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Determination of Total *N*-Nitroso Compounds by Chemical Denitrosation Using CuCl

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A method for the determination of total *N*-nitroso compounds (NOC) by chemical denitrosation and subsequent chemiluminescence detection of evolved NO is described. Denitrosation was accomplished with CuCl in HCl at 70 °C. The detection limit for *N*-nitrosoproline (NPRO) was 1 pmol. NO formation from NPRO was linear ($R^2 = 0.999$) from 4 pmol to 2 nmol. Among the possible interfering compounds tested, only *S*-nitroso compounds contribute any significant interference. This method had several advantages over other similar methods: (1) A commercially available one-piece reaction vessel and a NO analyzer with software were used. (2) NO release occurred rapidly and was easily measured and quantified. (3) Compared to HBr or HI, CuCl was more convenient to work with and safe. (4) CuCl was suitable for samples in aqueous and most organic solvents. The application of this method to food, personal care products, and human body fluids demonstrates its utility.

KEYWORDS: Total *N*-nitroso compounds; chemical denitrosation; chemiluminescence; CuCl; NO; Nitric Oxide Analyzer (NOA 280); Radical Purger

INTRODUCTION

Since the discovery of the carcinogenicity of nitrosamines, extensive research has been conducted to measure nitrosamines in food, personal care products, and tobacco. Techniques for measurement of volatile nitrosamines are now well-established. Development of analytical methods for measuring nonvolatile nitrosamines has been difficult and limited progress has been made in this area (*1*, *2*). It is often not practical or necessary to identify and measure each *N*-nitroso compound in a large number of complex samples, so a rapid and reliable screening tool would be valuable for analyzing this group of compounds in total.

The methods for determination of total *N*-nitroso compounds (NOC) are generally based on some means of chemical denitrosation. The evolved NO is reacted with ozone to form electronically excited nitrogen dioxide, which then decays back to the ground state with the emission of a photon. The chemiluminescent emission is detected and measured by a photomultiplier tube.

$$R_2N{-}NO \rightarrow R_2NH + NO$$

$$NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + h\nu$$

Two chemical denitrosation reagents are currently used. HBr in glacial HOAc with refluxing EtOAc, developed by Downes et al. in 1976 (*3*), is the standard and preferred reagent. It has been optimized and is widely used for total NOC determination in food (*4*−*6*), personal care products (*7*), and other uses (*8*−*11*). The method tolerates water only up to 10%. Denitrosation is inhibited above this point (*12*). The apparatus generally used with this method was constructed of common flasks and connectors but required disassembly and cleaning each time the reagent was changed.

A related chemical denitrosation reagent HI is freshly generated by the reaction between NaI and H_2SO_4 (*13*). Only limited applications of the HI method have been reported (*2*, *14*).

CuCl in HCl was first introduced as a denitrosation reagent in 1932 for recovery of amines from the corresponding nitroso derivatives via an amine copper chloride complex (*15*). Near quantitative evolution of NO was observed. In 1971, CuCl was used for the determination of nitrosamines in cigarette smoke condensate, based upon the conversion of the nitrosamines and subsequent analysis by gas chromatography of the resulting secondary amines (*16*). These studies focused on the recovery of the amine rather than the measurement of the evolved NO. The application of CuCl in HCl for the determination of NO from nitrosamines to estimate NOC has not been reported.

The objective of this study was to develop a rapid, simple, and more convenient method for measuring total NOC in many samples by using CuCl as the denitrosation reagent, a commercially available one-piece reaction vessel, and a NO analyzer.

