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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NITRITE IN ENVIRONMENTAL SAMPLES BY THE USE OF HYDRALAZINE

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SUMMARY

A high-performance liquid chromatographic (HPLC) procedure with UV and fluorometric detectors has been developed for the determination of nitrite by use of hydralazine. Hydralazine reacts with nitrite ion under acidic conditions at 37°C to form tetrazolo[5,1-*a*]phthalazine (Tetra-P) almost quantitatively. Without extraction, the determination of Tetra-P by reversed-phase HPLC was simple, specific, sensitive and reliable over the range 0.001–0.10 ppm of nitrite nitrogen. This procedure using hydralazine is one of the most useful methods for routine analysis of nitrites in foods, biological fluids and ambient waters.

INTRODUCTION

In previous papers[1–3] we reported that hydralazine (1-hydrazinophthalazine, HP), an effective depressor of hypertension, was transformed in human saliva to an acetylated product, 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP), together with a formylated one, *s*-triazolo[3,4-*a*]phthalazine (Tri-P). On the other hand, we also found that HP was nitrosated very easily to give tetrazolo[5,1-*a*]phthalazine (Tetra-P) under acidic conditions with nitrite ion, which is contained in human saliva. The Tetra-P formation from HP and sodium nitrite *in vitro* was very rapid in acidic buffer solutions (pH 1.1, 2.1 and 3.0) at 37°C[1–3], suggesting that appropriate choice of conditions might afford Tetra-P quantitatively in preference to MTP and Tri-P. Therefore, we developed a sensitive, accurate and reliable high-performance liquid chromatographic (HPLC) method which employs HP for the determination of nitrite ion in aqueous solutions; a part of this work has already been reported[4].

For nitrite analysis, colorimetric methods using sulphanilic acid[5] or sulphanilamide[6] have generally been used. Recently, gas chromatographic (GC) methods using *o*-phenylenediamine[7,8] or aromatic primary amines for the Sandmeyer reaction[9,10],

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and HPLC of nitrite ion itself without any derivatization[11–13], were reported. The colorimetric methods possess a lack of specificity which is crucially important for microanalysis because the sample is often turbid and slightly coloured. The GC methods generally require complex pre-treatments, and the methods using HPLC for nitrite itself have unsatisfactory detection limits.

In this paper we describe the usefulness of the HPLC method developed by us[4] for the determination of nitrite ion in environmental samples, namely in biological fluids, foods and ambient waters. We have succeeded in the detection of Tetra-P excreted in urine from a patient treated with HP, as has been reported in detail elsewhere[14].

EXPERIMENTAL

Chemicals

Reagent grade HP–HCl was purchased from Tokyo Chemical and reagent grade sodium nitrite from Wako. Tetra-P, MTP and Tri-P were prepared by known methods[15]. Other chemicals used were of reagent grade.

Apparatus and HPLC conditions

The liquid chromatograph (Toyo Soda, Tokyo, Japan) was equipped with an HLC-803A system, UV detector (UV-8), fluorometric (FL) detector (FS-970) and TSK GEL LS-410 ODS column (15 cm × 4 mm I.D.). The mobile phase (pH *ca.* 4.5) was 20% acetonitrile in 0.05 *M* KH_2PO_4. The solvent was degassed by ultrasonication before use. The flow-rate was 1.0 ml/min. The column eluate was monitored at 228 nm by the UV detector (0.02 a.u.f.s.), and at 228 nm (excitation) and above 340 nm or 370 nm (emission) by the FL detector (1.0 μA f.s.). The intensities were recorded by means of a two-pen recorder (Rikadenki Kogyo) and a Chromatopak C-RIA chromatography integrator (Shimadzu Seisakusho).

Sample preparation

A 200-ml solution of the food extracts was prepared from 10 g of food samples according to the procedure described in ref. 6. A 5-ml sample solution was prepared from 0.5 ml of human saliva or rat plasma according to the method of Shechter *et al.*[16].

Ambient waters were passed through a filter-paper, if necessary.

Standard nitrite solution

Dried sodium nitrite (197 mg) was dissolved in distilled water followed by dilution to 200 ml (the stock solution). A 2.0-ml volume of this stock solution was diluted with water to 200 ml, from which 5.0 ml were transferred to a 100-ml volumetric flask and diluted to volume with water (the work solution). The work solution was prepared just before the experiment. A 1-ml volume of the standard work solution contains 0.1 μg nitrite nitrogen (NO_2^--N) or 0.328 μg nitrite ion (NO_2^-).

