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Review

Methods for the determination of nitrite by high-performance liquid chromatography with electrochemical detection

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Abstract

A review on the use of high-performance liquid chromatography coupled with electrochemical detection (HPLC–ED) for the measurement of nitrite (NO_2^-) and nitrate (NO_3^-) is presented. HPLC–ED has been used for the determination of NO_2^- and NO_3^- in food, biological and environmental samples. Analysis of the current literature indicates that the measurement of NO_2^- and NO_3^- by the HPLC–ED procedure is more sensitive, selective and faster than methods based on UV absorption, photometry, fluorometry or chemiluminescence. © 1997 Elsevier Science B.V.

Keywords: Reviews; Nitrite; Nitrate; Inorganic anions

Contents

1. Introduction

Nitrite (NO_2^-) and nitrate (NO_3^-) are often used as additives for meat products. The principal function of these salts is to preserve food, as they provide highly effective protection against the food poisoning microorganisms [1–3]; they also give cured meats their characteristic colour and organoleptic properties [1]. Nevertheless, NO_2^- may react with amines to form carcinogenic nitrosamines in vivo and, consequently, it has become increasingly important to measure NO_3^- and NO_2^- levels in meat products [2]. Moreover, NO_2^- and NO_3^- are common environmental contaminants, thus it is crucial to monitor these compounds in surrounding samples such as water, soil, air and other environmental compartments, as indices of organic and inorganic contamination [4,5]. In recent years it has become of interest to measure

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NO_2^- and NO_3^- in biological samples. Analysis of NO_2^- and NO_3^- derived from the metabolism of nitric oxide (NO) represents a proven strategy for determining NO participation in a wide range of physiological and physio-pathological processes, such as smooth muscle relaxation, platelet inhibition, immune regulation, neurotransmission, atherosclerosis, arterial hypertension, hypercholesterolemia and *diabetes mellitus* [6,7]. Nitric oxide is formed in small amounts in vivo and it is rapidly oxidized to NO_2^- and NO_3^- [8], making the direct measurement of NO difficult. Thus, one of the approaches in studying the metabolism of NO in tissues is the evaluation of NO_2^-/NO_3^- in biological fluids. A number of methods are available for measuring NO_2^- and NO_3^- both in biological fluids and in food or environmental samples. The majority of the methods developed for the determination of NO_2^- and NO_3^- involve spectrophotometric procedures, such as the traditional Griess method [9–12]. However, these procedures are often time-consuming, highly prone to interferences, and can be unreliable for some food and biological samples, due to the difficulty in obtaining a clear solution for final measurement, or to the abundant presence of chloride ions, especially in biological samples [5,13–15]. In addition, the sensitivity of these methods for NO_2^- is relatively poor and trace levels of these ions are often undetectable [13]. The use of high-performance liquid chromatography (HPLC) is potentially very attractive, as it is much more rapid, sensitive and selective than methods based on reduction/colorimetry [23,27,29]. During the last few years, several analytical procedures for the separation and detection of anions by ion-exchange or ion-pair reversed-phase HPLC have been developed, including anion detection by conductimetry, indirect photometry, UV absorption, fluorometry, chemiluminescence or electrochemical detection (ED) [14–23]. Many interferences are effectively eliminated especially with UV or ED; moreover, amperometric detection tends to be selective and very sensitive [21–23].

Since there are few reports on the electrochemical detection of NO_2^- and NO_3^- by HPLC, in this brief review we describe several methods that use HPLC with ED as a more sensitive, selective and faster procedure than classical ones.

2. Methods

ED methods used in HPLC include the following: conductimetric, direct current (d.c.) amperometric, pulsed and integrated amperometric, and potentiometric [24]. In this review, we illustrate ED methods for analysis of NO_2^- and NO_3^- by HPLC, with particular attention to amperometric detection. Amperometry is selective for oxidizable anions, thus it is available for the detection of NO_2^- and NO_3^- ions. Conductimetry is universal for ionic substances, the detectors are simple and inexpensive. They exhibit a rapid response and a wide linear dynamic range and are reasonably sensitive, provided that the conductivities of the analyte and the mobile phase are sufficiently different. However, conductimetric detection is inferior in sensitivity to the other detection modes [25]. Potentiometric, amperometric and coulometric detectors are selective and highly sensitive. Amperometric and coulometric detectors are more suitable, because of their sensitivity. They have a wide linear dynamic range, rapid response and are readily miniaturized to match micropacked and capillary columns, while potentiometric detection has the disadvantage of a slower response time and a less stable baseline [24,25].

