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High-resolution microanalysis of nitrite and nitrate in neuronal tissues by capillary electrophoresis with conductivity detection

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Abstract

Nitrites and nitrates are widely used reporters of endogenous activity of nitric oxide synthases (NOS), an important group of enzymes producing the gaseous signal molecule nitric oxide (NO). However, due to the great chemical heterogeneity of neuronal tissues, standard analytical protocols for evaluation of neuronal nitrite/nitrate concentrations are inefficient. We optimized a high-performance capillary zone electrophoresis (CZE) technique to analyze nitrite/nitrate concentrations in submicroliter samples from mammalian neuronal tissues. The measurements were made using a PrinCE 476 computerized capillary electrophoresis system with a Crystal 1000 contact conductivity detector. Isotachophoretic stacking injection of 1000- to 10 000-fold diluted samples, which had been pretreated with a custom-designed solid-phase microextraction (SPME) cartridge, was employed to assay micromolar and nanomolar nitrite and nitrate levels in the presence of the high millimolar chloride concentrations characteristic of many biological samples. In the presented technique, a 10-μl volume of diluted ganglionic sample was used for chloride removal and sample cleanup. The method yields high analytical performance, including good reproducibility, resolution, and accuracy. The limits of detection relative to undiluted sample matrix were 8.9 n*M* (0.41 ppb) and 3.54 n*M* (0.22 ppb) for nitrite and nitrate, respectively. In addition, this technique resolves other anions that are present in neuronal tissues at sub-nanomolar concentrations and can be broadly applied for high-throughput anionic profiling. In rat dorsal root ganglia, endogenous levels of nitrate (231±29 μ*M*; $n=6$) and nitrite (24–96 μ*M*) were found. These concentrations exceeded those previously found in neuronal tissue homogenates using different techniques.

Keywords: Nitrite; Nitrate

1. Introduction

The use of high-performance capillary electrophoresis (HPCE) to analyze subnanoliter sample volumes has resulted in a large number of successful bioanalytical applications. This technique is specifically advantageous for neuronal microchemical analysis due to the extreme cellular and chemical heterogeneity of neuronal tissues. Reduction of the sample volume enhances both the spatial and temporal resolution of analytical assays. At a certain level of volume reduction, sampling also becomes nondestructive to cells and tissues, which benefits cellular

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physiology, biomedical research and therapeutic diagnostics.

The present work deals with a medically important application of capillary zone electrophoresis (CZE), the profiling of near-trace levels of nitrite/nitrate in complex neuronal tissues. The absolute concentration and nitrite/nitrate ratio are widely used reporters of nitric oxide synthase (NOS) activity [1–3]. The three isoforms of NOS found in the nervous system are the main source of endogenously produced nitric oxide (NO), a radical gas known as an important regulator of hundreds of biochemical pathways and a component of many neuropathologies [4]. Endogenous NO is rapidly oxidized in a normoxic biological microenvironment, resulting in the formation of nitrites and nitrates [5–8]. The membrane-impermeable anions NO_2^- and NO_3^- are mainly accumulated locally (in the vicinity of NOS and NOS-containing cells) where they can have a variety of functional effects individually and also serve as a secondary source of NO that is used in several non-enzymatic pathways [9]. However, the lack of data on the spatial distribution of nitrites/nitrates in the nervous system greatly limits our understanding of NO-mediated pathways. The major problem is the absence of adequate analytical approaches for the microchemical analysis of nitrite/nitrate in small samples, which would represent specific metabolic domains of neuronal tissues.