Determination of nitrite

A 200-μl volume of HP–HCl dissolved in 1 *N* HCl (1 mg/ml) was added to 2.0 ml of the nitrite sample solution, the concentration of which was less than 0.10 ppm

NO_2^--N. After adjusting the pH to about 1.1, the mixture was incubated at 37°C for 30 min. A methanolic solution (100 μl) of phenobarbital (PB) (200 μg/ml) was added to the reaction mixture as an internal standard (IS), and 20-μl aliquots were injected directly for HPLC.

Calculations

The concentrations of the sample solutions were determined by interpolation from a calibration curve, which was constructed by plotting the peak area ratios of Tetra-P to PB (IS) *versus* the concentrations of NO_2^--N (0.001–0.10 ppm).

RESULTS AND DISCUSSION

Tetra-P formation from HP and nitrite

In previous papers [1–4], we reported that HP reacts with nitrite under acidic conditions at 37°C to form Tetra-P almost quantitatively. We now describe this reaction in more detail.

Effect of HP–HCl concentration. The effect of the HP–HCl concentration was examined in the range of 0.04–5.0 mg/ml using a 0.06 ppm nitrite-nitrogen solution (Fig. 1). The yields of Tetra-P were constant at HP–HCl concentrations above 0.2 mg/ml in the aqueous solutions. At concentrations above 5.0 mg/ml HP–HCl, broad tailing of the peaks due to the presence of the excess of HP–HCl made it difficult to determine the amounts of Tetra-P. Therefore, 1.0 mg/ml HP–HCl was employed for subsequent experiments.

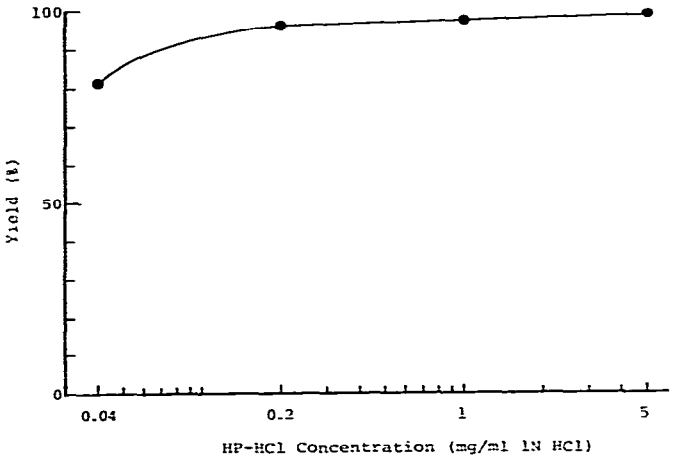

Fig. 1. Effect of HP–HCl concentration on the formation of Tetra-P. A 200-μl portion of HP–HCl dissolved in 1 *N* HCl was added to 2.0 ml of the nitrite aqueous solution (0.06 ppm NO_2^--N), and the mixture was incubated at 37°C for 30 min.

Effect of pH. The effect of pH was examined over the pH range 1.0–5.0 (Fig. 2). The test solutions were prepared as follows. Each 200-μl portion of HP–HCl aqueous solution (1 mg/ml) was added to 2-ml aliquots of 0.06 ppm NO_2^--N solution, which was prepared by mixing nitrite with 0.05 *M* phosphate buffer solutions (pH 1.5–5.0),