2.1. Determination of NO_2^- and NO_3^- in food and environmental samples

Lookabaugh and Krull [26] have applied ion-pair reversed-phase HPLC and the use of a PTFE knitted open tubular (KOT) reactor, which when wrapped around a UV source, provides a means of continuous, on-line photolysis. While NO_3^- is electrochemically unreactive under conditions used herein, an oxidative response could be photolytically induced from NO_3^-. This photolytic step elicits a photoreductive generation of NO_2^- from NO_3^- that reaches its maximum at pH 7 and at a flow-rate of 0.8 ml/min. Then, this derivatization step, combined with HPLC, permits the determination of both NO_2^- and NO_3^- by using oxidative amperometric detection with a number of samples (cured meats, smoked and fresh salmon, smoked cod, spiked water solutions) (Table 1). The chromatographic column used was an Econosil C_{18} reversed-phase column, 250 mm×4.6 mm I.D. Pre-

Table 1
Determination of nitrite in food and environmental samples by HPLC–ED

Sample	Column	Eluent (flow)	Electrode (voltage)	Detection limit	Ref.
Fish and cured meats	Econosil C_{18} reversed-phase (250×4.6 mm)	Methanol–phosphate buffer: 0.025 *M* KH_2PO_4 0.025 *M* Na_2HPO_4 5 m*M* tetrabutylammonium hydrogensulfate, pH 6.8 (0.8 ml/min)	Dual glassy carbon (1.1 and 1 V)	50 ppb	[26]
Meat products	Anion-exclusion/HS (100×4.6 mm)	5 m*M* sulfuric acid (0.8 ml/min)	Pt or glassy carbon (1 V)	1 ppb	[27]
Water and environmental samples	Anion-exclusion/HS (100×4.6 mm)	5 m*M* sulfuric acid (0.6 ml/min)	Pt (1 V)	0.1 ppb (water) 0–1.5 ppm (soil)	[28]
Meat	Poly(styrene–divinylbenzene) (PRP-1)	17.5% acetonitrile 82.5% aqueous (1 g/l) *tert*.-Bu_4NNO_3 (1 ml/min)	Glassy carbon (0.9 V)	0.6 ng/30 μl	[29]
Sea-water	Dionex AS4A (250×4 mm)	20 m*M* NaCl (2 ml/min)	Glassy carbon (1 V)	0.35 ppm	[5]

All potentials are quoted to the Ag/AgCl reference electrode.

liminary separations of NO_2^- and NO_3^- were carried out using a mobile phase consisting of 5 m*M* tetrabutylammonium hydrogensulfate (TBAHS) dissolved in a methanol–phosphate (10:90) buffer. Authentic mixtures (aqueous solutions) and a number of samples of smoked or fresh fish (salmon, cod), as well as cured meat products (beef/pork frankfurter, turkey bologna) were analyzed. Hydrodynamic voltammograms of NO_2^- indicated that an oxidative response is produced at a potential of 0.8 V (vs. Ag/AgCl) on a glassy carbon surface; for analytical use, working potentials of 1.1 V (W1) and 1.0 V (W2) were chosen. The linearity of response for NO_2^- was evaluated using UV detection, ED without photolysis, and ED with photolysis. In the case of NO_3^-, UV detection and ED with photolysis were used. ED was carried out using parallel dual electrodes maintained at 1.1 V and 1.0 V, respectively. This was done because by maintaining the electrodes at a voltage differential of 100–150 mV, it is possible to obtain current response ratios that will ideally lend an additional degree of sensitivity for the electrochemical detection scheme, since they should be characteristic of a particular analyte. For each analyte, linear responses were obtained for all detection schemes. A minimum detection level of 50 ppb was conservatively proposed for each analyte using either ED or UV detection. Solutions as low as 5–10 ppb yielded response, but the signals began to deviate from linearity. The accuracy of the analytical methodology was initially assessed through the use of authentic aqueous solutions of $NaNO_2$ and $NaNO_3$, essentially using simultaneous UV detection and post-column photolytic ED. Kim and Conca [27] used HPLC with ED which offers high specificity because only selected compounds are oxidized at the typical operating potential of an amperometric detector. The ion chromatography system was equipped with an anion-exclusion/HS column plus an anion-exclusion Ion-Guard cartridge and a Model 271 electrochemical detector with Pt working electrode, or a Wescan anion-exclusion Ion-Guard cartridge and HS column connected to a Waters Model 460 electrochemical detector with glassy carbon electrode

