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Development and validation of a sub-minute capillary zone electrophoresis method for determination of nitrate and nitrite in baby foods

Fabiana Della Betta, Luciano Vitali, Roseane Fett, Ana Carolina Oliveira Costa *

Department of Food Science and Technology, Federal University of Santa Catarina, Florianopolis, SC, Brazil

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ABSTRACT

This paper proposes an innovative sub-minute capillary zone electrophoresis method and a simple sample preparation procedure for simultaneous nitrate and nitrite determination. The novelty of the method is the simplicity of execution and the capacity to separate the analytes in less than 0.5 min. The BGE is composed of 10 mmol L^{-1} perchloric acid and 40 mmol L^{-1} β-alanine at pH 3.96. Thiocyanate was used as an internal standard. The method was validated following the Eurachem guidelines and applied to the analysis of 14 baby food samples. Of these samples, one had nitrate levels above that permitted by Brazilian legislation (250 mg kg^{-1}) and for all samples the nitrite concentrations were under the limit of quantification. The good analytical performance verified for this method indicates that it is suitable for implementation in food laboratories for the routine determination of nitrate and nitrite as an alternative to the official method provided by the AOAC.

1. Introduction

Vegetables are a good source of nutrients, but they are also a natural source of nitrate and nitrite [1], which are metabolites resulting from nitrogen uptake by plants. Nitrate accumulation is influenced by environmental factors, such as the amount and form of nitrogen application, light exposure, soil, harvest and temperature [2,3]. In addition to vegetables, nitrate is present in fertilizers, spices, sea salt and drinking water. Nitrite is generated as a consequence of nitrate reduction and thus the natural occurrence of the former is lower than that of the latter [4].

Nitrate is not toxic and can be excreted in the urine without adverse effects, but under conditions of low pH or due to the action of nitrate-reducing bacteria it can be reduced to nitrite, which can lead to methemoglobinemia (also called blue baby syndrome). This disease is particularly dangerous in infants less than 6 months old, when the immunological system is not yet entirely developed [1,5,6]. In older children and adults nitrate reduction can occur in the oral cavity and stomach, in contrast to infants where it occurs in the intestine, since the gastric pH is often higher compared to adults and older children and allows the growth of bacteria able to reduce nitrate to nitrite [7]. Some cases of methemoglobinemia associated with vegetable consumption by infants are reported in the literature [8,9].

According to Greer and Shannon [7] the consumption of vegetables with high nitrate content, such as green beans, carrots, squash, spinach and beet, by infants less than 3 months should be avoided. In Brazil, the national regulatory agency, the Brazilian National Health Surveillance Agency (Portuguese acronym ANVISA), establishes that nitrate content in ready-to-eat infant formulations must not exceed 250 mg kg^{-1} [10]. This legislation also indicates that products which contain spinach and beet must include the following warning on the label: “Contains spinach/beet and is not suitable for infants aged less than 3 months”. Although it is toxic, maximum limits for nitrite in these foods are not established by Brazilian legislation.

The method for nitrate determination in baby foods recommended by the Official Methods of Analysis of AOAC International is based on the reduction of nitrate to nitrite by cadmium, followed by the spectrometric determination of nitrate as nitrite [11]. This method is time consuming, especially the sample preparation step. Also, it can be used to determine only one analyte at a time and requires high amounts of solvents, some of them corrosive or toxic, which results in high amounts of residues, making it unsuitable for the routine analysis of large numbers of samples. Due to these disadvantages, interest in analytical techniques which could replace the classical methodology has been increasing. These techniques include ion chromatography

* Corresponding author. Tel. +55 48 3721 2975; fax: +55 48 3721 9943.
E-mail address: ana.costa@ufsc.br (A.C.O. Costa).

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[6,12,13], voltammetry [14], potentiometry [15], flow-injection analysis with spectrophotometric detection [16,17] and capillary electrophoresis [18–21].

Capillary electrophoresis (CE) is a versatile technique which can be used to analyze cationic, anionic and neutral compounds [22]. Furthermore, CE has been proposed as a powerful technique for anion analysis, since it provides very fast separations and high-resolution, and it requires small injection volumes (nL) and low amounts of reagents and samples, resulting in lower residue generation [23].

