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High-Resolution Capillary Electrophoresis of Nitrite and Nitrate in Biological Samples

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Summary

Nitrite and nitrate are widely used reporters of endogenous nitric oxide (NO) and nitric oxide synthase (NOS) activity, which are crucial for a broad spectrum of physiological and pathophysiological pathways. Because of the great variety in spatial expression and activity of NOS in animal tissues, a high-resolution analysis of nitrite/nitrate concentrations in very small biological samples, such as individual cells or homogeneous cell clusters, is required. A high-performance capillary zone electrophoresis (CZE) system, which includes a PrinCE-476 computerized capillary electrophoresis and Crystal-1000 conductivity detector, was optimized to analyze nitrite/nitrate concentrations in submicroliter samples of mammalian neuronal tissues and large individual cells of invertebrates. Solid-phase microextraction (SPME) and Isotachophoretic stacking (ITS) were used. The method is highly reproducible and yield excellent limits of detection (LODs): 8.9 n*M* (0.41 ppb) and 3.54 n*M* (0.22 ppb) for nitrite and nitrate, respectively, relative to undiluted samples.

Key Words: Capillary zone electrophoresis (CZE); biological sample; single cell; solid-phase microextraction (SPME); chloride cleanup; Isotachophoretic stacking (ITS); nitric oxide (NO); nitric oxide synthase (NOS).

1. Introduction

Nitrite and nitrate are widely used reporters of endogenous nitric oxide (NO), a biogenic free-radical molecule crucial to a broad spectrum of physiological pathways and disorders, including neuronal functions, vasodilatation, inflammation, pain, and macrophage-mediated intervention of tumors and infections. Endogenous NO, a subproduct of two mono-oxygenations of L-arginine to L-citrulline, catalyzed by NO synthases, is rapidly oxidized [half-life in normoxic biological systems is <30 s ***(1)***], resulting in the formation of nitrites and nitrates ***(2–5)***. NO gas freely diffuse through cellular membranes, leading to an increase in the NO_2^-/NO_3^- levels in the vicinity of NO-producing cells;

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however, the largest portion of NO oxidation products are accumulated intracellularly in ionic form as a result of a deficiency in membrane mechanisms for its retrograde transport. Intracellular NO_2^-/NO_3^- mediates a variety of functions by themselves and also serves as a substrate in several nonenzymatic reactions that form a pool of secondary NO sources, buffering intracellular NO levels ***(6)***. Because of the great spatial variety in the expression and activity of NOSs in animal tissues, a high-resolution analytical evaluation of nitrite/nitrate concentrations in very small biological samples such as individual cells or homogeneous cell clusters is required. Here, we describe a high-performance capillary zone electrophoresis (CZE) technique that is optimized to analyze nitrite/nitrate concentrations in submicroliter samples of mammalian neuronal tissues and large individual neurons of invertebrates. High-throughput automatic measurements were made using a PrinCE-476 computerized capillary electrophoresis system with a Crystal-1000 conductivity detector. To improve nitrite detection, samples were precleaned from interfering high concentrations of chloride ions using solid-phase microextraction (SPME) techniques. Samples were diluted 1000- to 10,000-fold to obtain optimal isotachophoretic stacking conditions and to reach an adequate sample volume (>5 µL), which is necessary for reliable automatic injection. The method is highly reproducible, yields excellent limits of detection (LODs) of 8.9 n*M* (0.41 ppb) and 3.54 n*M* (0.22 ppb) for nitrite and nitrate, respectively, relative to undiluted samples ***(7)***, and is simple enough to be changed for different capillary electrophoresis (CE) configuration and biological samples.

1.1. Capillary Electrophoresis

Capillary electrophoresis is a high-resolution (10^5–10^6 theoretical plates), ultrasmall-volume sample (10–100 nL), and fast (0.2–10 min) separation technique that offers remarkable advantages for analytical evaluation of practically all types of metabolite in biological samples. Separation is carried out in a 20- to 75-mm-inner diameter (ID) polyimide-coated fused silica capillary. The capillary is loaded with alkaline buffer that induces formation of charge-coupled binary layers by facilitating dissociation of silanol on the inner surface of the capillary. Electrophoretic drag of mobile inner-layer elecro-osmotic flow (EOF) depends on electrolyte concentration and pH. EOF-modifying additives are used to adjust separation properties. The sample can be loaded by hydrostatic force or pressure, or injected electrophoretically. The CZE coupled to a conductivity detector (CD) is the technique of choice to detect ultraviolet (UV)-silent inorganic ions, providing approximately a 10-fold better LOD compared to UV-light-based detection ***(8,9)***. Several ionic assays for distincting samples using ion chromatography and CE with a CD have been described ***(7,10–19)***

and a commercial CD system suitable for CE integration has recently become available ***(9)***.

