Photolytic Interface for High-performance Liquid Chromatography - Chemiluminescence Detection of Non-volatile *N*-Nitroso Compounds

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A photolytic interface between high-performance liquid chromatography (HPLC) and a chemiluminescence detector has been developed for the trace detection of non-volatile *N*-nitroso compounds in biological matrices. A chromatographic effluent containing separated *N*-nitrosoamino acids and *N*-nitrosamides is introduced into a glass coil with a purge stream of He and irradiated with ultraviolet light. Nitrogen oxide, cleaved by photolysis, is separated rapidly from the solvent through a series of cold traps and carried by the He into the reaction chamber of a chemiluminescence detector. The method is compatible with most types of HPLC, especially reversed-phase, and yields low-nanogram sensitivity for underivatised *N*-nitrosoamino acids and *N*-nitrosamides. The detection of a model *N*-nitrosamide, trimethylnitrosourea, in spiked porcine gastric fluid (42 μg l^{-1}), and of *N*-nitrosoproline and *N*-nitroso-1,3-thiazolidine-4-carboxylic acid, in spiked human urine (7–8 μg l^{-1}), is demonstrated.

Keywords: N-*Nitroso compounds; high-performance liquid chromatography - thermal energy analysis; N-nitrosamides*

The majority of over 300 *N*-nitroso compounds tested for carcinogenicity in laboratory animals have been positive.[1] Humans are exposed to *N*-nitroso compounds from a variety of sources including food, occupational environments, cosmetics and formation within the body.[2,3] Certain *N*-nitrosamides are also widely used as therapeutic anticancer agents.[4] For these reasons, there is considerable interest in the analysis of trace levels of these compounds in biological and environmental media.

Many *N*-nitrosamines can be analysed, either directly or after derivatisation, by gas chromatography coupled to a chemiluminescence detector and a commercial instrument, the Thermal Energy Analyser Detector (TEA),[5] has been introduced. The TEA is a modified chemiluminescence detector which relies on thermal cleavage of the N–N bond to produce a nitrogen oxide (NO) radical. The nitrogen oxide is reacted with ozone to produce excited nitrogen dioxide, which emits a photon on decay.[6] The photons are detected and amplified by a photomultiplier tube. There are two limitations to this system: first, the *N*-nitroso compounds must be volatile enough, or made volatile enough for gas chromatography, and secondly, they must yield nitrogen oxide on thermolysis. *N*-Nitrosamides and related compounds, unlike *N*-nitrosamines, typically rearrange on thermolysis to yield molecular nitrogen instead of nitrogen oxide and are only weakly detected by the TEA.[7] In addition, several *N*-nitrosamines that are of interest are not suitable for gas chromatography. High-performance liquid chromatography (HPLC) - TEA methods have been reported but mobile phases containing water give inconsistent results.[8]

Shuker and Tannenbaum[9] have described a method in which *N*-nitrosamides were cleaved photolytically by UV irradiation. The resulting nitrogen oxide was oxidised to nitrite, and reacted post-column with Griess reagent to form a chromophore which was detected spectrophotometrically at 541 nm. The sensitivity was 6–100 ng as injected depending on the specific *N*-nitroso compound. Fine *et al.*[10] have modified the pyrolysis chamber in a standard TEA so that *N*-nitrosamides release nitric oxide during pyrolysis. Sensitivities [signal to noise (*S*/*N*) 3 : 1] of less than 1 ng injected were reported for standards. Complete details of the instrument were not provided and the need for further development was noted. Singer *et al.*[11] used dilute acid to cleave *N*-nitrosamides and coupled the resulting nitrite to Griess reagent. A sensitivity of 50–1000 ng was reported. Sen and Seaman[12] have also cleaved the nitroso group chemically from *N*-nitroso compounds and have detected the nitrogen oxide by chemiluminescence using a modified TEA. The large dead volume of the system caused considerable peak broadening. Detection of *N*-nitrosamides by UV,[13] mass spectrometry after derivatisation[14] and denitrosation with subsequent detection of the amide[15] have also been proposed.

None of these methods satisfied our need for selectivity, sensitivity and simplicity so that we could screen large numbers of samples for *N*-nitrosamides and non-volatile *N*-nitrosamines. We therefore undertook the development of an instrument that was capable of selectively detecting *N*-nitrosamides and *N*-nitrosamines following separation by reversed-phase HPLC. The instrument was based on the photolysis of *N*-nitroso compounds (including *N*-nitrosamides[16]) and the highly selective chemiluminescence detection of the resulting nitrogen oxide.

