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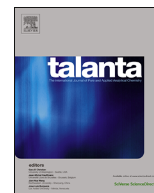

Development and validation of a sub-minute capillary zone electrophoresis method for determination of nitrate and nitrite in baby foods

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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.

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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

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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 $t_{m(\text{analyte})}/t_{m(\text{IS})}$, 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

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