1.2. Sample SPME Chloride Cleanup

A typical problem in CE of nitrite/nitrate in biological samples is a high concentration of chloride ions, which interfere with the injection and detection of bromate, nitrite, and nitrate because these ions have similar electrophoretic properties and elution times. Chloride interference becomes more critical if electrophoretic injection and especially isotachophoretic stacking (ITP) preconcentration of the sample is used. To improve nitrite determinations, chloride ions can be removed by passing the sample through a silver-form sulfonated styrene-based resin. Because commercial solid-phase microextraction (SPME) cartridges are not available ***(20)***, we designed a microcartridge and procedure suitable for cleanup of 5- to 50-µL samples by the spin-enforced SPME technique ***(7)***.

2. Materials

2.1. Components (see *Note 1)*

1. PrinCE-C 465 (PrinCE Technologies, Netherlands), a computerized CE system with a robotic sample injector.
2. DAx 6 for Microsoft Windows NT/98 data acquisition and analysis software (Van Mierlo Software Consultancy, Netherlands). The software is used to control the CE unit and 22-bit acquisition board (SCPA L42M, SCPA, DE) and to perform data analysis. A data acquisition ratio of 10–20 data point per second is used).
3. Crystal-1000 conductivity detector (Thermo Bioanalysis, cat. no. 9435 250 02001).
4. ConCap™ I fused silica capillary assembly, 70 cm × 50 µm (Thermo Bioanalysis, cat. no. CD220307-A01 or 9435 250 02211).
5. ConTip™ I conductivity sensor assembly (Thermo Bioanalysis, cat. no. CD219812-A01).
6. Small laboratory centrifuge (>1000 rpm).
7. 2-mL vial, vial holder, and 13-mm vial cap for inlet buffer and standards (PrinCE Technology, cat. no. 8035.001, 2520.011, and 2520.810).
8. 250-µL Vial and vial holder for samples (PrinCE Technology, cat. nos. 8030.010 and 2520.014).
9. MilliCupTM-HV filter unit for filtering and degassing CE buffer (Milipore SJHV, cat. no. M4710).
10. Tru-Sweep™ sonicator bath for degassing CE and cleaning reusable analytical components (Crest Ultrasonics).
11. Polimer Luer-lock 10 and 30-cm^3 syringes (*see* **Note 2**).
12. OnGuard II A®, 2.5-cm^3 buffer preparation cartridges (Dionex, cat. no. 057092).
13. OnGuard II Ag®, 2.5-cm^3 (Dionex, cat. no. 057090) (*see* **Note 3**).
14. Polymer 1/8-in. female Luer-lock (Small Parts, cat. no. U-1005109).

Table 1
Stock Solutions

Anions	Formula	F.W.	m*M* (1000 ppm)	mg/250 mL (1000 ppm)
Chloride	NaCl	58.44	28.2	412
Nitrate	$NaNO_3$	84.99	16.1	342
Nitrite	$NaNO_2$	69.00	21.7	374
Sulfate	Na_2SO_4	142.04	10.4	370
Fluoride	NaF	41.99	52.6	553
Phosphate	Na_2PO_4	141.96	10.5	374

15. 0.1- to 10-µL Filter tips (USA Scientific, cat. no. 1121-3810).
16. 1- to 20-µL Tips (*see* **Note 4**).
17. Falcon BlueMax 15-mL graduated tubes (Fisher, cat. no. 14-959-49D).