Although breastfeeding is recommended as the best feeding choice for infants under 6 months old, mothers are increasingly feeding their children with commercial baby food [24]. Since the first year of life is crucial for a child's development, the composition and quality of commercial baby food must be monitored to reduce the risk of exposure to nitrate and nitrite. Considering the disadvantages associated with the official method cited above, an alternative method needs to be developed to improve the analysis time, reduce the consumption of chemicals and simplify the sample preparation. Thus, the aim of this study was to develop and validate a sub-minute method of capillary zone electrophoresis (CZE) to determine the nitrate and nitrite levels in baby food using a factorial design to optimize the sample preparation method. The development of the separation method by CZE was performed using the PeakMaster software and the validation of the new method was performed according to the Eurachem guidelines [25].

2. Material and methods

2.1. Reagents and solutions

All solutions were prepared using analytical grade reagents and deionized water (Milli-Q, Millipore, Bedford, MA, USA). Sodium nitrate and nitrite as well as potassium thyocianate (> 99%) were purchased from Merck (Darmstadt, Germany). Perchloric acid reagent (70%) and β-alanine (> 99%) were obtained from Sigma-Aldrich (St. Louis, CO, USA). Standard solutions (1000 mg L^{-1}) of nitrate, nitrite and thiocyanate and stock solutions (100 mmol L^{-1}) of perchloric acid and β-alanine were prepared daily and stored at 4 °C until analysis. Other reagents used during the experiments were sodium hydroxide (Vetec, Rio de Janeiro, Brazil) and acetonitrile (Merck, Rio de Janeiro, Brazil).

2.2. Capillary electrophoresis system

The CZE assays were conducted in a capillary electrophoresis system (Agilent Techologies, model 7100, Palo Alto, CA, USA) equipped with a diode array detector, temperature-control device (maintained at 25 °C) and data acquisition and treatment software supplied by the manufacturer (HP ChemStation®). Before the first run the capillary was sequentially rinsed with 1.0 mol L^{-1} NaOH (30 min) and water (30 min). At the beginning of each day the capillary was conditioned by flushing with 1 mol L^{-1} NaOH (15 min) followed by a 15 min flush with deionized water and an electrolyte solution (15 min). Between runs the capillary was flushed for 0.5 min with BGE. At the end of each working day, the capillary was rinsed with 1 mol L^{-1} NaOH (10 min) and water (10 min) and then dried in air (2 min).

Separations were conducted in an uncoated fused-silica capillary of 32 cm (8.5 cm effective length × 50 μm I.D. × 375 μm O.D.). Direct UV detection set at 210 nm was used and the temperature was maintained at 25 °C. The standards and samples were introduced into the capillary using the short-end injection procedure with a hydrodynamic pressure of 50 mbar for 3 s. The separation voltage applied was −30 kV. The optimized background electrolyte (BGE) used in the proposed method was comprised of 10 mmol L^{-1} perchloric acid and 40 mmol L^{-1} β-alanine at pH 3.96. Thiocyanate, used as the internal standard (IS), was diluted to obtain a final concentration of 25 mg L^{-1}.

2.3. Samples

Fourteen different baby food samples of two brands (A – organic purees, $n=2$; B – other purees, $n=12$) were purchased from a local store and kept under refrigeration until the analysis. The main ingredients in the formulation were potato, pumpkin, carrot, beet, spinach, banana, meat and poultry.

2.4. Sample preparation

The sample preparation was carried out using a modified version of the method employed by McMullen et al. [6] using a 2^3 full factorial design with a central point. An overview of the experimental design is shown in Table 1 and responses were calculated using the following equation.

$$R = \frac{(A_{\text{nitrite}} + A_{\text{nitrate}})}{t_{\text{end of extraction}}} \quad (1)$$

where A_{nitrite} and A_{nitrate} are the areas of nitrite and nitrate, respectively, and $t_{\text{end of extraction}}$ is the total extraction time.

The optimized conditions were a 10 mL aliquot of hot deionized water (60 °C) added to a tube containing approximately 10 g of baby food. The tube was sealed and stirred in a vortex for 1 min. After cooling, the sample was diluted with deionized water in a 25 mL volumetric flask and clarified with 1 mL of acetonitrile (ACN). After mixing, the samples were subjected to centrifugation for 10 min at 4000 rpm. A 100 μL aliquot of the supernatant was diluted with 100 μL of internal standard (to give a final concentration of 25 mg L^{-1}) and then automatically injected into the CE system. Samples were prepared in triplicate.

2.5. Validation procedure and statistical analysis

The method was validated according to the Eurachem [25] guidelines employing assays with standard solutions and spiked samples, due to the unavailability of a blank sample. System suitability, linearity, matrix effects, selectivity, precision, accuracy, detection and quantification limits, and robustness were studied. The fitness-for-purpose of this method was assessed based on the results obtained for pre-established performance characteristics [26–28].