Experimental

Standard Compounds†

CAUTION: Nitroso compounds are potent animal carcinogens and must be handled with appropriate care. The *N*-nitrosoamino acids were synthesised by the method of Lijinsky *et al.*[17] except for MNTHZCA which was kindly donated by H. Ohshima. The *N*-nitrosamides, except for TMNU, were obtained from commercial sources and used without further purification; TMNU was synthesised by nitrosating trimethylurea (Alpha) at pH 2.5 in the presence of acetic acid. Analysis by HPLC (254 nm) gave a single peak.

Chromatography

Both ion-suppression [10 mM trifluoroacetic acid (TFA), pH < 2] and ion-pair (2 mM tetrabutylammonium dihydrogen-

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† *N*-Nitroso compound abbreviations used are as follows: HNPRO, 3-hydroxy-*N*-nitrosoproline; NSAR, *N*-nitrososarcosine; MNU, 1-methyl-1-nitrosourea; NPRO, *N*-nitrosoproline; NTHZCA, *N*-nitroso-1,3-thiazolidine-4-carboxylic acid; ENU, 1-ethyl-1-nitrosourea; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; MNTHZCA, 2-methyl-*N*-nitroso-1,3-thiazolidine-4-carboxylic acid; DMNA, dimethylnitrosoamine; TMNU, trimethylnitrosourea; NDPA, nitrosodipropylamine; and NPIP, *N*-nitrosopiperidine.

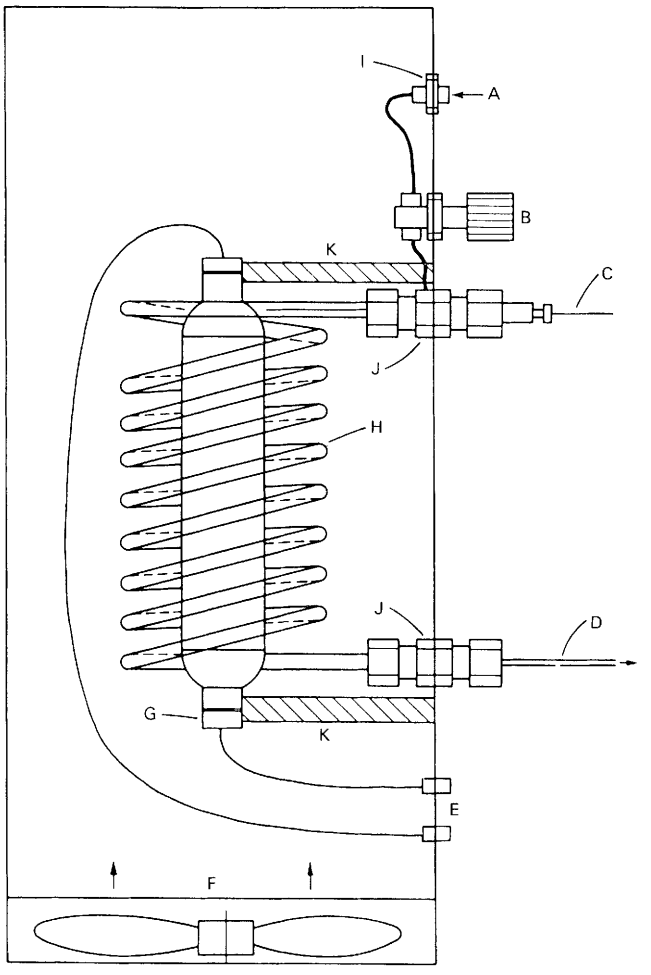

Fig. 1. Diagram of the HPLC - chemiluminescence interface. A, He inlet; B, analyser pressure adjustment (He flow-rate); C, effluent from HPLC column; D, 0.125-in stainless steel tubing from interface to traps and chemiluminescence detector; E, electrical connectors to ballast circuit; F, box fan; G, 200-W lamp; H, glass reactor coil; I, 1.59-mm bulkhead union; J, 0.64-cm bulkhead unions; K, Teflon support towers

phosphate, pH = 7) liquid chromatography were used to separate the *N*-nitroso compounds. Acetonitrile was used as an organic modifier with gradient elution. The flow-rate was 1 ml min^{-1}. Columns were a Brownlee Polymer RP (10 cm × 4.6 mm o.d., 10-μm particle size) or a self-packed Spherisorb ODS (15 cm × 4.6 mm i.d., 5-μm particle size, 12% loading). The Beckman HPLC system consisted of two 110B pumps, a 421A controller, a 210A injector and a 160 UV detector.