*2.2. Reagents and Stock Solutions (***see** ***Note 5)***

1. 1*M* NaOH cleaning solution for SPME cartridge and capillary cleanup. Prepare 200 mL solution by dissolving 8 g of NaOH in 200 mL of distilled water (DW).
2. 25 m*M* EOF modifier solution (TTAB). Prepare 250-mL solution by dissolving 2.10 g TTAB in DW. Apply ultrasonic agitation (*see* **Note 6**).
3. 12 m*M* LiOH leading solution. Dissolve 0.05 g of lithium hydroxide monohydrate in 100 mL of DW. A fresh solution should be prepared weekly.
4. 1000 ppm terminating solution. Dissolve 0.12 g of sodium octanesulfonate (monohydrate) in 100 mL of DW. A fresh solution should be prepared weekly.
5. Stock standards. Dilute reagent in 250 mL of DW (*see* **Table 1**, column 5).
6. Mixed standards for calibration (1/10/100/1000 ppb). Prepare 10 mL of 10 ppm mixed standard by pipetting 100 µL of each standard solution into 15-mL graduated tubes and add DW to reach 10-mL volume. Add 900 µL of DW into four 2-mL sampling vials. Pipet 100 µL of 100 ppm mixed standard into the first vial and mix the solution by gentle pipetting it three times. Sequentially dilute mixed standard by transferring 100 µL of solution to each next vial after mixing it in previous vial. (Use fresh tips for each solution transfer.) Replace DW by 10 ppm terminating solution if one is used for sample preparation. The mixed standard can be stored for a month.

3. Methods

3.1. Preparation of 250 mL of CE Buffer

The CE buffer with the EOF-modifying solution of tetradecyltrimethylammonium hydroxide includes 25 m*M* arginine, 81.5 m*M* borate, and 0.5 m*M* TTAOH, pH 9.5, with 1 *M* NaOH. TTAB is converted to TTAOH by replacing bromide ions with hydroxyl ions using ion-exchange cartridge OnGuard II A.

1. Dissolve 1.09 g Arg and 1.25 g boric acid in 250-mL polypropylene flask filled with approx 100 mL DW.
2. Attach OnGuard II A cartridge to a 10-cm^3 syringe with plunger removed, backfill with approx 10 mL of DW, reinsert plunger, and pump all DW volume through the cartridge.
3. Detach cartridge, remove plunger, and repeat **step 2** except for backfilling syringe with 10 mL of 1*M* NaOH.
4. Repeat **step 2** with DW to wash residual NaOH.
5. Repeat **step 2** with 10 mL of 25 m*M* TTAB, first disposing 5 mL and collecting the next 5 mL into the polypropylene flask from **step 1**.
6. Add DW to reach 250-mL volume.
7. Degas and filter buffer solution by passing through a 22-µm membrane filter using a vacuum pump and appropriate filtering assembly. New buffer solution should be prepared daily.

3.2. Preparation of Chloride SPME Cartridges

1. Open OnGuard Ag cartridge using pliers and collect resin on a waxed balance paper and then in an Eppendorf tube. The resin from one OnGuard Ag cartridge is enough to prepare about 200 SPME cartridges (*see* **Fig. 1**). Backload approx 3.5 mg of the resin into 0.1- to 10-µL filter tips, which then will be used as SPME cartridges (*see* **Note 7**).
2. Attach the SPME cartridge to a plastic syringe using a 1/8-in. female Luer-lock and slowly (approx 10 s) pump about 0.1–0.2 mL of 1*M* NaOH.
3. Repeat **step 2**, pumping about 0.5 mL of DW.
4. Balance cartridges in centrifuge using 1- to 20-µL tips and 1.5-mL Eppendorf tubes as a cartage support (*see* **Fig. 2**).
5. Spin cartridges for 30 s to remove residual DW. Ready SPME cartridges can be stored and used during several days; however, the best results are obtained with a freshly prepared cartridge.

3.3. Sample Preparation

1. Acquire liquid or solid samples following an appropriate procedure ***(7,21–25)***.
2. Estimate volume of samples and resuspend samples in DW 1:1,000 to 1:100,000 final sample dilution and at least 10 µL volume (*see* **Note 8**).
3. Break down tissue by freezing and ultrasonic agitation (*see* **Note 9**).
4. Backload diluted samples into SPME cartridge–sample vial assembly, balance, and spin for approx 30 s (*see* **Fig. 1**, *positions 5* and *6*).
5. Deposit sample vial into a holder, seal holder with a cap, and place in a sampling carousel from PrinCE.

3.4. CE Running Protocol (see *Note 10*)

1. Deposit a set of analysis-ready samples, along with a set of four gradually diluted standards (*see* **step 5** of **Subheading 3.2.**) into a sampling carousel from PrinCE.