Ion chromatography (IC) is commonly used for trace analysis of inorganic ions if a large sample volume is available. The IC technique, often combined with an on-line sample preconcentration cleanup procedure, has been used to measure low nitrite/nitrate concentrations in various matrices, including ultra-pure deionized water [10], drinking water [11–13], and biological fluids and tissues [14–18]. Nevertheless, the advantages of IC are diminished in assays of samples such as individual neurons and small neuronal clusters. The obvious method of choice is CZE, which has already been employed successfully to measure nitrite and nitrate in biological samples [19–26]. Most of these applications utilize a UV-based detection technique to determine non-UV-absorbing substances, although it is obviously not a method of preference due to its relatively low sensitivity [27,28]. The technique of choice to detect inorganic and small organic ions is conductivity detection (CD), which provides approximately 10 times better limits of detection (LOD) [29,30]. Several ion assays using IC and CE with CD have been described [2,3,11,15,18,27,31–34] and a commercial CD system suitable for CE integration has recently become available [35]. However, these tools have never been applied to nitrite/nitrate analysis in the nervous system.

Here we describe a high-resolution CZE technique for the sampling and evaluation of ionic profiles in small and specific samples from rat dorsal root ganglia (DRG). Our goals were (1) to evaluate a new approach for anion analysis of biological tissue samples based on the combination of capillary zone electrophoresis and direct outlet conductivity detection, (2) to optimize this technique for the determination of the nitric oxide-related metabolites, nitrite and nitrate, and (3) to optimize this technique for the automatic analysis of ultra-small samples so that it would be suitable for the analysis of small neuronal clusters and individual neurones. The described CZE technique is effective for the indirect monitoring of alterations in NO production from small ganglia or even individual neurons in specific brain regions.

2. Experimental

2.1. Instrumentation

A computerized PrinCE-C 465 system with a robotic sample injector (PrinCE Technologies, Netherlands) coupled to a Crystal-1000 conductivity detector (ThermoBioanalysis, CA, USA) was used. Separation was performed using a 70 cm (50 μm I.D.×360 μm O.D.) fused-silica capillary (Polymicro Technologies, AZ, USA) with an outlet conductivity cell encapsulated in two individually modified coupling connectors [ConTip, ConCap (both from Orion Research, Boston, MA, USA)] [35]. DAx 6 for Microsoft Windows NT/98 data acquisition and analysis software (Van Mierlo Software Consultancy, Netherlands) was used to control the CE and 20-bit acquisition board as well as data recording and analysis. The data acquisition ratio was 20 datapoints s^{-1}.

2.2. Reagents

Analytical-grade chemicals were obtained from Sigma (St. Louis, MO, USA). The background electrolyte was an arginine–borate buffer (ABB) with an added electro-osmotic flow (EOF) modifying solution of tetradecyltrimethylammonium hydroxide (TTAOH) (25 m*M* arginine, 81.5 m*M* borate and 0.5 m*M* TTAOH, pH 9.5). TTAOH was prepared by converting the bromide salt (TTAB) into the hydroxyl form using a styrene-based, anion-exchange resin cartridge (On-Guard A, Dionex, CA, USA). The buffer was filtered through a 22-μm membrane. Fresh electrolyte was prepared daily and degassed with combined vacuum–ultrasonic agitation prior to use.

2.3. Animals

Adult male Sprague–Dawley rats (90–110 g) were anesthetized with halothane. Following decapitation, the spinal cord was rapidly removed, and the dorsal root ganglia (DRG) were dissected free. Three pairs each of cervical, thoracic and lumbar ganglia were isolated and blotted on filter paper. Ganglia were washed for 5 s in isoosmotic sucrose solution and subsequently processed for further analysis. All animals were housed in quarters approved by the American Association for Accreditation of Laboratory Animal Care, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee.

2.4. Sample acquisition

Several optical scans of each sample were transmitted to a computer during the washing procedure using a Pixera CCD (WPI, Sarasota, FL, USA) camera and an Olympus SZX12 stereomicroscope. Sample dimensions were obtained from the digital pictures using standard ''measure'' macros with Scion image 4.2b software (PC version of NIH image; Scion, MD, USA). The sample volumes V were calculated from these dimensions, assuming that ganglia are oblate spheroids ($V = 4/3\pi R_1 R_2 R_3$). Alternatively, sample volumes were evaluated by liquid displacement. For such evaluations, ganglia were deposited on patches of Parafilm, blotted, and immersed in a small volume of distilled water (DW) which was extruded from a calibrated glass capillary. The sample with the DW was immediately drawn back into the capillary. The sample volume was calculated from the displacement of the liquid volume inside the capillary. The capillary surface was coated by dipping into Sigmacot solution (Sigma) to prevent sticking of the sample to the glass. Finally, samples were diluted 1000- to 10 000-fold with DW, deposited in Eppendorf tubes and autolyzed by flash freezing cycles and ultrasonic agitation.