2.5.1. System suitability

In this study, the system suitability was tested considering the relative standard deviation (RSD) of the means obtained from 10 consecutive injections of a standard mixture (using the IS). Repeatability was evaluated using the corrected peak area calculated as $\text{area}_{(\text{analyte})}/\text{area}_{(\text{IS})}$, the corrected migration time $t_{\text{m(analyte)}}/t_{\text{m(IS)}}$, and the resolution ($R_s$).

Table 1
Experimental design.

Variables	Factor	Level		
Full 2^3 factorial design		−1	0	+1
1	Weight, g	5	7.5	10
2	Agitation, min	1	5	10
3	[ACN], mL	1	2	3

2.5.2. Linearity and matrix effect

Linearity was established from the standard solution and matrix calibration curves, employing six concentration levels prepared in the range of 5–55 mg L^{-1}, with increments of 10 mg L^{-1}, and three replicate injections were performed at each level, randomly, as suggested by Thompson et al. [28]. The matrix calibration curve was obtained using the standard addition method, due to the matrix complexity and the unavailability of a blank sample [29].

After an exploratory fit by linear regression, residual plots were examined to verify the existence of patterns and the presence of points which lay out of the range (outliers). After the visual identification, other potential outliers were identified by applying the Grubbs test [30] until no outliers were detected. The residues were also evaluated for violations of the assumptions of normality employing the Shapiro–Wilk test [31], homoscedasticity by applying the Cochran test [32], independence through the Durbin–Watson test [33] and the fitting of an appropriate model (*F*-test), at the 99% confidence level [34]. The matrix effect was assessed comparing the slopes obtained for the standard solution and matrix calibration curves using the *t*-test ($\alpha=0.01$) [28].

2.5.3. Selectivity

In order to check the selectivity of the proposed method, the PeakMaster software was used. With this important analytical tool it was possible to simulate the presence of compounds in the baby foods which could potentially interfere in the analysis. For the optimized BGE (10 mmol L^{-1} perchloric acid and 40 mmol L^{-1} β-alanine at pH 3.96) the following parameters were inputted into the software: the compounds of interest (nitrate and nitrite); voltage (30 kV); total capillary length ($L_{tot}=320$ cm; $L_{det}=8.5$ cm); injection polarity (negative); no marker flow; selection of the ionic strength correction; and signal input (direct). Other anions that can be found in baby foods were also added to the simulation (bromide and sulfite). The selectivity was established from the near baseline separation of the compounds in the simulated electropherogram because of the unavailability of these anions.

2.5.4. Precision and accuracy

To assess the repeatability the intra-day precision was determined by three consecutive injections of nitrate, nitrite at six concentration levels and the I.S. at 25 mg L^{-1}. The intermediate precision (inter-day precision) was assessed by analyzing three preparations of standard solutions, over three days with three consecutive injections. The apparent recovery was investigated through the mean obtained for the three independent replicates of a sample spiked at three levels in the range used for the calibration curves.

2.5.5. Limits of detection and quantification

The limit of quantification (LOQ) was considered as the concentration below which the method could not operate with an acceptable precision (signal/noise ≥ 10). The limit of detection (LOD) was the lowest concentration of nitrate or nitrite that was detectable in all replicates, but was not quantifiable, that is, distinguishable from zero (signal/noise ≥ 3). These limits were established based on the mean obtained for three independent replicates.

2.5.6. Robustness

The robustness of the CZE method for the nitrate and nitrite quantification was assessed using the method proposed by Youden and Steiner [35]. Since the nitrite concentrations were under the LOQ value in all samples, the test was performed using samples spiked with a standard solution. Seven analytical parameters were selected and small variations were induced in the nominal values of the method. Eight runs were then performed to determine the influence of each parameter on the final result. The seven analytical parameters employed at the nominal values under conditions with small variations included voltage separation (30 and 29 kV); pH of the BGE (3.96 and 3.87); cartridge temperature (25 and 26 °C); injection pressure (50 and 48 mbar); wavelength (210 and 212 nm); flush time between runs (30 and 20 s); and injection time (3 and 4 s).

For each combination, three injections of three samples (pumpkin-based puree, vegetable-based puree and chicken, vegetable and pasta-based puree) were carried out. The results obtained for each combination were nitrate and nitrite content (mg kg^{-1}); nitrate and nitrite ratio (analyte area/internal standard area); migration time (t_m); and peak symmetry and resolution (R_s). To determine the influence of variations in each parameter on the final result, the mean of the four values corresponding to the nominal conditions was compared to the mean of the four values corresponding to the altered conditions. Youden's test can be applied, to establish the parameters that have the greatest influence on the final result of the analysis and gain more rigorous control of the variations in these parameters that may occur during routine analysis [27,35].