MATERIALS AND METHODS

1. Apparatus. A Nitric Oxide Analyzer (NOA 280) equipped with a Radical Purger reaction vessel (**Figure 1**) from Sievers Inc. (Boulder,

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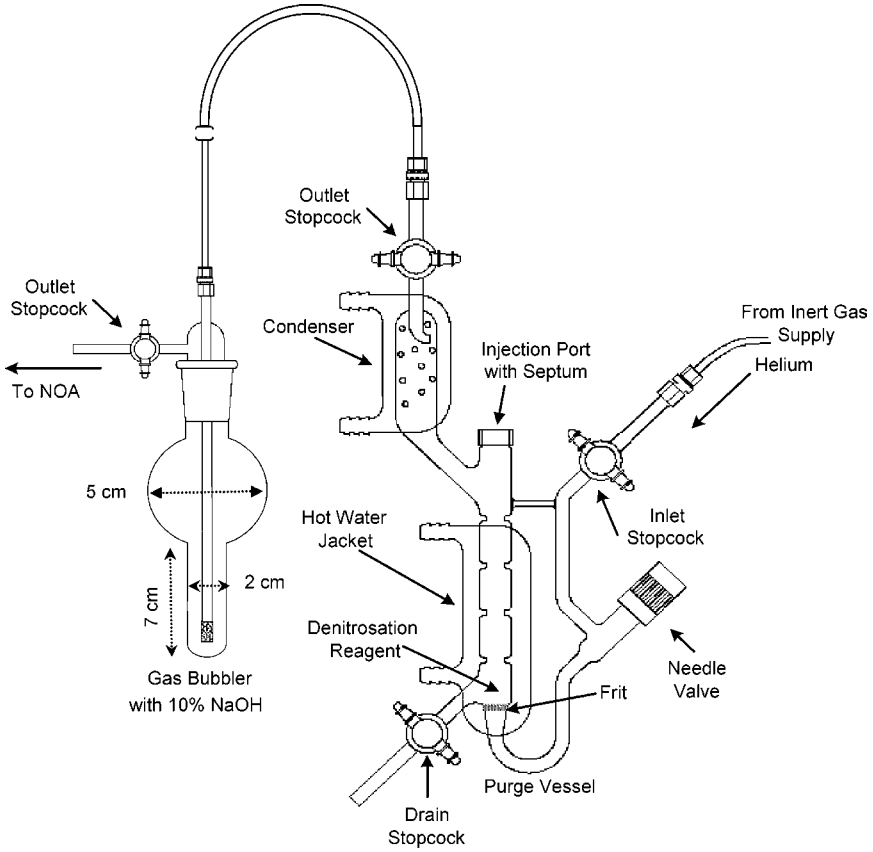

Figure 1. Sievers Radical Purger (*30*). (Figure is used with permission of Sievers Instrument, Inc.)

CO) was used for NOC determination. Heating of the reaction chamber and cooling of the condenser were accomplished by an EX-111 bath/circulator and an RTE-111 refrigerated bath/circulator from Neslab Inc. (Portsmouth, NH). Additional equipment was obtained as follows: Tissue-Tearor (Biospec Products, Bartlesville, OK); nutating mixer Orbitron Rotator II Model 260250 (Boekel Scientific, Feasterville, PA) and IEC HN−SII centrifuge (International Equipment Co., Needham Heights, MA).

2. Reagents. CuCl, sulfamic acid (SA), and *n*-butyl nitrite were obtained from Acros Organics (Fairlawn, NJ). NaOH, 10% w/v, was obtained from LabChem, Inc. (Pittsburgh, PA), and HCl was obtained from Fisher Scientific (Pittsburgh, PA). Deionized water was obtained from a MilliQ Plus ultrapure water system equipped with a QPAK 1 Purification Pack from Millipore Corp. (Bedfore, MA). 2-Methyl-2-nitrosopropane, 1-nitropropane, acetone oxime, creatinine, nitroguanidine, cyclohexanone oxime, 3-hydroxypyridine *N*-oxide, nitrosobenzene, *N*-nitroso-*N*-methylurea (MNU), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosoglutathione (GSNO), isoamyl nitrite, and *n*-butyl nitrite were purchased from Sigma (St. Louis, MO). Nitrobenzene, *N*-nitroso-*n*-propylamine, *N*-nitrosodiphenylamine, and *N*-nitrosodipyrrolidine were purchased from Ultra Scientific (Kingstown, RI). *N*-Nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)butanone (NNK), and *N*-nitrosoproline (NPRO) were purchased from ChemSyn Laboratories (Lenexa, KS).