(Table 1). Detector voltage for either the Pt or glassy carbon electrode was set to 1.0 V vs. Ag/AgCl reference electrode. Kim and Kim [28] applied ion-exclusion chromatography with ED for the analysis of drinking water, rain and lake water and soil samples to eliminate interferences from a complex mixture containing excess amounts of other common ions that could interfere with NO_2^-. Using an anion-exclusion/HS column coupled to an electrochemical detector with a Pt working electrode, and Ag/AgCl reference electrode with sulfuric acid 5 m*M* as eluent, they obtained a hydrodynamic voltammogram between 0.6 and 1.2 V, in which the maximum sensitivity was achieved at 1.0 V (Table 1). To demonstrate the selectivity of the ion-exclusion chromatographic separation and ED for NO_2^-, they analyzed solutions containing chloride, nitrate, sulfate, phosphate, carbonate, bromide, fluoride and nitrite, with the detector voltage set to 1.0 V. Since most common anions are not oxidized at the same potential, and some anions are not detected by ED at 1.0 V, they obtained chromatograms in which none of the common anions interfered with NO_2^-. Thus, they used these conditions to analyze some environmental and soil samples, achieving high selectivity and sensitivity. Newbery and de Haddad [29] used an Hamilton PRP-1 poly(styrene–divinylbenzene) copolymeric column and a mobile phase of 17.5% acetonitrile and aqueous *tert*.-Bu_4NNO_3 with a glassy carbon electrode set at 0.9 V for the determination of NO_2^- in aqueous solutions containing a range of other species (Table 1). No interferences were assessed by injecting samples of test ions such as Na^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, SO_4^{2-}, NH_4^+, Mn^{2+}, Fe^{2+}, Pb^{2+}, Cl^-, I^-, PO_3^{2-}, SO_3^{2-}, ascorbate and others. Pastore et al. [5] obtained good results by using HPLC with amperometric detection for the analysis of sea-water samples, in the presence of a large excess of chloride (Table 1). Using a 250 mm×4.0 mm AS4A column in conjunction with a AG4A guard column and an amperometric detector with a glassy carbon working electrode, a Pt counter electrode, and an Ag/AgCl/saturated KCl reference electrode, set to 1.0 V, they demonstrated that it is possible to detect small concentrations of NO_2^- in the presence of a large amount of chloride, with a limiting ratio of about 715 000/1 Cl^-/NO_2^-, without difficulty.