2.5. Chloride cleanup by solid-phase extraction

The native chloride peak in most biological samples is so large that it masks the nitrite and nitrate peaks. To improve nitrite determination, chloride anions were removed by passing the sample through a silver-form sulfonated styrene-based resin using laboratory made micro-cartridges suitable for cleanup of 5–50 μl samples by the spin-enforced solid-phase extraction technique (SESPE). Resin was obtained from an OnGuard-Ag cartridge (Dionex, Sunnyvale, CA, USA). Approximately 3.5 mg of the resin was back-loaded into 0.1–10 μl filter tips (USA Scientific, FL, USA), which were then used as SESPE cartridges (see Fig. 2). The cartridges were pre-cleaned by perfusion with approximately 1 ml of 1 *M* NaOH and 3–5 ml of 18 MΩ DW using disposable 5 ml syringes and plastic assembly (see Fig. 2, left). Residual DW was spun down using a Personal Microcentrifuge (USA Scientific), and evaporated in air for 1–2 h at room temperature. The cartridges were inserted into larger 10–100 μl tips to prevent surface contamination and to separate waste during spinning (see Fig. 2). Pretreated cartridges were inserted into sampling vials loaded with 10–20 ml of diluted neuronal samples and spun for approximately 30 s, allowing sample passage through the cleanup column. Sodium chloride (100 n*M*) was then added to the final assay sample to generate an internal reference peak, which was used to qualify other ions.

2.6. Separation and analysis

A sample set, along with a set of gradually diluted standards, was injected and analyzed in an automatic

mode using identical capillary treatment and separation conditions. The sample was introduced into the capillary by isotachophoretic-stacking injection (−6 kV for 20 s) to a preloaded plug of high-mobility electrolyte (pressure-loaded 12 m*M* LiOH, 50 mbar for 12 s). The separation conditions were: −28 kV, 3–5.5 μA, 22±0.5 °C, running time 15 min. The capillary was precleaned with 1 *M* NaOH (2000 p.s.i. for 2 min), washed with DW (2000 p.s.i. for 3 min) and loaded with buffer (2000 p.s.i. for 3 min) prior to each run. The separation protocol was optimized for the analysis of inorganic anions at 0.1–100 μ*M* concentration in a nanoliter injection volume [2,3,36].

Anions were identified by absolute retention times and retention times relative to chloride. Some samples were analyzed a second time after spiking with nitrite or nitrate standards to validate the quantification of these ionic species. The data analysis combined high-frequency cut-off filtering and baseline reconstruction/subtraction with a five-datapoint moving average algorithm, followed by an automatic peak qualification and quantification protocol, which depends upon an initially created standard database. Ion concentrations were determined from relative peak areas and calibration slopes using DAx 6 software. A four-point calibration database with nonlinear point-to-point interpolation was used for quantitative analysis. Finally, electropherograms were exported into Windows Metafile vector format (*.wmf) and assembled into representative graphs.

2.7. Evaluation of analytical performance

The 3σ method was used to determine limits of detection and quantification (LOD and LOQ, respectively) [37]. The theoretical maximum LOD/LOQ was calculated from computed standard deviations of blank replicates (SD, $n=7$) and the calibration slope m of low concentration standards in DW ($LOD_{max}=3\ SD/m$ and $LOQ_{max}=10\ SD/m$). Standard deviation values were computed from exported raw data of selected electropherograms using Sigma Plot software (SPSS, Richmond, CA, USA). A similar procedure was utilized to determine the LOD/LOQ of ions loaded from an in situ matrix, except that blank data were substituted by raw data from a normal sample separation with digitally subtracted ion peaks. The precision (reproducibility) and accuracy of the method were evaluated by computing a relative standard deviation (RSD) and error values of the determined ion concentrations in a commercial standard ion mixture (IC Instrument Check Standard # 6; SPEX CertiPrep, NJ, USA).

