	201.23
CI slope	128.19–151.49	136.24–154.61	192.03–202.96	173.61–228.85
Intercept	39.87	414.17	14.78	72.76
CI intercept	−31.67 to 111.42	361.67 to 466.67	−77.61 to 107.16	−305.90 to 451.42
Standard error	55.25	53.73	104.43	178.69
r	0.99221	0.99449	0.99884	0.99069
Low-concentration-level CE method				
Slope	2.181	2.271	2.459	2.499
CI slope	2.122–2.240	2.257–2.284	2.427–2.495	2.446–2.553
Intercept	−0.05312	0.54540	−0.3176	0.1890
CI intercept	−0.074 to −0.033	0.531 to 0.560	−0.049 to −0.014	0.169 to 0.209
Standard error	1.358	0.966	0.076	0.899
r	0.99926	0.99997	0.99929	0.99943

[a] CI = confidence interval; *r* = correlation coefficient.

of 0.037 and 0.034 μg/ml for nitrate and nitrite, respectively. For the reference method a detection limit of 0.040 and 0.020 μg/ml was obtained for nitrate and nitrite, respectively.

The LOQ is considered as the concentration at which the signal obtained is sufficiently precise and accurate for quantitative purposes. For the high-concentration-level CE method an LOQ of 1.05 μg/ml [R.S.D. ($n = 4$) = 3.83%] and 1.04 μg/ml [R.S.D. ($n = 4$) = 8.52%] was obtained for nitrate and nitrite, respectively. For the low-concentration-level CE method the LOQ was around 0.1 μg/ml for both nitrite [R.S.D. ($n = 6$) = 4.21%] and nitrate [R.S.D. ($n = 6$) = 7.37%]. An electropherogram obtained at this level for nitrite and nitrate is shown in Fig. 1. The accuracy data are discussed later. The spectrophotometric method had an LOQ of 0.10 μg/ml for both nitrate [R.S.D. ($n = 4$) = 12.15%] and nitrite [R.S.D. ($n = 4$) = 2.94%], respectively.

The low-concentration-level CE method is more sensitive than the high-concentration-level method because injection is performed by electromigration, which results in a sample stacking effect. This leads to a better detection limit and peak shape [23,24]. Additionally, the runs were performed at lower voltage, which decreased the generation of noise due to temperature effects. As mentioned earlier, the injection of samples containing large amounts of chloride and sulphate by this method did not adversely affect the separation.

3.3. Repeatability and time-different intermediate precision measure

High-concentration-level CE method

The repeatability (within-day precision) of the high-concentration-level CE method and the spectrophotometric method was determined by analysing a sample of spinach, endive and pars-

ley six times. These three vegetables represent high, medium and low concentration levels of nitrate, respectively. The residual standard deviations values of the methods are given in Table 4. As can be seen, both methods show adequate repeatability considering the concentration of nitrate in the samples. No significant difference could be detected, at a significance level of 5%, between the variances of the two methods (two-sided F-tests). This was the case for all the concentration levels. The results for the repeatability obtained for the CE method are

Table 4
Results for the precision of the high-concentration-level CE and the spectrophotometric method

Run	Repeatability					
	Spinach		Endive		Parsley	
	Ref.[a]	CE	Ref.[a]	CE	Ref.[a]	CE
1	2.986	3.500	1.770	1.448	0.214	0.276
2	3.265	3.350	1.659	1.388	0.224	0.291
3	3.014	3.475	1.734	1.440	0.230	0.298
4	3.207	3.575	1.727	1.490	0.232	0.286
5	3.340	3.375	1.735	1.558	0.220	0.272
6	3.168	3.550	1.632	1.570	0.216	0.268
Mean	3.163	3.471	1.709	1.482	0.223	0.282
S.D.	0.139	0.091	0.052	0.071	0.007	0.012
R.S.D. (%)	4.409	2.633	3.067	4.808	3.224	4.114
F-value	2.329		1.849		2.609	
F-crit. 5%[b]	7.15		7.15		7.15	
Day	Time-different intermediate precision measure[c]					
	Spinach		Endive		Parsley	
	Ref.[a]	CE	Ref.[a]	CE	Ref.[a]	CE
1	3.387	3.500	1.770	1.448	0.252	0.276
	3.231	3.350	1.659	1.388	0.299	0.291
2	3.173	3.395	1.539	1.328	0.214	0.293
	3.345	3.405	1.564	1.388	0.224	0.274
3	3.625	3.200	1.528	1.493	0.274	0.324
	3.078	3.125	1.479	1.305	0.243	0.278
4	3.331	3.525	1.628	1.520	0.320	0.295
	3.343	3.625	1.802	1.538	0.337	0.270
5	2.986	3.325	1.665	1.293	0.311	0.327
	3.265	3.300	1.463	1.353	0.316	0.302
Mean	3.276	3.375	1.626	1.405	0.275	0.293
S.D.	0.177	0.150	0.110	0.090	0.044	0.020
R.S.D. (%)	5.403	4.459	6.778	6.378	16.044	6.889
F-value	1.384		1.512		4.779	
F-crit. 5%[b]	4.03		4.03		4.03	

Concentrations in mg/g.
[a] Reference spectrophotometric method.
[b] Two-sided.
[c] For duplicate determinations.

comparable to those for the reference method, and are more or less in agreement with the results that had been obtained in previous studies [25].

