HPLC determination of three *N*-nitrosamine compounds including *N*-nitrosodiethanolamine (NDELA) using HPLC with post-column photochemical and chemical derivatization

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N-Nitrosamine compounds are naturally produced when an amine or amino derivative comes in contact with a nitrosating agent. *N*-nitrosamine compounds have been detected in a wide variety of foods treated with nitrite including cheese, fish, bacon and other cured meats, and other products regulated by the F&DA including: tobacco smoke, chewing tobacco, beer, toiletry and cosmetic products, including shampoos and cleansers. They are found in tanneries and plants manufacturing pesticides, rubber products and tires as well as fish processing plants, foundries, dye-making plants and research labs. Rubber baby bottle nipples and pacifiers may also contain small amounts of *N*-nitrosamine compounds (1).

N-Nitrosamines such as *N*-nitrosodiethanolamine (NDELA) have long been recognized as a class of hazardous compounds. "UK Department of Trade and Industry categorizes nitrosamine as more toxic in more animal species than any other category of chemical carcinogen" (1).

In 1977, when testing for *N*-nitrosamine contamination of cosmetics first began, NDELA was detected in 27 of the 29 cosmetic lotions and creams tested with levels ranging from 10 ppb – 50 ppm (2). NDELA can be formed in cosmetics when diethanolamine (DEA) in the form of triethanolamine, alkanolamide, or other amino compounds combine with a nitrosating agent, possibly 2 -bromo-2-nitro-1,3-propanediol. It has been shown that DEA is absorbed through the skin and may be a delivery agent for NDELA (3).

The potential presence of *N*-nitrosamines in the environment, foods and cosmetics make detection and accurate quantitation essential for public safety.

Various analytical methods have been developed for the determination of *N*-nitrosamine compounds including: GC w/ MS, GC w/ Thermal Energy Analyzer (TEA), HPLC w/TEA and HPLC w/MS. Most of these methods require post-column derivatization with toxic chemicals, expensive analytical equipment and time intensive clean-up procedures (4). In 2009, the International Standards Organization approved and published an analytical method for the detection and determination of NDELA in cosmetics using HPLC with post-column photolysis and chemical derivatization. The method is both highly sensitive and relatively simple (5). By employing the Aura post-column photochemical reactor, PHRED™, and post-column chemical derivatization unit, EPOCOD™, Aura Industries has evaluated the ISO method and has demonstrated that it provides quick, accurate and economically feasible determination of NDELA and other *N*-nitrosamine compounds.

Chemistry of the reaction

NDELA and the other N-nitrosamine compounds assessed have no natural UV absorption at the wavelengths of interest. A three-step derivatization process was used to produce an absorptive product in the UV/Vis range (Figure 1). The first step is the photolysis of an *N*-nitrosamine molecule in the presence of water to photolytically cleave off the nitroso functional group and release a nitrite ion. The second and third steps of diazotization and coupling, known as the Griess reaction, was first reported by Johann Peter Griess in 1879 as a method for the analysis of nitrite ion (NO_2^-) (6). The nitrite ion reacts under acidic conditions with sulfanilamide ($C_6H_8N_2O_2S$) to form a diazonium cation ($HO_3SC_6H_4$–

$N^{+}\equiv N$), which is subsequently coupled to an aromatic amine; we used *N*-(1-naphthyl)ethylenediamine ($C_{12}H_{14}N_2$) to produce a red–violet colored, water-soluble azo dye ($HO_3SC_6H_4–N\ N–C_{10}H_6NH_2$) (λmax ≈540 nm). A schematic of the dual derivatization apparatus coupling an Aura PHRED™ with the delivery of the post column chemical derivatization agent (the Griess reagent) with an Aura EPOCOD™ is detailed in Figure 2.

Methods and Materials

HPLC Parameters:

Column: Supelco reverse phase C18 Column, 15 cm x 4.6 mm, 5 µm particles
Temperature of column: 40°C maintained with column jacket CJB-10 (Aura Industries, Inc.) and circulating water bath
Mobile Phase: 0.02 M ammonium acetate solution pH 7.6. Phosphate buffer at pH 7.6 can also be substituted
Flow rate: 0.5 mL/min
Injection Volume: 100 µL

Post-Column Conditions:

Photochemical reactor:

PHRED™ with Knitted Reactor Coil 5 M with 0.25 mm ID (KRC 5-25). KRC 10-25 and KRC 15-25 were also used for evaluating the appropriate amount of UV exposure necessary for complete photolytic cleavage (Aura Industries, Inc.)