3. Results and discussion

3.1. Sample acquisition

To improve analytical performance a commercial conductivity detection system [35] providing better LOD for inorganic ions than other detection techniques was used [30]. Using an indirect sampling procedure, several sample-conditioning steps were incorporated to optimize the determination of nitrite/nitrate anions in neuronal tissue; these included sample dilution in DW, chloride solid-phase microextraction (SPME) cleanup, and isotachophoretic (ITP) stacking of the diluted samples (Fig. 1).

Crucial for indirect sampling techniques is precise volume determination. Initially, sample volumes were determined from their visual dimensions (see Experimental). However, due to the complex geometry of the DRG samples, the volume calculation was difficult and resulted in a standard error of 7.6% (based upon the inconsistency of calculated values, $n=5$ per sample). Subsequently, the ganglionic volume was determined by liquid displacement (Fig. 2). This technique resulted in better precision and accuracy; the corresponding normalized values were 0.8 and 1.5%, respectively (based on the assay of spherical micro-beads with known volumes, $n=12$). Although both of these techniques are suitable for the determination of nanoliter sample volumes, the second procedure is superior for DRG assay and was used in all final measurements. The first technique may be superior, however, for calculation of the volume of isolated cells with spherical shape. The average DRG sample volume used in this experiment was 12 nl.

3.2. Solid-phase microextraction (SPME) cleanup

A typical problem in nitrite/nitrate determinations of biological matrices with CZE and CD is interfer-

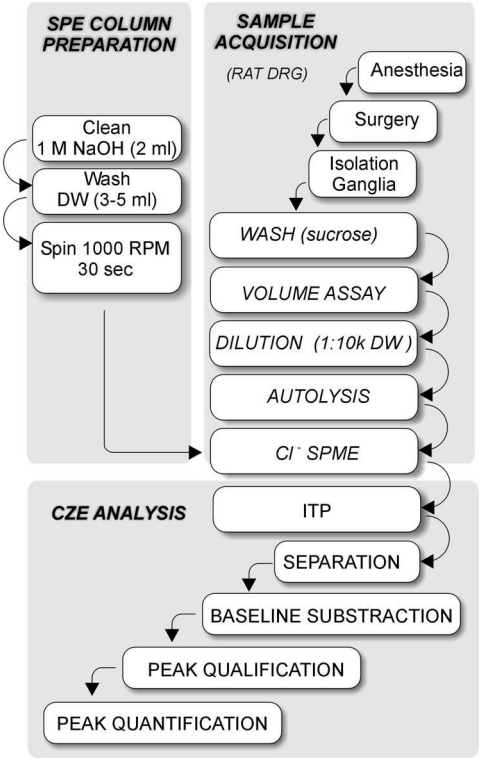

Fig. 1. Flow-chart diagram of sample handling and analytical procedures. Abbreviations: DRG, dorsal root ganglia; DW, 18 MΩ deionized water; ITPS, isotachophoretic stocking; SPEC, solid-phase microextraction cleanup.

ence from the high natural concentrations of chloride anions, which mask and reduce the injected quantity of other ions. Chloride interference becomes even more critical if electrophoretic injection and, especially, isotachophoretic stacking (ITP) preconcentration steps are used. Bromate, chloride, nitrite and nitrate slightly differ in their electrophoretic mobilities; thus, high chloride activities (100–450 m*M*) in biological samples interfere with the electrophoretic injection and detection of nitrites and nitrates known to be present at much lower concentrations (typically $<$1–100 μ*M*). Under these conditions, manipulation of the pH (7–9.5) and concentration of TTAOH (0.5–2 m*M*) does not improve the separation. In contrast, the removal of chloride anions from the sample matrix using a solid-phase extraction (SPE) technique has been shown to dramatically improve IC determinations of bromate traces in drinking water [12], nitrate in a coral skeleton [38]