3. Results and discussion

3.1. Background electrolyte optimization for CZE method

Nitrate and nitrite have good absorption in the ultraviolet region (UV) at 190–225 nm and thus the simulations were performed using the direct detection method. In the selection of an appropriate background electrolyte for the nitrate and nitrite determination, several characteristics were considered, including good buffering capacity and adequate co- and counter-ions, to minimize the peak asymmetry and anomalous dispersion effects. According to Merusi and co-workers [29] nitrate and nitrite have very close electrophoretic mobility (μ_{eff}) values, but at low pH the nitrite mobility can be reduced, because at this pH nitrite is not fully dissociated and in this case nitrites move more slowly than nitrates. The co-ion selected was perchlorate ($\mu_{eff}=-69.8\times10^{-9}$ m^2 V^{-1} s^{-1}), since its mobility is similar to that of the analytes ($\mu_{eff}=-74.10\times10^{-9}$ m^2 V^{-1} s^{-1} and -74.60×10^{-9} m^2 V^{-1} s^{-1}, for nitrate and nitrite, respectively) which minimizes the peak asymmetry in the electromigration dispersion (EMD). Also, the co-ion does not adsorb in the ultraviolet region. The internal standard (I.S.) chosen was thiocyanate, which is an anion that absorbs in the UV region and its mobility is close to that of the analytes ($\mu_{eff}=-68.5\times10^{-9}$ m^2 V^{-1} s^{-1}). The separation pH is around 4, so the counter-ion selected was β-alanine, which has a pKa of 3.43 and therefore provides buffering capacity for the BGE.

The ideal separation conditions were defined through simulations carried out in the PeakMaster 5.1 software, fixing the β-alanine concentration and varying the concentration of perchlorate, as shown in Fig. 1. The best BGE composition was 5 mmol L^{-1} perchlorate and 20 mmol L^{-1} β-alanine, which gives a low EMD for all analytes and the pH was adequate for the separation. One disadvantage is that the buffer capacity is very close to the limit (<10) with this composition, but this problem can be solved by doubling the amounts of the BGE compounds employed to 10 mmol L^{-1} perchloric acid and 40 mmol L^{-1} β-alanine.

The PeakMaster simulations were found to be a very helpful tool in developing a fast method for nitrate and nitrite analysis, reducing the number of experiments and amount of reagents required. The method has a good peak symmetry and resolution, and was able to separate nitrate, nitrite and thiocyanate in less