2.2. Determination of NO_2^- and NO_3^- in biological samples

Gorbunov and Esposito [23] have developed a rapid and simple method for determination of NO_2^- from rat cerebellar synaptosomes as a tool for studying NO metabolism. They used ED to measure NO_2^- concentrations in the supernatant of synaptosomal preparation. The separation of NO_2^- was obtained by using a reversed-phase co-poly(styrene–divinylbenzene) (PLRP-S) equipped with a LiChrosorb RP-8 guard column, connected to a Model 5100A Coulochem detector with a Model 5010 analytical cell with the potential of the porous graphite electrode maintained at 0.4 V, relative to a H_2/H^+ ion couple reference electrode (Table 2). The mobile phase was as follows: sodium dihydrogenphosphate solution (5 m*M*, pH 3.3±0.03) containing 0.15% 1-heptanesulphonic acid and 0.1% acetonitrile at a flow-rate of 1 ml/min pumped at room temperature. A pH over 4 was chosen to prevent S-nitrosothiols formation for NO_2^- binding to the thiol groups of proteins [23]. To activate NO production in synaptosomes, the membranes were incubated in Krebs–Ringer solution (pH 7.4) for 1 h in the presence of 0.5 m*M* NADPH, 100 μl L-arginine and 100 μ*M* NMDA, then the reaction was stopped by adding a concentrated solution of sodium hydroxide up to a final pH of 9.0, which is optimal for hydrolysing S-nitrosothiols. After precipitation and separation of proteins by centrifugation at 12 000 *g* for 30 min at 4°C from, aliquots of supernatant (20 μl) were injected into the chromatograph for analysis. Standard solutions of $NaNO_2^-$ gave an optimum linear response over the concentration range of 1.5–30 μ*M*, the correlation coefficient being 0.999. Also Kaku et al. [22] have used HPLC–ED to measure NO synthase activity (Table 2). This method was highly sensitive and elicited a steep peak of NO_2^- without any influence from other ED-detectable substances in the enzyme preparation. After homogenization of cerebella, supernatant fraction was obtained by centrifugation and dialysed against homogenizing buffer to remove endogenous L-arginine. First, they tried to detect authentic NO_2^- using a 100 m*M* phosphate buffer (pH 7.4) mobile phase eluted through an ODS column coupled to a glassy carbon electrode set at 0.75 V. The peak area

Table 2
Determination of nitrite in biological samples by HPLC–ED

Sample	Column	Eluent (flow)	Electrode (voltage)	Detection limit	Ref.
Cerebellar synaptosomes	Co-poly(styrene–divinylbenzene) reversed-phase (PLRP-S) (150×4.6 mm)	5 m*M* NaH_2PO_4, pH 3.3 0.15% 1-heptanesulphonic acid 0.1% acetonitrile (1 ml/min)	Porous graphite (0.4 V) (H_2/H^+ ion couple reference electrode)	n.d.	[23]
Cerebellar cytosol	Silica based anion-exchange (TSK-GEL IC-Anion SW) (50×4.6 mm)	10 m*M* NH_4Cl 30% methanol (1 ml/min)	Glassy carbon (0.9 V) (Ag/AgCl reference electrode)	5 p*M*	[22]
Human blood	Anion-exchange LC A08	40 m*M*/l NaCl acetonitrile–methanol–water (70:10:20, v/v) (1 ml/min)	Glassy carbon (0.7 V) (reference electrode not specified)	100 n*M*/l	[30]

n.d.=Not determined.

of NO_2^- increased linearly with voltage, reaching a plateau at 0.85 V. In order to asses the usefulness of this HPLC–ED method for the quantitative determination of NO_2^- in the measurement of NO synthase activity, the production of NO_2^- from L-arginine was examined, and the results obtained were compared with those obtained by the diazo-coupling method using Greiss reagent and the isotopic method, in which [^{14}C]-L-arginine was used as the substrate and [^{14}C]-L-citrulline measured as the product. Since it is very difficult to detect NO_2^- in blood because of its rapid metabolism within erythrocytes, Preik-Steinhoff and Kelm [30] have recently developed a method to prevent NO_2^- degradation during sample preparation which allows a sensitive measurement of NO_2^- by high-performance anion-exchange chromatography coupled with ED (Table 2). A 1.5 ml sample of human blood was added to an equal volume of 0.1 *M*/l sodium hydroxide solution (stop solution), after that the mixture's pH was set to 7.0 with a standard volume of 1 *M*/l phosphoric acid. Next, the mixture was centrifuged at 14 000 *g* at room temperature for 5 min. The supernatant was ultrafiltered with Centrisart cut-off 10 000 for 15 min at 2000 *g* and 20°C. The ultrafiltrate was either injected into the HPLC system or further diluted depending on co-eluting oxidizable contaminations. Using the described sample preparation, NO_2^- and NO_3^- in human blood were clearly separated by an anion-exchange analytical column kept at a constant temperature of 20°C. The analytical column was connected to a UV detector set at 220 nm for detection of NO_3^- and to an electrochemical detector ESA Coulochem 5200A for detection of NO_2^- with an amperometric analytical cell 5040 maintained at 0.7 V with a range of 1 or 5 nA gain depending on the sensitivity needed, and flowed with a mobile phase consisting of acetonitrile–methanol–water (70:10:20, v/v) and 40 m*M*/l NaCl in the final solution. In these conditions a linear relation between the peak area and the concentration of standard $NaNO_2$ and $NaNO_3$ was obtained in the range of 100–1500 n*M*/l and 5–50 μ*M*/l, respectively. Since the major problem in blood sample preparation is the rapid metabolism of NO to NO_2^- and NO_3^-, protein contamination, and the presence of chloride and bromide ions that interfere with the ED or UV signal, this methodology gave good response with an extraordinarily low detection limit of 3 n*M*/l in aqueous solution and 60 n*M*/l in human blood which is below the concentrations expected under normal or pathological conditions in vivo.