Photolysis Apparatus

The photolysis apparatus is shown in Fig. 1. The effluent of the chromatographic column was transferred to the photolysis coil (H) via a microbore stainless steel tube (C). The end of the stainless steel transfer line was fed into the photolysis coil approximately 3 cm after passing through a 0.64-cm Swagelok bulkhead union (J) and a laboratory-built 0.64 cm × 1.59 mm reducer. The 0.64-cm union was modified into a T by silver soldering a 1.59 mm o.d. stainless-steel tube into the side in order to mix the carrier gas (A; He) with the column effluent. The photolysis coil (H) consisted of a 3 m × 0.64 cm o.d. × 1 mm i.d. borosilicate glass tube coiled to a diameter of 6 cm and a width of 12 cm. The carrier gas was adjusted using a fine metering valve (B; Nupro, Model M-2MA) to give a total analyser pressure of 1.7–2.0 Torr (with an ozone pressure of 0.9 Torr). A 200-W mercury vapour lamp (G; Model 654A-0100, Canrad-Hanovia, Newark, NJ, powered by a 34245-101 ballasted circuit) was vertically mounted in the centre of the photolysis coil on Teflon towers (K). The entire photolysis apparatus was mounted inside an aluminium box in which a cooling fan (F) had been mounted in the bottom and several air holes had been drilled in the top. The ballast was powered through a thermal safety circuit (Model HTLC1, Cannon Instrument Company, State College, PA) in order to prevent overheating in case of failure of the cooling fan. The temperature of the air leaving the box was 30–40 °C. An optional power attenuator (PAESAR, Lutron, Cooperstown, PA) was used to vary the wattage of the ballasted circuit between 120 and 200 W.

The effluent of the photolysis coil (D) was directed to a series of cold traps by 0.32 cm o.d. stainless steel tubing. The first two traps were 100-ml round-bottomed flasks. The inlet tube was located near the neck of the first trap which was cooled by ice. The inlet in the second trap was located midway between the geometric centre and the neck of the flask. The second trap was cooled by a dry ice - acetone bath. In both cases the outlet tube was above the inlet tube. The third trap was a 0.64 cm o.d. stainless steel U-tube cooled to −159 °C with liquid nitrogen. The first and second traps were sealed with tapered Teflon stoppers fitted with O-rings.

The nitrogen oxide produced in the photolysis coil was detected using the chemiluminescence reaction chamber of a Model 543 Thermal Energy Analyser (Thermedics, Woburn, MA) by by-passing the pyrolyser and valving portions of the instrument. In some instances, the chromatographic column effluent was first directed through a UV detector (Beckman, Model 160) in series with and ahead of the photolysis coil.

Results and Discussion

N-Nitrosamines and *N*-nitrosamides absorb in the regions of 300–380 and 380–430 nm, respectively. Borosilicate glass is transparent in these regions. On photolysis the *N*-nitroso group is cleaved and nitrogen oxide liberated.[16] The nitrogen oxide is carried to the reaction chamber of the chemiluminescence detector by the helium carrier gas and reacted with ozone, thus producing the chemiluminescence reaction. It was necessary to purge the HPLC effluent with helium as it passed through the photolysis coil so that the nitrogen oxide would be removed from the solution as it was formed. When the HPLC effluent was photolysed prior to being mixed with the helium, only a very weak response was seen, presumably because the nitrogen oxide was oxidised to non-volatile oxides (*e.g.*, NO_2).