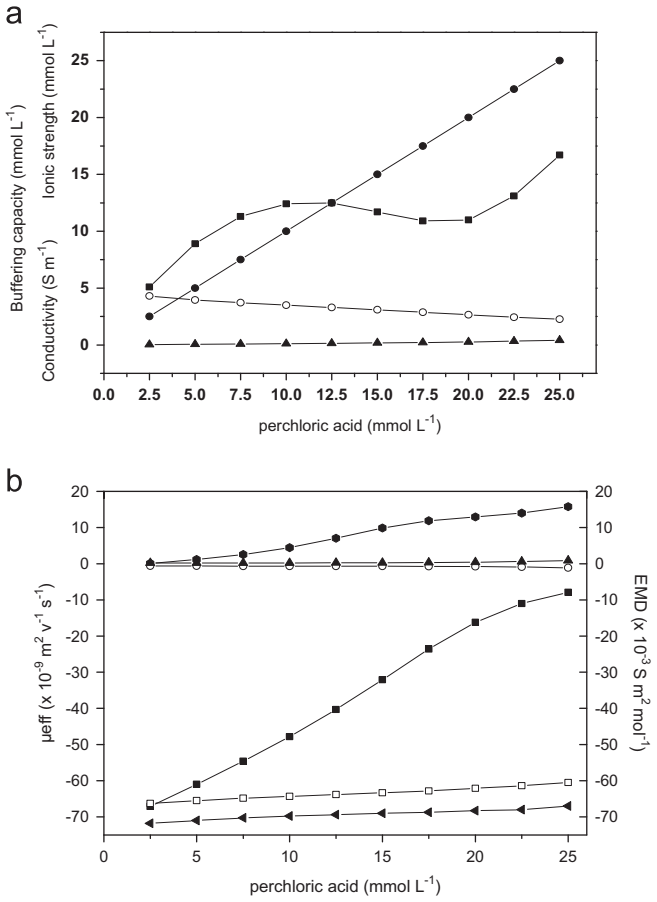

Fig. 1. BGE optimization in PeakMaster 5.1 with fixed β-alanine concentration (20 mmol L^{-1}) and varying the perchloric acid concentration (2.5–25 mmol L^{-1}). (a) Electrolyte parameters. Legends: (○) pH; (●) ionic strength; (▲) conductivity; (■) buffering capacity. (b) Effective mobility and EMD versus perchloate. Legends: (○) EMD nitrate; (●) EMDnitrite; (▲) EMD thiocyanate; (■) μ_{eff} nitrite; (◂) μ_{eff} nitrate; and (□) μ_{eff} thiocyanate.

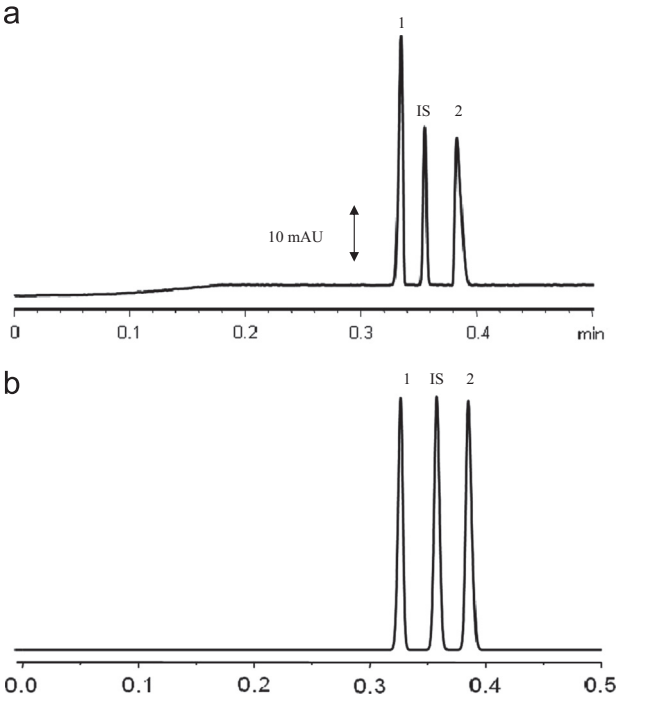

Fig. 2. Electropherogram of the analytes in standard solutions (a) and the simulation analysis carried out in PeakMaster 5.1 (b).

than 0.5 min. A high degree of similarity was observed between the simulated and experimental electropherograms for the standards, verifying showing that the separation conditions were chosen correctly. Fig. 2 shows an electropherogram of the analytes in standard solutions (a) and the simulated analysis (b).

3.2. Factorial design used for the extraction of nitrate and nitrite in baby foods

The optimized extraction method was based on the nitrate and nitrite solubility in hot water, followed by protein precipitation with ACN, centrifugation, dilution and injection into the capillary electrophoresis system. The application of a low volume of ACN replaced the use of borax and zinc sulfate solution, wich are recommended in the AOAC official method. Regardless of the sample fat content, an additional step for defatting was not required, since fat did not interfere in the analysis.

In order to find the optimum extraction procedure with the shortest sample preparation time, a 2^3 full factorial experimental design with a central point was applied. The variables affecting the nitrate and nitrite extraction were investigated based on previous experiments performed by McMullen et al. [6], who proposed a method for vegetable and fruit-based baby food analysis using ion chromatography. The variables investigated were sample weight (g); agitation time (min) and ACN volume for protein precipitation (mL). All variables were evaluated at two levels: low (−1) and high (+1) and with a central point (0) (see factors in Table 1). Other variables, such as the sample/water ratio used for the extraction, final dilution and centrifugation time were maintained fixed. The experiments were performed randomly and the response obtained from Eq. (1) for the levels studied (data not shown). Eq. (1) considers the extraction yield for nitrate and nitrite with the minimal time of extraction to enhance the sample analysis throughput.

The best condition for the sample preparation was identified as 10 g of sample, 1 mL of ACN and 1 min of agitation. The use of a factorial design improved the method optimization, reducing the number of experiments required in an appropriate procedure. The optimized extraction method was found to be effective for the determination of nitrate and nitrite in baby foods. It is a simple and fast procedure, providing high analysis throughput, which is important for routine laboratory analysis and requires a lower amount of reagents than the official AOAC method.