3. Conclusions

One of the most tedious problems in measuring NO_2^- and NO_3^- in food or biological and environmental samples are the matrix interferences that may hamper the determination of the substances of interest. Usually, two of the simplest and easiest sample preparation methods namely dilution or filtration, are not suitable with some samples. Especially in the case of NO_2^- and NO_3^-, filtration or dilution are often inapplicable because of loss, dilution or alteration of analytes in the samples containing small quantities of these ions, or due to the inappropriate use of membrane filters such as those manufactured from mixed esters of cellulose acetate and nitrate, or other filters from which NO_3^- is leached during the filtration process. Handling of the sample may sometimes introduce imprecisions, and the success of the analysis is often dependent on successful sample preparation. In some cases, an optional solid-phase extraction clean-up step is necessary, but it is difficult to achieve reproducible recoveries of each analyte. In the case of NO_2^-, sample manipulations need to be kept to a minimum, due to the instability of this ion that oxidizes to nitrous acid and/or NO_3^-. Classical colorimetric methods often require long time extraction, and interferences in cured meats, caused primarily by added reducing compounds, are not completely eliminated by long time extraction or the high temperature required to oxidize these compounds; in addition, these reducing compounds will interfere with colour formation during the diazotisation method for NO_2^- analysis. Interferences by most inorganic anions are eliminated by UV detection, but NO_3^- or chloride ions could still interfere if they are present in large quantities and UV detection might also introduce interferences by some organic contaminants. In UV detection there are often broad negative (or positive) peaks due to the chloride ion eluted before NO_2^-, and to eliminate this problem, a membrane suppressor is required. Thus, membrane suppressor strongly reduces broad negative peaks due to the chloride ion eluted before NO_2^-. Amperometric and coulometric detectors are selective and highly sensitive, and have a wide linear dynamic range and rapid response. The selectivity of amperometric detection is due to their capability to oxidize only selected compounds, at the typical operating potential of an amperometric detector (between 0.4 and 1.2 V), thus ion-exchange or reversed-phase chromatography coupled with amperometric detection can very well be utilized in the analyses of complex samples to determine NO_2^- and NO_3^- which is indirectly detected by means of the photolytic step. When using an electrochemical (amperometric) detector, it is usually necessary to operate at an applied voltage which gives an acceptable response from materials of interest without generating an high standing current from the eluent. A particularly interesting value of ED in providing selectivity and sensitivity is that when it is applied to the examination of body fluids, it indeed simplifies the chromatogram when there are several unresolved UV-absorbing species or negative peaks due to the presence of chloride. Furthermore HPLC–ED systems can be used to measure NO synthase activity and can be applied to study the effect of compounds on the enzyme activity in various tissues and cells. However, it is necessary to periodically wash the electrode to maintain a constant high sensitivity. In conclusion, ion-exchange chromatography is ideal for separation of NO_2^- from other weak acids as well as from strong acid anions. Amperometric detection offers further selectivity due to the fact that the standard reduction/oxidation potentials vary among different analytes. The combination of these two selective processes yields an extremely high overall specificity.

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