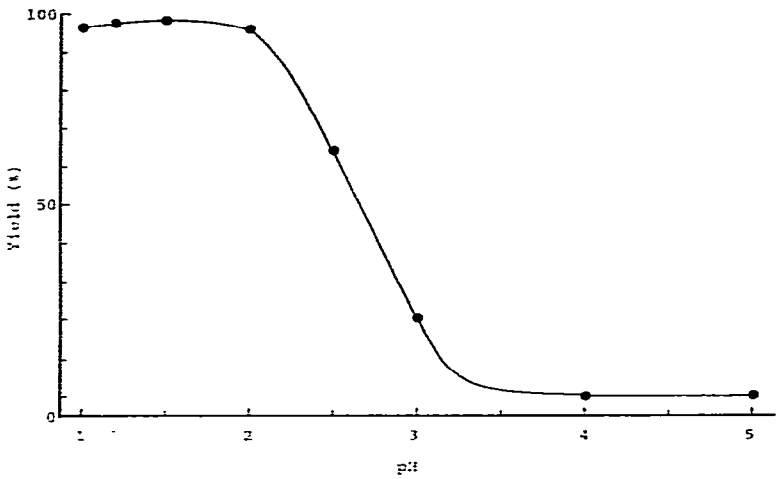

Fig. 2. Effect of pH of the reaction mixture on the formation of Tetra-P. A 200-μl portion of HP-HCl aqueous solution (1 mg/ml) was added to 2.0 ml of the nitrite buffer solution (0.06 ppm NO_2^--N), and the mixture was incubated at 37°C for 30 min.

0.1 *N* HCl or 0.25 *N* HCl. Constant yields of Tetra-P were obtained in the range of pH 1.0–2.0. Therefore, pH *ca.* 1.1 was adopted.

Effects of temperature and time. The effect of temperature was investigated in the range of 0–50°C. The rates and amounts of Tetra-P formation are dependent on the reaction temperature and the time (Fig. 3). When the reactants were incubated at 37°C or 50°C, a maximal yield was reached after about 15 min in each case. At room temperature (22°C), yields of Tetra-P of higher than 90% were detected after about 45 min. When the determination was performed on extracts from foods and biological fluids, the formation of undesirable by-products, MTP and Tri-P, increased with increasing temperature and time. Therefore, a procedure at 37°C for 30 min was

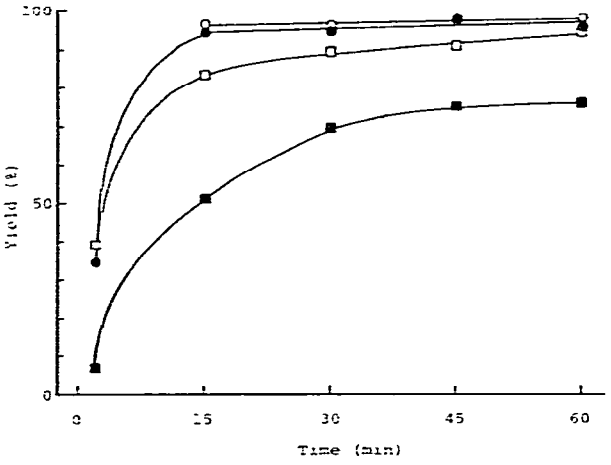

Fig. 3. Time course of Tetra-P formation at 50°C (○), 37°C (●), room temperature (22°C) (□) and in an ice-bath (■).

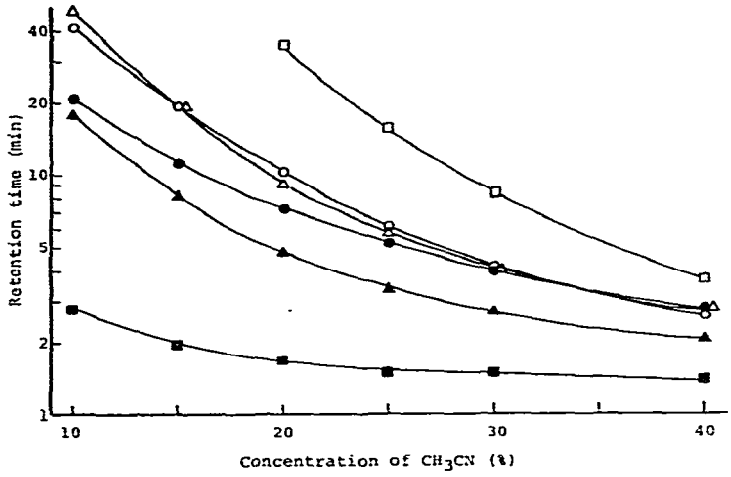

Fig. 4. Effect of acetonitrile concentration on the retention times of PHT (□), PB (○), Tetra-P (●), HP (■), MTP (△) and Tri-P (▲), respectively. HPLC conditions: column, TSK-GEL LS-410 ODS, 15 cm × 4 mm I.D.; mobile phase, 10–40% acetonitrile in 0.05 *M* KH_2PO_4; flow-rate, 1.0 ml/min.

adopted in the subsequent experiments. In order to prevent further side reactions, the reaction mixture was stored in an ice-bath until required for HPLC.