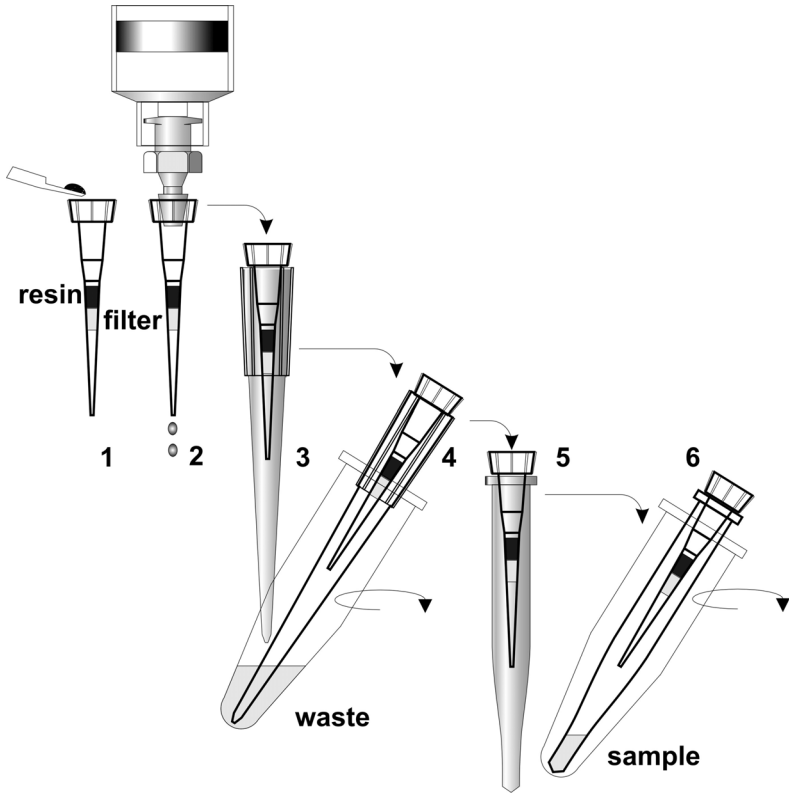

Fig. 1. Solid-phase microextraction-chloride cleanup cartridge preparation *(1–4)* and sample cleanup *(5, 6)* procedures. SPME cartridge is inserted into a 1- to 20-µL tip and sampling vial at 3 and 5, respectively, are shown.

2. Enter CE Global Configuration, and Crystal 1000 Configuration values according to manufacturer instructions.
3. Enter "Run Table" values (run number; separation method, sample/standard location).
4. Enter CE "Running Method" values (*see* **Table 2**).
5. Precondition CE capillary: Load capillary with 1*M* NaOH prior to run and flush it with DW after 5 min (*see* **Note 11**).
6. Postcondition CE capillary: After analysis, flush capillary sequentially for 2 min with CE buffer and then for 2 min with DW (*see* **Note 12**).
7. Refresh running buffer after each 10 separation cycles by switching to another running buffer vial.
8. Examples of electrophoretograms for different sample types are presented in **Fig. 2**.

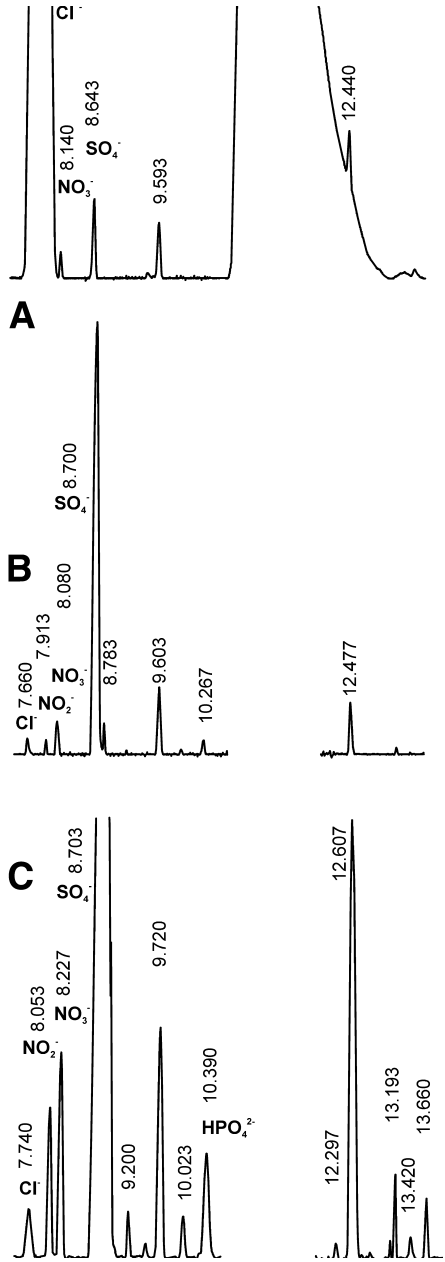

Fig. 2. Representative electropherograms of different tissue samples (modified from **ref. *7***). **A–C**: 10,000-fold diluted rat dorsal root ganglia before **(A)** and after **(B)** SPME chloride removal. Absolute retention times along with anion identities are shown. **B** and **C:** different ITP staking injection intervals, 3 and 12 s, respectively.