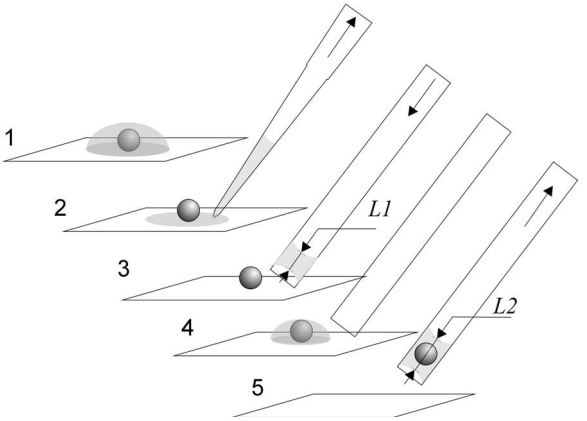

Fig. 2. Determination of sample volume by equivalent liquid displacement. The sample volume is given by $V_s = V_2 - V_1 = \pi r^2(l_2 - l_1)$, where l_1 and l_2 are the distances between the meniscus before and after sample withdrawal (plates 3 and 5, respectively). 1, Sample in a drop of sucrose solution; 2, blotting; 3 and 4, a known volume of DW is added; 5, sample is drawn back into the capillary tube.

and nitrite/nitrate in perfusate samples from isolated rat tumor tissue [15,16]. Previously, two membrane-based SPE sample cleanup systems have been used in CE experiments: (1) a polytetrafluoroethylene (PTFE) membrane impregnated with ion-exchange media [39], and (2) a Novo-Clean IC-Ag membrane impregnated with polystyrene–divinylbenzene sulfonated cation-exchange resin beads in a silver form that are encapsulated in a filter holder with Luer locks (Alltech, IL, USA) (Fig. 3). However, both of these commercial designs require relatively large sample volumes to be treated for sufficient sample recovery. It has been reported that this method ''likely involved sample contamination'' [17]. A custom device for Cl-SPE cleanup has also been described, in which an ~20 mg OnGuard-Ag resin was weighed into a sampling vial prior to sample loading [15]. Although efficient measurement of nitrite and nitrate in rat tumor tissues was achieved by high-performance ion chromatography [15], the large volume requirement of this cleanup procedure precludes its use for analysis of small neuronal samples.

Here, we introduce a simple cleanup procedure for the treatment of 10–20 μl liquid samples which utilizes cartridges prepared by back-loading disposable 0.1–10 μl filter tips (USA Scientific) with 10

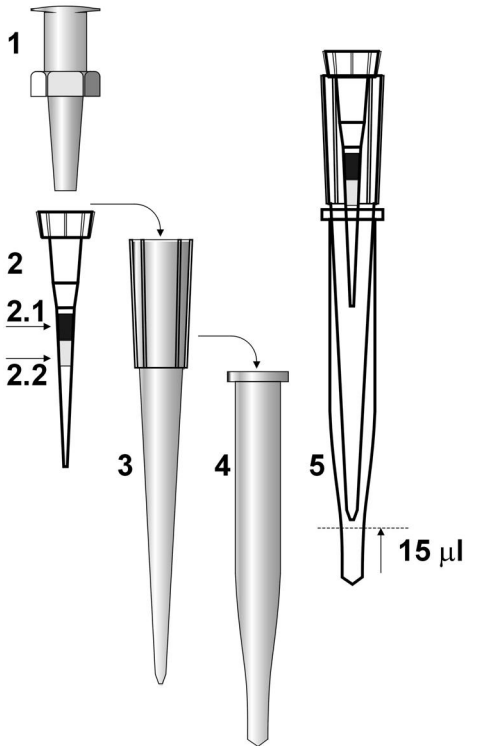

Fig. 3. Solid-phase extraction (SPE) chloride cleanup cartridge and its functional assembly with an injection vial. 1, Luer lock for cartridge cleaning with disposable syringe; 2, 0.1–10 μl filter tip cartridge; 2.1, OnGuard-Ag resin bead plug; 2.2, filter plug; 3, 1–20 μl beveled tip used as a cartridge support; 4, injection vial; 5, functional assembly for spin-enforced sample cleanup, upper levels of 15 μl liquid are shown.