The time-different intermediate precision (the term recommended by ISO for the between-day precision [26]) was assessed by analysing the samples of spinach, endive and parsley in duplicate during five days by both methods. The results are also shown in Table 4. As can be observed, the R.S.D. values of the CE method are generally lower, indicating a better time-different intermediate precision. When the variances were compared with F-tests, a significant difference was detected between the variances of the methods at low concentration level (parsley sample). A careful observation of the data in Table 4 reveals that the AOAC method results in higher nitrate levels for parsley starting from day 4. Therefore, the significant difference of the variances for the determination of nitrate in parsley is probably due to a measurement error.

Low-concentration-level CE method

The repeatability of the low-concentration-level CE method was investigated by determining three concentration levels of nitrate and nitrite six times. In Table 5 the results obtained with the weighted calibration procedure are given. As can be seen, this resulted in acceptable R.S.D. values. Considering the time-different intermediate precision, the weighted calibration procedure also resulted in an acceptable precision at the three concentration levels for both nitrite and nitrate. The results are given in Table 5. The R.S.D. values at the three concentration levels were also acceptable for quantitative purposes.

3.4. Accuracy

By comparing the slopes of an external and a standard addition calibration line, one can detect a possible influence of matrices. The slopes of both lines were compared by a t-test. There was no significant difference detected at the α-level of 5% between the slopes for either the reference method (nitrate, $0.05 < p < 0.10$; nitrite, $0.5 < p < 0.9$) or the CE method (nitrate, $0.05 < p < 0.10$; nitrite, $0.3 < p < 0.5$). This indicates that there were no relative systematic errors that influence the accuracy of the determination. In the comparison of the slopes of the weighted calibration lines from the low concentration level CE method by means of a t-test (α-level of 5%), no significant difference was detected (nitrite, $0.05 < p < 0.10$, nitrate, $0.5 < p < 0.9$). Some statistics for the calibration lines are given in Table 3. As no nitrite was detected by either the CE or the spectrophotometric method in any of the samples in this study, three concentration levels of nitrite were added to a (blank) sample. These samples were analysed six times and the results (weighted procedure) are presented in Table 6. As can be observed, the recovery of the spiked samples was within the range $100 \pm 10\%$. Therefore, one can conclude that the low-concentration-level CE method had an acceptable accuracy.

To investigate the accuracy further, the results obtained by the high-concentration-level CE method were compared with those of the reference method. When doing so it is sometimes not sufficient to look only at the numerical results, and a visual evaluation is recommended [27–29]. In addition to regression procedures, other visualization procedures have also been described [27,28] for method comparison. The percentage differences between the results of two methods are plotted against the mean of the two obtained values (Bland and Altman plot [29]). This was applied to evaluate the agreement between the results obtained for nitrate by the two techniques (Table 2). Good agreement was observed between the results of the two methods (orthogonal regression). The absence of a systematic proportional error was tested by a t-test ($\alpha = 5\%$), which confirmed that the slope of the orthogonal regression line was not significantly different from 1. Fig. 3 shows the Bland and Altman plot. The mean of the percentage differences in the experimental results between the two methods is slightly above the expected mean zero. However, this difference was not found to be signifi-

Table 5
Results for the precision of the low-concentration-level CE method with weighted regression

Repeatability at three concentration levels

Run	Nitrite			Nitrate		
	0.1 μg/ml	0.5 μg/ml	1.6 μg/ml	0.1 μg/ml	0.5 μg/ml	1.6 μg/ml
1	0.1045	0.4807	1.5606	0.0934	0.4541	1.6041
2	0.1181	0.4757	1.5805	0.0997	0.4976	1.5928
3	0.1125	0.4821	1.6010	0.1009	0.5046	1.5844
4	0.1128	0.4731	1.6318	0.1179	0.4750	1.6208
5	0.0993	0.4887	1.6670	0.1016	0.4932	1.6245
6	0.1070	0.5006	1.6262	0.1008	0.4868	1.6946
Mean	0.1090	0.4835	1.6112	0.1024	0.4852	1.6202
S.D.	0.0068	0.0100	0.0384	0.0082	0.0183	0.0396
R.S.D. (%)	6.20	2.07	2.38	7.98	3.76	2.45