Effects of mobile phase-pH and acetonitrile concentration on the separation and detection of Tetra-P

A reversed-phase column packed with TSK GEL LS-410 ODS was used. Increasing the concentration of acetonitrile in phosphate buffer solution shortened the retention time of Tetra-P. When the concentration was greater than 25%, however, it became difficult to separate Tetra-P from the two by-products, MTP and Tri-P (Fig. 4).

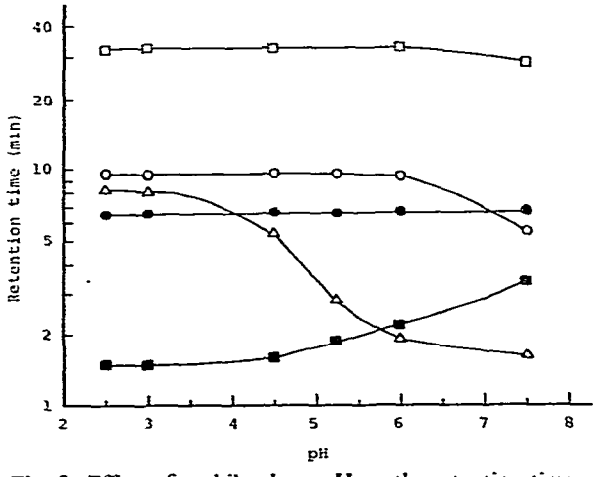

Fig. 5. Effect of mobile phase pH on the retention times of PHT (□), PB (○), Tetra-P (●), HP (■) and sorbic acid (△). HPLC conditions: column, TSK-GEL LS-410 ODS, 15 cm × 4 mm I.D.; mobile phase, 20% acetonitrile in 0.05 *M* phosphate buffer (pH 2.5–7.5); flow-rate, 1.0 ml/min.

In the range of pH 2.5–7.5 in the mobile phase, the retention time of Tetra-P is constant, but below pH 3.0 a large peak due to sorbic acid, a food preservative, overlaps that of Tetra-P (Fig. 5). A satisfactory result was obtained when the reaction mixture was eluted with 20% acetonitrile in phosphate buffer (pH 4.5–5.25).

PB was adopted as IS instead of phenytoin (PHT), used in our previous work[4], because the retention time of PHT was too long under the elution conditions.

The UV absorption spectrum of Tetra-P in 0.05 *M* KH_2PO_4 is given in Fig. 6. UV and FL monitoring of Tetra-P was not only very sensitive but also effective for the identification of Tetra-P, MTP and Tri-P. The detection limits of Tetra-P by UV and FL detection were 0.25 and 0.06 ppb NO_2^--N in aqueous solution (signal-to-noise ratio = 5).

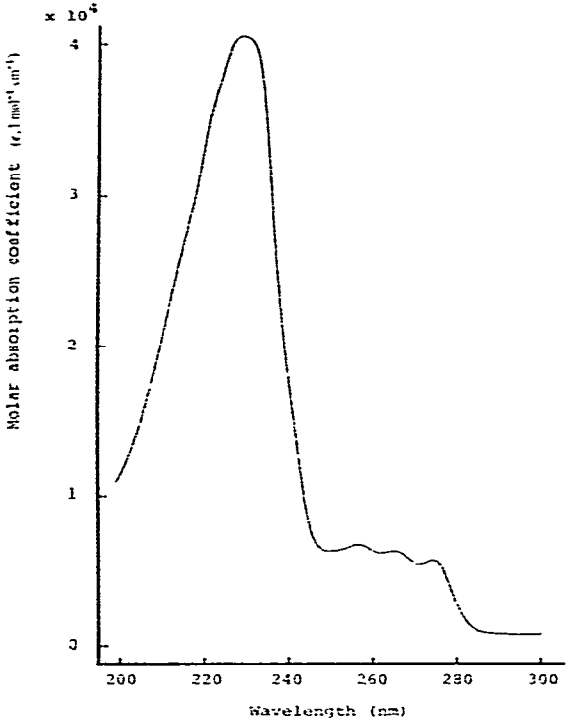

Fig. 6. UV spectrum of Tetra-P in 0.05 *M* KH_2PO_4.