The denitrosation reagent was prepared fresh daily by addition of 20 mL of 6 N HCl (graduate cylinder) to 1.0 g of CuCl in a 2 oz brown wide mouth bottle. CuCl was purchased in small lots (5 g) and stored at room temperature in a desiccator over Drierite. Unused portions were discarded after 1 month.

3. Determination of Total NOC. The determination of NO from *N*-nitroso compounds was accomplished by a modification of the procedure for nitrate analysis (*17*) using a lower temperature and CuCl in the purge vessel. The denitrosation reagent (4 mL) was placed in the purge vessel and maintained at 70 °C. The condenser temperature was kept at −5 °C. The pressure at the chemiluminescence reaction cell inside the NOA was maintained at 6.0 ± 0.2 Torr. The samples being analyzed were injected into the purge vessel through a septum at the top. NO was released and swept by the helium stream to the detector. The NOA was protected from acid vapor and liquid contamination by the presence of a trap containing 10% NaOH solution and a filter after the purge vessel. The amount of NO was calculated on the basis of the peak area from each injection.

Calibration standard solutions of NPRO were prepared with deionized water at the concentrations 40, 100, and 400 nM and 1, 2, 10, and 20 μM and were stored frozen (−5 °C) until needed. A calibration curve was obtained by injecting 100 μL of each standard into the purge vessel. One calibration run was sufficient for each 20 mL batch of CuCl solution.

4. Sample Preparation. The sample preparations for food (*4*, *5*, *18*), cosmetic products (*7*, *19*), and human body fluids (*9*, *10*, *11*, *20*) have been described in detail previously. We adapted many of these protocols as described below.

Meat Products. Meat products were purchased from local stores in Richmond, VA, stored in a refrigerator, and used within 1 week. The meat was cut with scissors and mashed with a mortar and pestle and 2 g was homogenized by a Tissue-Tearor in 6 mL of 100 mM SA. (This amount of SA was sufficient to eliminate residual nitrite and prevent artifact formation.) The mixture was shaken on a nutating mixer Orbitron Rotator II at full speed for 1 h and centrifuged (2000 rpm) for 10 min using a IEC HN-SII centrifuge. The supernatant was decanted and filtered through a 0.45 μm PVDF membrane filter. The aqueous meat extract (250 μL) was injected into the Sievers Radical Purger.

Sauces, Beer, and Urine. Sauces and beer were also purchased locally. Urine samples from normal subjects were freshly collected. A 400 μL sample was then treated with 1600 μL of 100 mM SA, allowed to stand for at least 10 min and filtered through a 0.45 μm PVDF membrane filter. The filtrate (250 μL) was injected into the Sievers Radical Purger.

Cosmetic Products. Shampoo and lotion were purchased locally. A 1 g sample was dissolved in 2 mL of 70:30 (v/v) tetrahydrofuran:water. A 400 μL aliquot was then treated with 1600 μL of 100 mM SA,