Instrument Design

The power of the 200-W lamp was attenuated with an electronic device (PAESAR) in order to determine the effect of various wattages on the response. A wattage of 120 reduced the response for NPRO by about 40% of that of the 200-W lamp. A wattage of 260 (using an attenuated 450-W lamp) did not significantly reduce the response to *N*-nitrosamines compared to 450 W, but 450 W slightly reduced the response from *N*-nitrosamides, possibly due to thermal degradation at the higher operating temperature (70–80 °C). The higher wattage lamp gave an unacceptably high base line when urine or gastric juice samples were analysed, especially when non-volatile amine ion-pairing reagents were used in the mobile phase. We believe that the power of the lamp may be analogous to the pyrolyser temperature of the TEA detector in that the selectivity of the instrument is related to the temperature of the pyrolyser. Higher wattage lamps and/or temperatures may produce nitrogen oxide from compounds in addition to those containing the *N*-nitroso group (*e.g.*, *C*-nitroso and nitro compounds).

We investigated several sizes and types of Teflon [tetrafluoroethylene (TFE), perfluoroalkoyl (PFA), and fluorinated ethylene polypropylene (FEP)] microbore tubing for irradia-

tion coils with wall thicknesses of 0.15–0.41 mm. The Teflon resulted in a loss of response after only 2–3 weeks of use with the higher wattage lamp. The response could not be recovered by washing the tubing and could only be recovered by replacing the tubing with new material. Others have also noted that Teflon can be degraded under intense UV irradiation.[18] These problems were overcome by the use of thick-walled glass capillary tubing. Glass tubes of lengths between 0.6 and 3.6 m were tested. Lengths of less than 3 m gave a decreased response while longer tube lengths did not result in an increased response. The inside diameter of the tubing was important for chromatographic resolution. Inside diameters of >2 mm required a solvent flow-rate of at least 2–5 ml min^{-1} in order to produce acceptable peak shapes. Tubing of 1 mm i.d. gave good peak shapes at a flow-rate of 1 ml min^{-1}. The flow of solvent had to be in the direction of gravity or the resulting solvent bumping within the coil resulted in pressure surges in the analyser and an erratic base line.

Fig. 2 compares the chromatograms of 2.4–14 ng each of an eight-component standard that was routed first through a UV detector, then the photolysis detector. The UV detector was not sensitive enough to produce acceptable peaks. However, the photolysis - chemiluminescence detector showed all peaks to be eluted within 8 min with peak widths of 30–60 s. The configuration of the glass coil is not an optimum for efficiency; however, the fact that the system was operated at 1–2 Torr and that the ultimate analyte was volatile made up for the lack of efficiency. Comparison of peak widths at half height from the UV and chemiluminescence detectors indicated that the loss of efficiency was less than 10–20%.

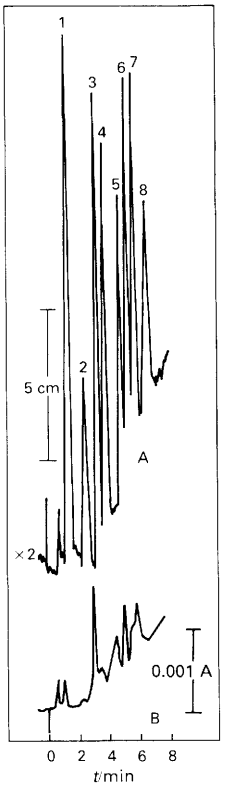

Fig. 2. Chromatogram obtained by routing the effluent of the HPLC column first through a 254-nm UV detector, then to the photolysis inlet. Compounds are: 1, HNPRO; 2, NSAR; 3, MNU; 4, NPRO; 5, NTHZCA; 6, ENU; 7, MNNG; 8, MNTHZCA. (A) Chemiluminescence detection and (B) 254-nm UV detector placed in series. Column, Brownlee Polymer RP; mobile phase, 10 mM TFA containing MeCN. Gradient elution: 0–7% MeCN at 1% min^{-1}, hold 5 min, then 7–22% MeCN at 1.5% min^{-1}, hold 3 min; 20-µl injection, 2.4–14 ng component; lamp power, 260 W

The maximum sensitivity ($S/N > 3:1$) of the chemiluminescence detector was less than 1 ng for *N*-nitrosoamino acids (Fig. 2). *N*-Nitrosamides were somewhat less responsive, with limits of detection of approximately 10 ng. These limits are higher than those for volatile *N*-nitrosamines, which typically have thresholds of detection of <0.5 ng injected when analysed by gas chromatography - TEA.