Fig. 7 shows HPLC chromatograms of a mixture of authentic samples of Tetra-P, MTP and Tri-P, and of the reaction mixtures in water and in fish sausage extract.

Calibration curve

A linear relationship between the peak area ratio of Tetra-P to PB and the concentrations of nitrite-nitrogen was obtained (Fig. 8). The reproducibility of the method was examined by analyzing samples whose concentrations of NO_2^--N were 0.001, 0.01 and 0.10 ppm (N = 5). The corresponding coefficients of variation were 15.8, 4.2 and 0.9%, respectively.

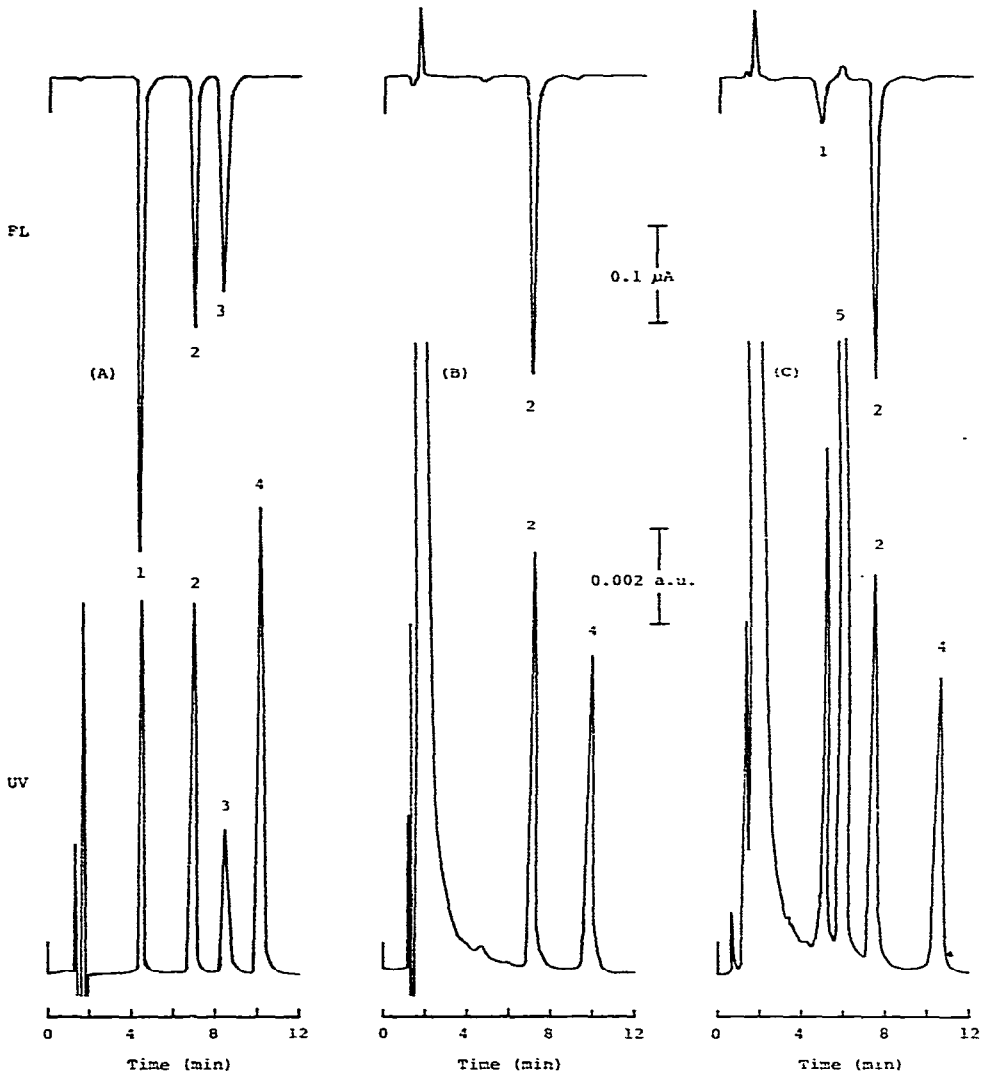

Fig. 7. HPLC chromatograms of authentic samples (A), and of the reaction mixture of HP–HCl and nitrite in water (B) or in fish sausage extract (C), each being spiked with 0.06 ppm NO_2^--N before the reaction. Peaks: 1 = Tri-P; 2 = Tetra-P; 3 = MTP; 4 = PB (internal standard); 5 = sorbic acid. HPLC conditions: column, TSK-GEL LS-410 ODS, 15 cm × 4 mm I.D.; mobile phase, 20% acetonitrile in 0.05 *M* KH_2PO_4; flow-rate, 1.0 ml/min. Detection, UV 228 nm, 0.02 a.u.f.s. (bottom); fluorescence, excitation = 228 nm, emission >370 nm, 1.0 μA f.s. (top).

Application to the determination of environmental nitrites

The results of the determinations of nitrites in foods, biological fluids and river-water are given in Table I. The recoveries were calculated from the yields of Tetra-P based on the addition of an extra 0.06 ppm NO_2^--N to each sample solution. The fish sausage extract contained very small amounts of nitrite although it was coloured pink. In such a case, this HPLC method is very favourable for microanalysis as regards specificity and reliability.

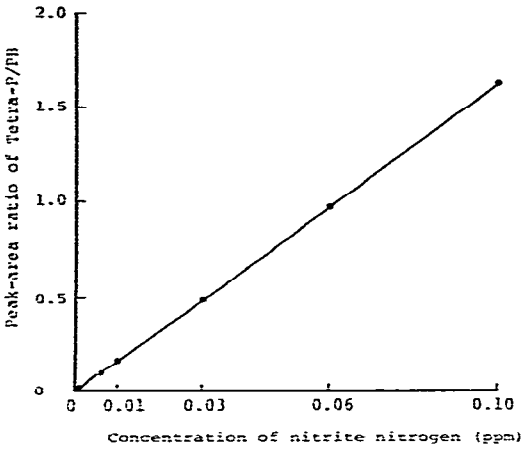

Fig. 5. Calibration curve for nitrite. Details as in the text.