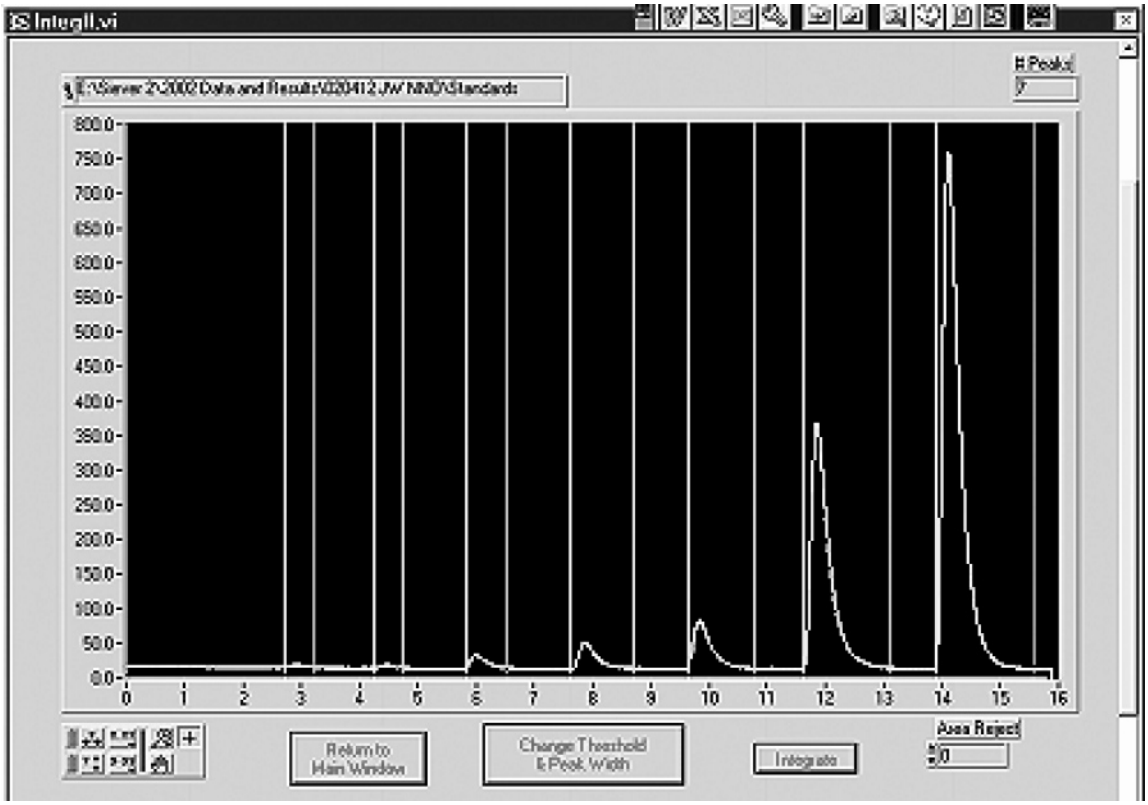

Figure 2. Data Display: NPRO Standards. (The two vertical lines adjacent to each peak signify the beginning and end of automatic integration.)

allowed to stand for at least 10 min, and filtered through a 0.45 μm PVDF membrane filter. The filtrate (250 μL) was injected into the Sievers Radical Purger.

5. Safety. Nitrosamines are known to be human carcinogens. Appropriate precautions, such as the use of gloves and fume hoods, should be exercised.

RESULTS AND DISCUSSION

The NOA and Radical Purger combination (**Figure 1**) from Sievers Inc. is typically used for nitrite and nitrate analysis using VCl_3 and NaI to release NO. These methods are described in detail in the instrument manual (*17*). The nitrate analysis was adapted for total NOC determination as follows: (1) 5% (w/v) CuCl solution in 6 N HCl at 70 °C was used to release NO from NOC; (2) a customized gas bubbler (**Figure 1**) containing NaOH was used to prevent any foam from being carried over to the NOA. The utility of this method was achieved by the use of CuCl in conjunction with this commercially available equipment. The NOA and one-piece Radical Purger allowed for efficient processing and high sample throughput. Once the cooling and heating baths reached the desired temperatures, the CuCl/HCl reagent was placed in the purge vessel. The pressure at the chemiluminescence reaction cell inside the NOA was adjusted and maintained at 6.0 $\pm$ 0.2 Torr. A steady baseline was obtained within 10−30 min, and the system was ready for sample analysis and stable for the entire day. By manipulating the stopcocks on the purge vessel, one could easily replace the reagent and clean the vessel without disassembly. A stable baseline was reestablished within minutes. The lack of extraneous volume within the gas stream produced sharp and easily integrated NO peaks. As can be seen from the captured screen display of NPRO standards shown in **Figure 2**, the release of NO typically occurred and dissipated in less than 2 min, allowing the additional samples to be injected every 3−5 min. The software supplied by the manufacturer of the NOA was used to detect and quantify the peaks automatically. NO formation from NPRO was linear ($R^2 = 0.999$) from 4 pmol to 2 nmol. The detection limit of this method was 1 pmol of NPRO ($S/N = 3:1$).