Table 2
Running Method (for 50-μm Bore, 500-mm-Length Capillary)

Procedure	Inlet vial	Pressure (mbar)	Voltage (kV)	Time (s)
1. Clean	1*M* NaOH	2000	0	100
2. Wash	18 *M*Ω DW	2000	0	100
3. Fill	Buffer load	2000	0	100
4. Stacking plug	12 m*M* LiOH	30	0	10
5.1 Sample injection[a]	Sample load	0	–5	5[b]
5.2 Sample injection[a]	Sample load	20	0	10[b]
6. Separation	Running buffer[c]	0	–20	900

[a]Value for electrophoretic and hydrodynamic injection 5.1 and 5.2, respectively, are shown.
[b]Average values of injection time for 1:1000 diluted samples are shown.
[c]This step is synchronized with data acquisition start.

3.5. Data Analysis

1. Here, we provide a brief summary of analysis procedure for DAx 6 software, which includes CE control and many special futures for automatic analysis of electrophoretograms (*see* **Note 13**).
2. Open electrophoretograms of four dilutions of mixed calibrations standard in a DAx "Graphic Window."
3. Construct and subtract baseline sequentially selecting individual electrophoretograms.
4. Select 10-ppb graph and annotate peaks and their concentrations. Anions in standards can be identified by relative-to-chloride retention times: chloride < nitrate < nitrite < sulfate < fluoride < phosphate < carbonate (carbonate from air).
5. Sequentially select 1, 100, and 1000 ppb and run "Detect Peak" analysis procedure.
6. Use the data tag peak menu to display "Peak List" for each of the measurements you want to include in the calibration.
7. Enter peak annotations if they are absent and correct concentration values in the "Peak List."
8. Save calibration procedures, which can be used with the same capillary length and CE protocols for an extended period of time.
9. Subtract baseline and run automatic analysis procedure for each sample.
10. Ion concentrations are determined from relative peak areas and calibration slopes using DAx 6 software. A four-point calibration database with linear or nonlinear point-to-point interpolation is used for quantitative analysis. Finally, electrophoretograms are exported into "Windows Metafile" vector format (*.wmf) and assembled into representative graphs (*see* **Fig. 2**).

4. Notes

1. Suppliers' URL: Dionex, www.dionex.com; PrinCE Technologies, www.princetechnologies.nl; Sigma-Aldrich, www.sigmaaldrich.com; SCPA, www.scpa.de; Van Mierlo Software Consultancy, www.dax.nl/mierlo/vmsc; Small Parts, www.smallparts.com.
2. Syringes are used to prepare buffer components and SPME cartridge cleanup.
3. OnGuard II Ag resin is used for preparing chloride SPME cartridges.
4. The 1- to 20-µL tips are used as a support and protect SPME cartridges.
5. Analytical-grade chemicals (99+%, A.C.S.) and water (>18 MΩ) must be used throughout.
6. The TTAB solution can be stored for 1 mo at room temperature. Do not refrigerate to avoid precipitation.
7. OnGuard-Ag resin can be stored in the dark.
8. In our experience, the terminating additive may improve separation if the sample dilution is < 1:1000; if dilution is > 1:1000, analytical-grade DW can be used. Diluted samples can be stored in Eppendorf tubes at –20°C for several days.
9. Most samples are broken down by hypo-osmotic shock, which provides sufficient conditions for equalizing of ionic concentrations. However, ultrasonic agitation and sample freezing can be beneficial in some instances.
10. Detailed PrinCE- and Cristal 1000-specific setup procedures can be found in the manufacturers' instructions.
11. Preconditioning is obligatory for new and previously dried CE capillaries.
12. Postconditioning increases the lifetime of CE capillaries. In the PrinCE interface, preconditioning and postconditioning of the separation capillary can be included as first and last lines in the "Run Table."
13. A detailed manual and demonstration of newer version of DAx CE is available at www.dax.nl/daxce. Alternatively, appropriate acquisition system and data analysis software can be used.

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