3.3. Method validation

3.3.1. System suitability

Before obtaining the figures of merit in the validation experiments, the system used for the analysis must be shown to provide data of acceptable quality. Therefore, suitability tests need to be performed. The system suitability test is carried out to verify the adequate working of an instrument used for analytical measurements, and can demonstrate that the system is able to produce accurate and reproducible data [36,37]. This parameter was evaluated using the corrected peak area calculated as $area_{(analyte)}/area_{(IS)}$, corrected migration time $t_{m(analyte)}/t_{m(IS)}$ for nitrate and nitrite, and the resolution for nitrate, thiocyanate and nitrite. Table 2 shows the data obtained.

As shown in Table 2, the % RSD values for the instrumental precision were 0.21 and 0.89 for the corrected migration time and 2.48 and 3.42 for corrected peak area for nitrate and nitrite, respectively. These values demonstrate that the instrumental system is suitable for use in the validation procedure.

Table 2
Analytical performance of the proposed CZE-UV method.

Parameter	*n*	Nitrate	Nitrite
Resolution (R_s, nitrate:thiocianate)[a]	10	1.9	
Resolution (R_s, thiocianate:nitrite)[a]	10		2.0
Instrumental precision – corrected peak area (RSD, %)	6	2.48	3.42
Instrumental precision – corrected migration time (RSD, %)	6	0.21	0.89
Intra-day precision – corrected peak area (RSD, %)	6	3.85	4.30
Intra-day precision – corrected migration time (RSD, %)	6	1.32	0.95
Inter-day precision – corrected peak area (RSD, %)	18	6.95	5.45
Inter-day precision – corrected migration time (RSD, %)	18	3.36	3.81
Linearity – linear range (mg L^{-1})		5–55	
Linearity – slope		0.0923	0.0860
Linearity – intercept		0.0953	0.0735
Linearity – coefficient of regression, R^2		0.997	0.997
F[b]	18	193	2927
LOQ (mg L^{-1})		0.30	0.49
LOD (mg L^{-1})		0.09	0.15
Matrix-matched[c]			
t	6	9.67	1.16
p		0.001	0.31
Solvent[c]			
t	6	3.29	1.45
p		0.031	0.22

n: number of observations, t: t-statistic, p: significance.

[a] $R_s = 2(t_n - t_{n-1})/(w_n + w_{n-1})$, where t is the migration time (min) and w is the baseline peak width.

[b] F critical: 1.16.

[c] Residual homoscedasticity evaluation by test t for solvent and matrix-matched calibration curves (t critical: 4.60).

3.3.2. Matrix effect and linearity

The assumptions of linearity were confirmed for both curves: standard solutions and standard addition solutions. The matrix effect was assessed by comparing the slopes for the standard and standard addition solutions using the F and t-tests for three of the 14 samples, and in one case a matrix effect was observed (Table 2). Thus, the standard addition curve must be used for the quantification. For the samples that did not show a matrix effect it was possible to conclude that the two curves originated from the same signal, and the standard curve can therefore be used in the quantification. All of the regression assumptions were tested and confirmed for 5–55 mg L^{-1}, the curves demonstrating linearity in this range.

The profiles of the residual plots showed no obvious trends that demonstrated departure from linearity or heteroscedasticity. The confidence intervals for the residues suggested the presence of possible outliers, which were not confirmed by the Grubbs test. The residues were considered normally distributed in the Shapiro–Wilk test, according to the table of critical values for $n=18$ and $W=0.858$, and the W values for the samples were higher than the critical W values (vegetable-based puree, 0.871 and 0.946; meat and vegetable-based puree, 0.926 and 0.934; and banana and milk-based puree, 0.909 and 0.942 for nitrate and nitrite, respectively) confirming the normality assumption. The homoscedasticity was confirmed in the Cochran test, since the statistics were not significant ($p > 0.01$), and the residue independence was confirmed applying the Durbin–Watson test ($p > 0.01$). The results obtained for the tests performed to verify the normality, homoscedasticity and independence of residuals indicated that the use of the ordinary least squares (OLS) method was appropriate in the case of nitrate and nitrite, as well as for the standard and the standard addition solutions.

3.3.3. Selectivity

There are many ionizable species in baby food samples, such as bromide and sulfite, which could potentially interfere with the determination of nitrate and nitrite by CZE, since these compounds are in ionic form in the BGE and show absorptivity in the UV range (at 210 nm). Therefore, it is important to maximize the method selectivity prior to the analysis and this can be carried out by exploiting the versatility of CZE. In the case of nitrate and nitrite determination, only anionic species, including bromide and sulfite, can be detected, but these do not interfere in the nitrate and nitrite determination. This was confirmed by simulations carried out in the PeakMaster (data not shown).