The capacity of the CuCl solution to produce NO was not diminished by 5−10 injections. However, the liquid level in the purge vessel increased from the added volume of injected samples and must be drained and replaced when liquid began to physically carry over to the trap.

This method had several advantages over other similar methods: (1) A commercially available one-piece reaction vessel and an NO analyzer with software were used. (2) NO release occurred rapidly and was easily measured and quantified. (3) Compared to HBr or HI, CuCl was more convenient to use and safe. (4) CuCl was suitable for samples in aqueous and most organic solvents (except CH_2Cl_2 and $CHCl_3$). The use of CH_2Cl_2 and $CHCl_3$ was not recommended in this method because their presence produced an unstable baseline.

A proposed mechanism of NO release by CuCl is shown in **Figure 3**. CuCl is sparingly soluble in water but is readily soluble in HCl, due to the formation of a copper dichloride complex $[CuCl_2]^-$ (*21*). It has already been suggested by Jones and Kenner that CuCl could transfer an electron to the nitroso nitrogen of a protonated nitrosamine to release NO and the amine (*15*). Alternatively, nucleophilic attack of either chloride ion or water molecule would release the amine and nitrosyl chloride (NOCl) or nitrous acidium ion (*22*, *23*) ($H_2NO_2^+$). Reduction of the latter by CuCl will produce NO.

This method was developed to analyze *N*-nitroso compounds, so it is important to ensure that the results were indeed due to the decomposition of *N*-nitroso compounds and not due to the decomposition of other interfering sources, such as *C*-nitroso, *O*-nitroso (*O*-nitroso compounds are often referred to as nitrite esters), *S*-nitroso, *C*-nitro, and other organic nitrogen compounds. In addition, nitrite and nitrate occur in food and

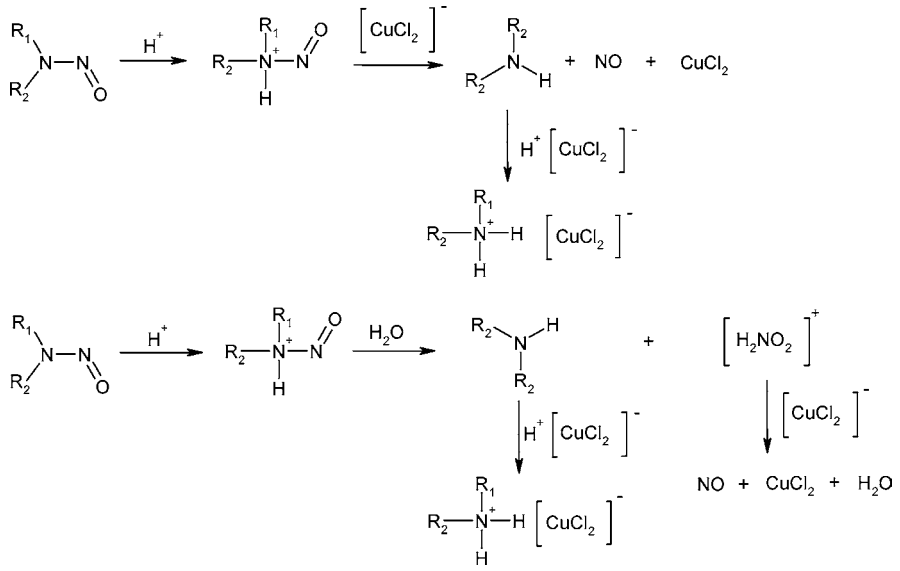

Figure 3. The proposed mechanism of NO release by CuCl.