The molar response ratios for several *N*-nitrosoamino acids, *N*-nitrosoureas and *N*-nitrosamines are given in Table 1. Standard solutions (*ca.* 0.1 mmol) in acetonitrile were injected (10 µl) into the photolysis coil after passing through a 20.3 cm × 1.6 mm o.d. stainless-steel wide-bore tube, in order to mix the sample with the mobile phase. The mobile phase composition was varied from 100% aqueous to 100% organic modifier and the lamp was set at the lowest power (120 W). At a solvent concentration of MeCN + 10 mM TFA (90 + 10), *N*-nitrosoamino acids had the highest molar response, while *N*-nitrosamides and *N*-nitrosamines typically had reduced relative molar responses (DMNA or MNU, 9%; TMNU, 20%; MNNG, 40%). Increasing the aqueous portion in the mobile phase to 90% reduced the response of the *N*-nitrosoamino acids by an order of magnitude, while the response of the other *N*-nitroso compounds decreased less dramatically. Table 1 shows the relative molar response ratios for all the compounds tested, in mobile phase concentrations that are practical for ion-suppression chromatography. Addition of TFA (10 mM) to the aqueous portion did not have any effect on relative response; however, it improved flow through the coil when 100% H_2O was used. The sensitivity of the system, coupled to the 10–100-fold increase in injection volume of HPLC compared to GC, results in an over-all sensitivity for HPLC that is greater than that of GC - TEA. The response was linear ($r^2 > 0.99$) between 1 and 100 ng of NPRO injected. Above 100 ng the response fell off. Repeated injections of NPRO gave a coefficient of variation of <5%.

The Brownlee Polymer RP column gave sufficient resolution for quantifying *N*-nitrosoamino acids in the ion-suppression mode but did not resolve the *syn*- and *anti*-isomers.[17] The ODS column in the ion-pair mode separated *syn*- and *anti*-isomers but produced unacceptable base-line noise at higher lamp wattage. The ion-pair reagent also resulted in a build-up of a residue on the inner surface of the glass coil.

N-Nitrosoamino Acids in Urine

N-Nitrosoamino acids are components of mammalian urine at the 2–300 µg l^{-1} level, and have been used as indicators of the endogenous formation of *N*-nitroso compounds.[19,20] The analysis of large numbers of samples for these compounds has

Table 1. Relative molar response ratios*

Solvent	*N*-Nitroso compound											
% MeCN†	NPRO	HNPRO	NSAR	NPIP	NTHZCA	MNTHZCA	TMNU	MNU	ENU	MNNG	DMNA	NDPA
0	1.0	1.1	—	1.0	1.5	—	1.0	0.8	—	1.6	0.5	—
10	1.4	1.4	1.1	1.1	1.7	1.4	1.2	0.9	0.5	1.7	0.6	0.6
25	1.7	1.7	1.2	1.4	2.5	2.1	1.2	1.0	0.7	2.1	0.6	0.6

* NPRO (0% MeCN) = 1.
† Balance, 10 mM TFA or water.

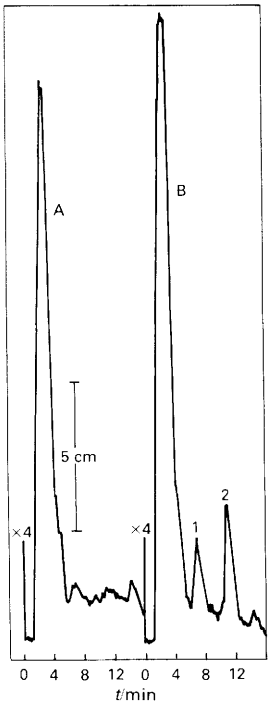

Fig. 3. A 2000-µl injection of (A) unspiked human urine and (B) spiked human urine [7.5 and 8.0 µg l^{-1} of (1) NPRO and (2) NTHZCA respectively]. Column and mobile phase as in Fig. 2; gradient elution: 5% MeCN, hold 2 min, increase to 20% MeCN at 3% min^{-1}; lamp power, 120 W