3.3.4. Precision and accuracy

The intra-day precision was determined through consecutive injections carried out randomly for each level of the calibration curves for nitrate and nitrite. Each point (6 levels) was prepared with three replicates of independent standard solutions. The repeatability (intra-day precision) for the migration time corrected (%RSD) were better than 1.32 for nitrate and 0.95 for nitrite. The repeatability for the corrected peak area was less than 3.85% for nitrate and 4.30% for nitrite. The %RSD results for the intermediate precision (inter-day precision) for three consecutive days were also obtained with three replicates of independent standard solutions. For the corrected migration time the values were better than 3.36% for nitrate and 3.81% for nitrite and for the corrected peak area the results were 6.95% and 5.45% for nitrate and nitrite, respectively.

The method accuracy was established through recovery tests at six concentration levels covering the linear range of the method. The means for the apparent recovery were 70–101, 75–104 and 80–135% for nitrate and 75–101, 91–109 and 60–89% for nitrate, for the meat and vegetable-based puree, vegetable-based puree and banana and milk-based puree samples, respectively. The AOAC recommends a range of 70–120% for ppb and low ppm levels [38], therefore the samples presented good recovery results, except for the banana and milk-based puree which provided lower values, possibly due to the complexity and matrix effect associated with this sample.

3.3.5. Limits of detection and quantification

The LODs were 0.09 mg L^{-1} and 0.15 mg L^{-1} and the LOQs were 0.30 mg L^{-1} and 0.49 mg L^{-1} for nitrate and nitrite,

respectively. The detection limits are close to those of other methods reported in the literature, which range from 0.3 to 0.5 mg L^{-1} and 0.8 to 1.6 mg L^{-1} for nitrate and nitrite, respectively [28], and are even lower in some cases.

3.3.6. Robustness

Youden's test is a reliable method to evaluate the robustness of analytical methods by means of an experiment design, which involves seven analytical parameters combined in eight tests. Using the criteria of Youden's test, the electrophoretic method was shown to be highly robust regarding the nitrate contents when variations in seven analytical parameters were introduced. The greatest variation in nitrate content was 1.6%, when the wavelength and temperature were altered, and for nitrite the greatest variation was 1.1%, when the pressure was altered, values considered to be very low and not significant in routine analysis.

3.4. Determination of nitrate and nitrite in baby food

After being optimized and validated the proposed method was applied to determine the nitrate and nitrite contents of commercially available baby food samples ($n=14$). The quantification results are given in Table 3 and the electropherogram of the two samples can be observed in Fig. 3.

The banana-based puree had the lowest nitrate level (8.84 mg kg^{-1}), which was an expected result since fruits have lower levels of nitrate than vegetables, because nitrate is exclusively transported by the xylem and also when the metabolic requirement is exceeded the plants tend to accumulate nitrate mainly in their roots and above-ground parts [2].

For the 14 baby food samples analyzed, the highest nitrate levels were presented by the two organic purees, in contrast with reports that the nitrate contents of organic foods are lower than those of conventional foods [39]. According to Pardo-Marín and co-workers [3] the nitrate concentration in vegetables is dependent on environmental factors such as the light conditions, soil type, temperature, humidity, harvesting time, storage time and nitrogen source. The higher nitrate levels in the organic samples may be explained by the ingredients used in the formulation (for both samples the main ingredients were vegetables with a high nitrate content), poor quality control (brand A) or the fact that these two samples were produced in winter and the lower light intensity could have led to an increase in nitrate.

Other samples formulated with ingredients like beet, spinach and carrots also contained high amounts of nitrate, since these vegetables have naturally high nitrate concentrations. With the exception of the organic pumpkin-based puree (247.70 ± 6.52), all samples analyzed presented nitrate concentrations below the limit established by ANVISA (250 mg kg^{-1}) and in all samples the nitrite concentrations were under the limit of quantification. The limit established for nitrate in Brazil is higher than that of the European Union (EU), which establishes 200 mg L^{-1} as the maximum [40], and according to this limit three samples would be considered inadequate for consumption.

McMullen et al. [6] determined nitrate and nitrite levels in baby food using ion chromatography (IC). Nitrite was not detected in any of the samples tested and the nitrate levels ranged from 96 to 1084 mg L^{-1}, values higher than those obtained in this study.