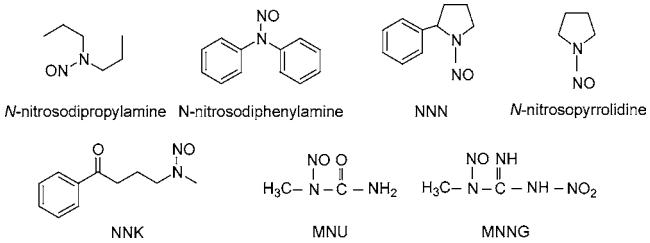

Figure 4. *N*-Nitroso compounds for selectivity test.

Table 1. NO Yield from NOC by CuCl/HCl with and without Treatment with SA[a]

chemical	NO yield % without SA	NO yield % with SA
N-nitrosodiphenylamine	86.0	54.1
N-nitrosopyrrolidine	88.8	84.2
N-nitrosodipropylamine	88.0	71.2
NNN	80.7	64.0
NNK	91.4	82.5
MNU	59.0	44.0
MNNG	74.0	63.5

[a] Solutions of nitrosamines were made at 5 μg/mL concentration in deionized water. For testing with and without SA, 200 μL of solution was mixed with 1800 μL of 100 mM SA and 1800 μL of deionized water, respectively, and 100 μL of solution was injected into the reaction vessel. MNU and MNNG solutions were made at 5 μg/mL concentration in methanol. For testing with and without SA, 200 μL of solution was mixed with 1800 μL of 100 mM SA and 1800 μL of methanol, respectively, and 100 μL of solution was injected into the reaction vessel.

biological matrixes and their presence alone may interfere with the analysis. The compounds for our selectivity study were chosen on the basis of previous literature (*24*−*27*) and commercial availability. All of the *N*-nitroso compounds, including nitrosamines and nitrosamides (**Figure 4**), produced a positive detector response (**Table 1**) under the experimental conditions. Most nitrosamides were unstable in an aqueous medium, especially at pH >5 (*28*, *29*). When MNU or MNNG was dissolved in water, the resulting integrated NO peak area decreased as a function of time, probably due to decomposition. Therefore, MNU and MNNG were analyzed as a methanol solution.

Nitrate interfered with total NOC measurements by irreversibly shifting the baseline upward, probably due to a very slow conversion to NO (**Figure 5**). The baseline change was proportional to the amount of nitrate in the sample. The peak became a plateau when nitrate was present at a high concentration and integration was then rendered impossible. Only replacing the CuCl reagent could restore the baseline to a normal level. The interference from nitrate could be minimized by subjecting the sample to anion-exchange resin as described in the previous literature (*10*, *13*, *19*).

Table 2. Reduction of NO Evolution with Treatment with SA[a]

chemical	NO yield (%) without SA	NO yield (%) with SA
sodium nitrite	103.7	BDL[b]
n-butyl nitrite	86.2	BDL
isoamyl nitrite	79.7	BDL
tert-butyl nitrite	86.1	BDL
2-methyl-2-nitrosopropane	4.5	0.1
nitrosobenzene	1.3	BDL
1-nitropropane	0.1	BDL
nitrobenzene	1.5	BDL
acetone oxime	1.6	BDL
cylcohexanone oxime	0.8	BDL
creatinine	0.2	BDL
3-hydroxypyridine *N*-oxide	1.7	BDL
S-nitroso-*N*-acetylpenicillamine	67.8	3.3
GSNO	82.6	32.0

[a] Solutions of the compounds were made at 5 μg/mL concentration in deionized water. For testing with and without SA, 200 μL of aqueous solution was mixed with 1800 μL of deionized water and 1800 μL of 100 mM SA, respectively, and 100 μL of solution was injected into the reaction vessel. [b] BDL, below detection limit.