mm^3 of Dionex OnGuard-Ag resin (Fig. 2). These cartridges were effective for the treatment of 100- to 10 000-fold diluted DRG samples. To clean larger volumes or more concentrated samples, the resin bead volume must be increased. The cartridge performance is summarized in Table 1.

Table 1
Cl^- cleanup SPME cartridge performance based on the cleanup of 10 μl of 0.1 m*M* standards

Parameter	Average recovery		RSD (%)
	(%)	*n*	
Sample volume	82	12	1.2
Chloride	0.1	8	1.3
Nitrite	96.5	8	2.9
Nitrate	98.3	8	2.1
Phosphate	89.4	5	3.5
Sulfate	115.3	8	4.7

3.3. Optimization of isotachophoretic (ITP) stacking

Although 1000-fold dilution of neuronal tissue samples allowed effective chloride removal with the

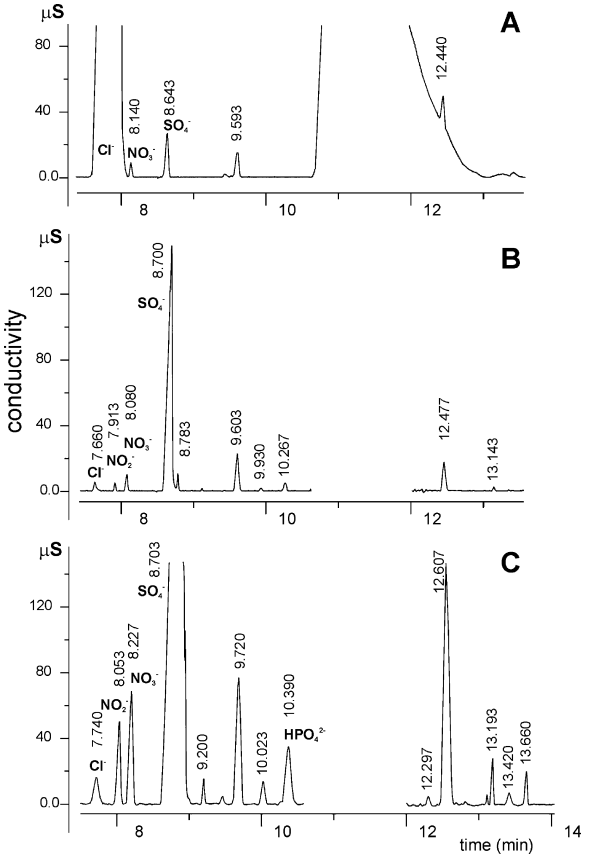

Fig. 4. Electropherograms obtained for a 10 000-fold diluted rat dorsal root ganglia before (A) and after (B) solid-phase microextraction of chloride anion from the sample matrix. (C) Same sample as (B) after longer ITP stacking injection. Absolute retention times are shown along with anion identities. Separation was performed at −20 kV in a 0.7 m length 50 μ*M* bore fused-silica capillary filled with 25 m*M* arginine–81.5 m*M* borate–0.5 m*M* TTAOH buffer at pH 9.5. ITP stacking injection −5 kV, 0.05 min for (A) and (B) and 0.20 min for (C), was used. The large hydrogencarbonate peak (A, R_t 11–12 min) was subtracted from traces (B) and (C) using a baseline subtraction algorithm (DAx 6.0, Van Mierlo Software Consultancy, Netherlands).