Time-different intermediate precision

Day	Nitrite			Nitrate		
	0.1 μg/ml	0.5 μg/ml	1.6 μg/ml	0.1 μg/ml	0.5 μg/ml	1.6 μg/ml
1	0.1045	0.4807	1.5606	0.0934	0.4541	1.6041
	0.1181	0.4757	1.5805	0.0997	0.4976	1.5928
2	0.0755	0.4662	1.5568	0.1039	0.4597	1.5773
	0.0867	0.5018	1.5430	0.1109	0.4781	1.4916
3	0.0785	0.4679	1.5549	0.1038	0.4598	1.3620
	0.0897	0.5033	1.5411	0.1108	0.4782	1.4921
4	0.1183	0.4917	1.8212	0.1001	0.5137	1.7092
	0.0988	0.5301	1.5867	0.0999	0.4613	1.4793
5	0.1003	0.4933	1.5547	0.0713	0.5015	1.6657
	0.0998	0.4788	1.6435	0.1147	0.4934	1.5544
6	0.1026	0.5109	1.6664	0.1272	0.4909	1.7132
	0.1041	0.5452	1.7379	0.1127	0.5238	1.6357
Mean	0.0981	0.4955	1.6123	0.1040	0.4843	1.5731
S.D.	0.0135	0.0244	0.0891	0.0137	0.0229	0.1040
R.S.D. (%)	13.76	4.92	5.52	13.13	4.72	6.61

cant ($\alpha = 5\%$) by a t-test. The spread of the data points in Fig. 3 does not show a specific trend in the data, indicating also the absence of a systematic proportional error. Compared with the other samples, points 11 and 22, corresponding with the vegetable broccoli (Table 2), shows deviant behaviour when the evaluation is performed by the percentage difference. Nevertheless, globally one can conclude that the results for the determination of nitrate by the two methods are comparable.

4. Conclusions

The proposed CE methods are linear in the described ranges and have an acceptable precision and accuracy for the determination of ni-

Table 6
Results for the recovery of nitrite added to a blank vegetable sample

0.1 μg/ml added		0.5 μg/ml added		1.0 μg/ml added	
Found (μg/ml)	Recovery (%)	Found (μg/ml)	Recovery (%)	Found (μg/ml)	Recovery (%)
0.0867	86.70	0.4854	97.07	0.9512	95.12
0.0934	93.39	0.4879	97.57	1.0759	107.59
0.0946	94.56	0.5066	101.32	0.9601	96.01
0.0967	96.73	0.4863	97.25	1.0091	100.91
0.0926	92.64	0.4898	97.96	1.0417	104.17
0.0970	96.98	0.4879	97.59	0.9152	91.52
Mean	93.5016		98.1282		99.2210
S.D.	3.7587		1.5953		6.0642
R.S.D. (%)	4.02		1.63		6.11

trates and nitrites in vegetables. The LOD of the high-concentration-level CE method is too high for the determination of nitrite, but for the determination of nitrate in the vegetables this is not a problem. The concentration of nitrate in the samples is generally sufficiently high for accurate determinations. For nitrite, however, a lower LOD is achieved with the low-concentration-level CE method. An LOD of less than 50 μg/l is sufficiently low for determining dangerous amounts of nitrite considering the acceptable daily intake of nitrite. Therefore, the proposed CE methods appear to be acceptable for the determination of nitrates and nitrites in vegetables. The major advantage of the CE methods is that they are extremely fast, as a run requires only ca. 5 min, whereas in the spectrophotometric method up to 1 h can be required. This compensates for the disadvantage of having two methods in the CE technique for the determination of nitrate and nitrite. Another advantage is that one can determine several anions simultaneously.

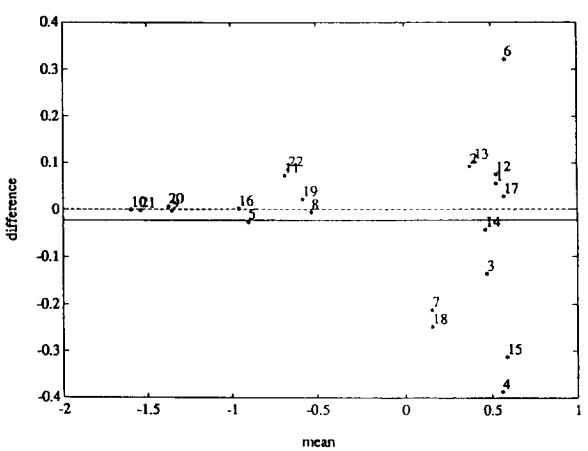

Fig. 3. Visual evaluation by the Bland and Altman plot for comparison of the results of nitrate determination obtained by the high-concentration-level CE method and the reference spectrophotometric method. The dashed and continuous lines represent the expected and the found means of the percentage difference in the experimental results, respectively. In order to observe the spread at low concentrations (around zero), the values of the mean concentration levels have been transformed to a logarithmic scale.

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