After we had reported a brief summary of our method[4], an alternative procedure which was based on the same reaction but using GC was described by Tanaka and co-workers[17–19]. Their method gave very satisfactory results, that is, very high recoveries of nitrites in foods, milk and blood, and excellent detection limits (0.02 ppm, GC-FID; 0.3–4.0 ppb, GC-ECD). The sensitivity of the GC-ECD method is comparable to that of our method. However, the GC method requires more complex pre-treatments —extraction, drying, evaporation and alumina column chromato-

TABLE I

ANALYTICAL RESULTS OBTAINED BY THE PROPOSED METHOD

Sample	NO_2^--*N added (ppm)*	*Found*			*Mean (ppm)*	*Recovery (%)*
		ppm	*ppm*	*ppm*		
Fish sausage*	0	0.02	0.02	0.02	0.02	
	1.2	1.20	1.17	1.17	1.18	97
Fish ham*	0	0.41	0.40	0.40	0.40	
	1.2	1.48	1.46	1.47	1.47	89
Wiener*	0	1.44	1.23	1.32	1.33	
	1.2	2.43	2.39	2.39	2.40	89
Tomato*	0	0.02	0.02	0.04	0.03	
	1.2	1.00	1.00	0.99	1.00	81
Spinach*	0	0.08	0.06	0.08	0.07	
	1.2	1.06	1.13	1.05	1.08	84
Saliva (human)**	0	1.55	1.56	1.57	1.56	
	0.6	2.15	2.15	2.15	2.15	98
Plasma (rat)**	0	0.02	0.01	0.01	0.01	
	0.6	0.60	0.59	0.60	0.60	98
River-water	0	0.004	0.004	0.004	0.004	
	0.06	0.065	0.065	0.066	0.065	102

* A 200-ml sample solution was prepared from 10 g of a sample.

** A 5-ml sample solution was prepared from 0.5 ml of a sample.

graphy as clean-up— all of which is unnecessary in our HPLC procedure. The most striking characteristics of the HPLC method are the high sensitivity and the simple procedure. In conclusion, the described method is considered to be one of the most useful methods for routine analysis of nitrites in environmental samples.

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