Although 1000-fold dilution of neuronal tissue samples allowed effective chloride removal with the

described micro-cartridge, additional dilution of DRG samples was necessary to obtain a sufficient volume for consistent sample loading in automatic or semi-automatic injection modes. We found that a 10-μl sample in the standard sampling vial (Fig. 2) is sufficient for consistent ion quantification with the PrinCE 465 and Crystal 1000 system (nitrate RSD = 2.1%, $n = 8$), whereas a 5-μl sample is insufficient (nitrate RSD = 38.3%, $n = 3$). Sample dilution also produces CE peak sharpening due to ion stacking at the sample–carrier electrolyte interface and therefore provides better LOD values [40]. High field stacking pre-concentration is the commonly used optimization strategy for CZE analysis of diluted ion samples, leading to a 50–1000-fold increase in sensitivity [41]. To increase the analytical performance for the analysis of diluted neuronal tissue samples we use the EOF-modifying additive TTAOH [42]. We also preloaded a plug of high ion mobility leading electrolyte (12 m*M* LiOH) to further increase the efficiency of ITP stacking and therefore improve the LOD value.

3.4. Analytical performance

Fig. 4 shows typical electropherograms obtained for 10 000-fold diluted DRG samples after SPME chloride cleanup and different ITP stacking conditions. The nitrate concentrations in different DRGs were found to be relatively consistent (231 ± 29 μ*M*, value ± RSD, $N = 6$). In contrast, nitrite concentrations varied in the samples, with maximal and minimal concentrations of 96 and 24 μ*M*, respectively, being detected in different DRGs.

Our technique provides good analytical performance with standards and natural matrices (summarized in Table 2). The maximum attempted dilution (100 000-fold, $n = 7$) was still very effective for nitrite/nitrate quantification in DRG samples after SPME chloride cleanup. Calculations from the dilution value and a convenient volume (10 μl) suggest that the described technique allows the determination of nitrite/nitrate concentrations at normal physiological levels in relatively small samples of 100 pl (10^{-4} mm^3), and therefore suggests the possibility of single-cell analysis using the described technique.

In summary, a combination of CZE with conductivity detection, efficient chloride removal, sampling dilution, and isotachophoretic (ITP) stacking significantly reduces the amount of biological tissue needed for anion analysis to provide a reliable technique for the evaluation of anionic profiles of small neuronal samples and even of individual cells. Dorsal root ganglia represent a specialized population of primary mechanosensory and nociceptive neurons. Thus, the reported high concentrations of nitrate and nitrite in these structures (the highest reported from mammalian neuronal tissues to date) reflect a high level of NOS activity and indicate an important role for NO signaling in mammalian tactile reception and pain mechanisms.

Table 2
Analytical performance of anion determinations

Anion	Parameter								
	LOD^S (n*M*)	LOD^S (ppb)	LOD^N (n*M*)	LOD^N (ppb)	Precision[a]		Reproducibility[b]		Accuracy[c] (%)
					(%)	RSD %	(%)	RSD %	
Chloride	1.41	0.05	–	–	97.1	2.2	98.0	3.1	99
Nitrite	4.34	0.20	**8.90**	**0.41**	94.6	6.4	78.8	12.1	98
Nitrate	1.87	0.16	**3.54**	**0.22**	96.2	3.2	95.2	4.0	95
Sulfate	1.09	0.12	**1.61**	**0.18**	94.1	4.2	96.2	4.6	98
Phosphate	11.9	1.29	–	–	97.1	5.8	82.1	12.0	72

Note: limit of detection in standard (LOD^S) mixture and natural (LOD^N) tissue matrices; RSD, relative standard deviation. Values in bold type are after chloride SPME removal.

[a] From quantification samples of three standard concentrations: 10^{-5}, 10^{-6}, and 10^{-7} *M*.

[b] From subsequent 3-day quantification of a 10^{-6} *M* sample.

[c] From quantification of IC Instrument Check Standard # 6. SPEX CertiPrep.

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