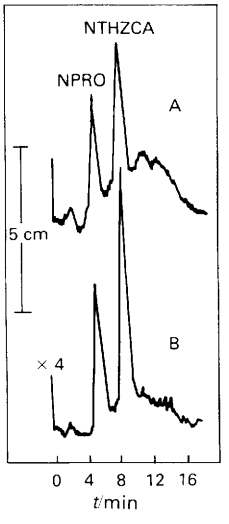

Fig. 4. Chromatogram of 10 ml of (A) human urine that had been spiked with 7–8 µg l^{-1} of NPRO and NTHZCA, and (B) a standard solution of 0.15–0.16 µg ml^{-1} of NPRO and NTHZCA. Column and mobile phase as in Fig. 2; gradient elution: 5–20% MeCN at 7.5% min^{-1}; 100-µl injection; lamp power, 120 W

been undertaken in several biochemical epidemiology studies. The current methodology is to extract and methylate the *N*-nitrosoamino acids to make them amenable to gas chromatography (GC) - TEA. The selectivity of the detector suggested that *N*-nitrosoamino acids could be analysed directly without concentration. A 2000-µl injection of spiked (*ca.* 8 µg l^{-1} of NPRO or NTHZCA) urine, which had been passed through a C_{18} solid-phase extraction (SPE) high-capacity cartridge (Baker), gave a significant response (Fig. 3).

Fig. 4 is a chromatogram of an extract from human urine that had been spiked with NPRO and NTHZCA, at 7.5 and 8.0 µg l^{-1}, respectively. Urine (10 ml) at pH 6–7 was passed through a C_{18} SPE high-capacity cartridge that had been

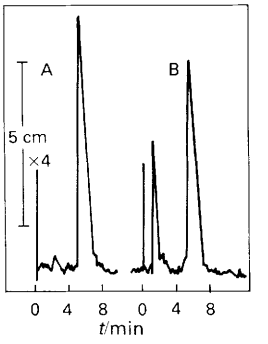

Fig. 5. Chromatogram of a standard solution of TMNU (A: 17 ng injected) and an extract of (B) porcine gastric fluid that had been spiked with 42 µg l^{-1} of TMNU. Column as in Fig. 2. Isocratic elution with MeCN + 10 mM TFA (15 + 85); 20-µl injection; lamp power, 120 W

pre-wetted with MeOH and H_2O. The cartridge was washed with two volumes of water and the combined eluent acidified (1% $H_2NSO_3NH_4$ in 1.8 M H_2SO_4) to pH 1, extracted with ethyl acetate (6 × 25 ml), washed with saturated NaCl and dried over Na_2SO_4. The ethyl acetate layer was vacuum concentrated, blown to dryness with N_2, redissolved in 10 mM TFA (0.50 ml) and filtered through glass-wool. The sample was injected in the HPLC column (100-µl sample loop) and eluted with MeCN + 10 mM TFA (5 + 95), with a gradient (2% min^{-1}) to MeCN + 10 mM TFA (20 + 80). The apparent low recovery of NTHZCA (approximately 60%) was not investigated but was probably due to the known instability of this compound. This procedure allows the analyses to be carried out without the need for derivatisation.

N-Nitrosamides in Gastric Fluid

The endogenous formation of *N*-nitrosamides has been suggested as a factor in the etiology of human gastric cancer.[21] *N*-Nitrosamides are also used therapeutically as antitumour agents. They are not amenable to TEA detection due to thermal rearrangement to liberate N_2. Krull *et al.*[13] have investigated HPLC - UV as a method for *N*-nitrosoureas in blood. The limit of detection was 100–200 µg l^{-1} and, in blood cells washed with saline, unknown peaks interfered.

Fig. 5 shows a chromatogram of an extract of porcine gastric fluid which had been spiked at 42 µg l^{-1} with TMNU. This compound was used because it is relatively stable compared to other *N*-nitrosamides. Centrifuged gastric fluid was acidified (HCl) to pH 3–3.5, centrifuged and passed through a pre-wetted (CH_3OH, H_2O, 2 mM TFA) C_{18} SPE high-capacity cartridge. The cartridge was rinsed with 3 ml of 2 mM TFA, vacuum dried and eluted with 5 ml of ethyl acetate. The ethyl acetate was dried (Na_2SO_4), reduced to dryness (N_2) and the residue suspended in 0.50 ml of 2 mM TFA. Recovery of the spike was 84% with a limit of detection of approximately 2–5 µg l^{-1}. This could be improved easily by increasing the sample size and/or the 20-µl injection volume.

We are presently using this detector to investigate the occurrence, formation and stability of non-volatile *N*-nitroso compounds, particularly *N*-nitrosamides and related compounds in a variety of biological fluids and food samples.

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