Nitrite and *O*-nitroso compounds gave false positive responses with CuCl, but they were totally eliminated by the addition of SA (**Table 2**), presumably according to the scheme below (*11*).

$$NH_2SO_3H + RONO \rightarrow N_2 + H_2SO_4 + ROH$$

(R = H or alkyl)

C-Nitroso, *C*-nitro, and other organic nitrogen compounds used in this study are shown in **Figure 6**. These compounds evolved NO in an amount that was ≤4.5% of the theoretical yield from the nitroso group (**Table 2**). After treatment with SA, the NO yield decreased to less than 0.2%, and for most of the compounds, the evolution of NO was under the detection limit. Therefore, *C*-nitroso, *C*-nitro, and other organic nitrogen compounds did not interfere to any significant extent.

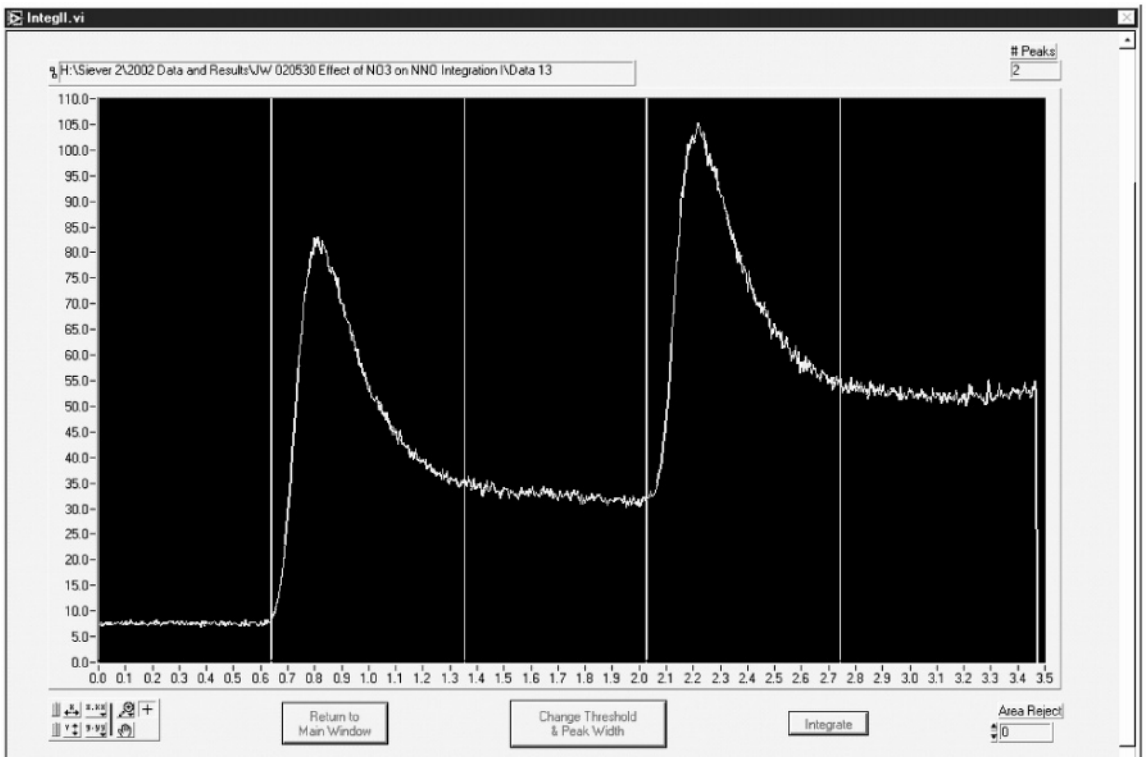

Figure 5. Interference from nitrate.