Levels of nitrate in vegetables and vegetable-based baby foods have also been determined in Valencia, Spain, and 1150 samples (1083 vegetables and 67 baby foods) were analyzed by high performance liquid chromatography (HPLC) in the period of 2000–2008 [3].The median levels of nitrate in the vegetable-based baby food was 60.4 ± 38.6 mg $kg^{-1}$and all nitrate levels were lower than the maximum level established by European Union legislation.

Table 3
Quantitative analysis of nitrate and nitrite in baby food.

Sample	Nitrate (mg kg^{-1})	Nitrite (mg kg^{-1})
Organic pasta and vegetable-based puree	222.68 ± 4.72	< LOQ
Organic pumpkin-based puree	247.70 ± 6.52	< LOQ
Vegetables and meat-based puree	63.87 ± 1.39	< LOQ
Vegetables and chicken-based puree	157.58 ± 4.37	< LOQ
Beet-based puree	205.07 ± 6.24	< LOQ
Chicken and vegetable-based puree	61.34 ± 1.82	< LOQ
Meat, vegetables and pasta-based puree	45.23 ± 3.94	< LOQ
Vegetables and meat-based puree	45.81 ± 0.52	< LOQ
Yolk, meat and vegetable-based puree	65.35 ± 0.81	< LOQ
Turkey, vegetables and rice-based puree	74.49 ± 2.66	< LOQ
Chicken, vegetables and pasta-based puree	33.86 ± 1.24	< LOQ
Banana and milk-based puree	8.44 ± 4.41	< LOQ
Vegetable-based puree	135.58 ± 2.58	< LOQ
Meat and vegetable-based puree	97.12 ± 7.41	< LOQ

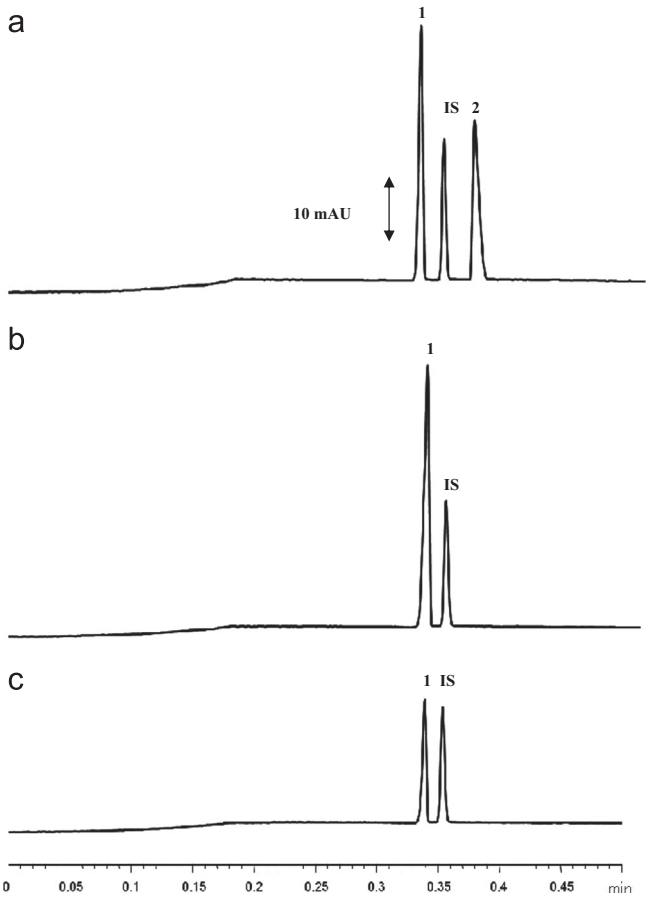

Fig. 3. Electropherograms obtained using the optimized method by CZE: (a) standard solution, (b) organic pumpkin-based puree baby food sample, (c) vegetables and chicken-based puree baby food sample. Legend: nitrate (1); thiocyanate – (IS); and nitrite (2).

4. Conclusions

The proposed CZE method showed good results for linearity, precision, accuracy, robustness and limits of detection and quantification, being suitable for the proposed purpose. Furthermore, the optimized sample preparation method was shown to be simpler, faster and of lower cost compared to the official method provided

by the AOAC. For these reasons this method has the potential for application as an alternative or complementary method for routine laboratory analysis to assess the nitrate and nitrite contents of commercially available baby foods. In this study most of the baby food samples did not show evidence of a risk to consumers, since most of them presented nitrate levels lower than the maximum limits established by Brazilian legislation. However, the constant monitoring of these compounds is important for the safety of the infant foods.

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