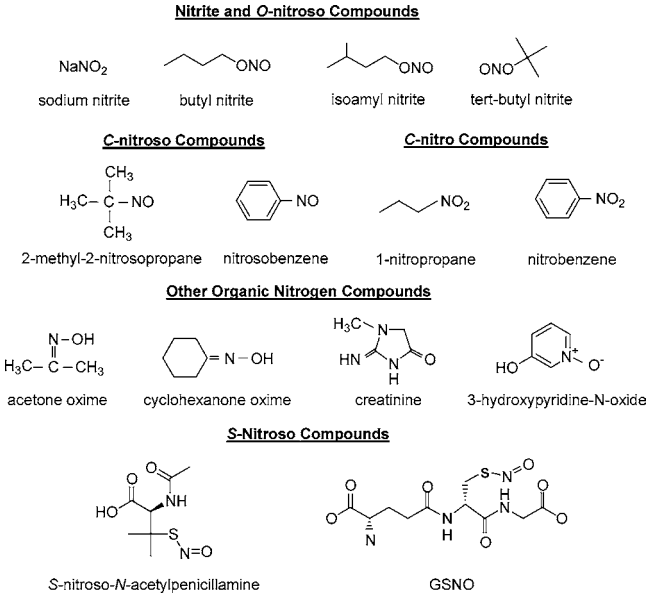

Figure 6. *O*-, *C*-, *S*-Nitroso, *C*-nitro, and other organic nitrogen compounds for selectivity test.

Two commercially available *S*-nitroso compounds (**Figure 6** and **Table 2**) gave positive responses with CuCl. After treatment with SA, the evolution of NO from *S*-nitroso-*N*-acetylpenicillamine was equivalent to 3% of the theoretical yield and was not a significant interference. But the release of 32% of the NO from GSNO was a concern. *S*-Nitroso compounds such as GSNO may interfere with the analysis, especially in biological matrixes. However, very few compounds other than NOC gave positive response. NOC could thus be selectively measured by CuCl, and this method was indeed suitable for the determination of total NOC. The only interference that could not be eliminated was the presence of significant amounts of sulfur compounds.

Table 3. Determination of Water Extracts of Food Products and Urine for NOC[a]

product	NOC (μmol/kg or μmol/L of analyte)
smoked beef A[b]	6.1
smoked beef B[b]	12.0
smoked ostrich	8.6
hamb	10.4
smoked ham[b]	6.7
hot dog[b]	1.5
pepperoni[b]	9.1
soy sauce A	1.5
soy sauce B	0.9
soy sauce C	11.8
soy sauce D	8.8
soy sauce E	BQL
teriyaki sauce	3.0
steak sauce	BQL[c]
Worcestershire sauce A	BQL
Worcestershire sauce B	BQL
hot sauce	BQL
urine A	1.3
urine B	1.1

[a] Results as presented are average of duplicate injections of one extraction which differ by $\leq$5%. [b] Results are presented as μmol/kg. All other results are presented as μmol/L. [c] BQL, below quantification limit (signal/noise = 9:1).

This method was used to measure water-extractable total NOC in food, cosmetic products, and human body fluids (**Table 3**). This method worked very well with meat products, sauces, and urine samples. Our data were comparable to the previous results using a HBr method (*27*). Some samples could not be easily analyzed (shampoo, lotion, and beer) due to excessive physical foaming. This caused peak broadening and often caused liquid to be carried out of the purge vessel and condenser into the NaOH trap. We speculated that the broadening of the peak was due to NO entrained by the foam. Further work on sample preparation would be required for these samples to eliminate the foaming issue.

In summary, this method describes the first application of CuCl in conjunction with a commercially available one-piece reaction vessel and NO analyzer with software supplied by the manufacturer for total NOC determination. The method was also shown to be applicable to total NOC analysis in meat, sauces, and urine samples. For cosmetic products and beer, further sample preparation techniques should be developed.

ABBREVIATIONS USED

NOC, *N*-nitroso compounds; NOA, nitric oxide analyzer; SA, sulfamic acid; MNU, *N*-nitroso *N*-methylurea; MNNG, 1-methyl-3-nitronitrosoguanidine; NPRO, *N*-nitrosoproline; GSNO, *S*-nitrosoglutathione; NNN, *N*-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)butanone; BDL, below detection limit; BQL, below quantification limit.

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