Chemical Derivatization

Masterflex® single speed peristaltic pump
Flow rate: 0.7 mL/min.
EPOCOD™ with mixing tee and KRC 5-50 reactor coil
Temperature of reactor coil: 50°C maintained with column jacket CJB-4 (Aura Industries, Inc.) and circulating water bath

Post-column chemical derivatization agent:

Griess reagent involves mixing of 2 parts. 1) 0.25g *N*-(1-naphthyl)ethylenediamine dihydrochloride diluted in 250mL water. 2) 4.0g sulfanilamide dissolved in 250mL of a 5% (mass/volume) aqueous solution of 85% orthophosphoric acid (may require heat and stir bar to dissolve). Mix the reagents together in a dark bottle and keep mixture away from light. Mixture can be used for five days. Store at 2–8°C.

Detection:

Milton Roy SM4000 programmable wavelength detector; λ: 540 nm

Chemicals:

Stepanol® WAT-K (a surfactant), Dowicil™ 200 preservative (Dow Chemical, Midland, MI), Glydant® XL-1000 preservative, and Germall® 115 preservative (Sutton laboratories, Chatham, NJ) were obtained from the manufacturers. All other chemicals and reagents were purchased from Sigma-Alderich. Poland Spring® Brand distilled water was used for reagent and sample preparations.

Standards and Sample Preparation:

A 1.0mg/mL primary stock solution (S_o) of nitrous acid was prepared by dissolving 10.0mg of sodium nitrite, 97% A.C.S. reagent in 10 mL of H_2O. Sequential dilutions with water were made of the S_o to have final concentrations of 5, 10, 20, 50 and 100ng/mL. These were used to make a calibration curve and test the linearity of response.

4.0µL of *N*-nitrosodiethanolamine (NDELA) was diluted in 4.0mL of water to prepare a primary stock solution (1mgNDELA/mL). The primary stock solution was stored in a dark test tube in 2–8°C for a maximum of 4 weeks. A secondary stock solution made from the dilution of 40µL primary stock

solution in 4.0mL of water (10µg NDELA/mL) and a tertiary stock solution of 40 µL of secondary stock solution diluted in 4mL of water (100ng NDELA/mL) were also made and stored in the dark at 2–8°C for a maximum of 1 week. Aliquots of tertiary stock solution were diluted with water to make working solutions of 1ng, 5, 10, 25, and 50ng NDELA/mL and used to create a calibration curve of NDELA. A portion of primary stock solution was used to spike cosmetic samples with a final concentration of 10µg NDELA/mL to estimate recovery rates. A portion of secondary stock solution was prepared with a final concentration of 250ng/mL for UV exposure analysis.

40µL of *N*-nitrosopyrrolidine standard and 40µL of *N*-nitrosodimethylamine standard were each diluted in 4mL of water to prepare separate primary stock solutions (10mg/mL of each). Secondary stock solutions were created by diluting 40µL primary stock solution in 4mL of water achieving a final concentrations of 100µg *N*-nitrosopyrrolidine/mL and 100µg *N*-nitrosodimethylamine/mL. Aliquots of secondary stock solutions of NDELA, 1-Nitrosopyrrolidine and 1-Nitrosodimethylamine were combined to form a working stock for the simultaneous determination of these three compounds. Serial dilutions with water were prepared to achieve 3 working stocks with final concentrations of: 2.07, 4.15 and 8.3 µg/mL for NDELA; 4.15, 8.3 and 16.6 µg/mL for *N*-nitrosodimethylamine; and 16.67, 33.3 and 50 µg/mL for *N*-nitrosopyrrolidine. All stock solutions and working stocks were kept in dark vials and stored at 2–8°C for up to 1 week. A portion of secondary stock solution was also prepared with water for UV exposure tests (final concentration of 2500ng/mL for *N*-nitrosopyrrolidine and 1250ng/mL for *N*-nitrosodimethylamine).

Cosmetic preparation:

2.0g of each water-soluble cosmetic was placed into a 50mL plastic centrifuge tube. 20mL of water was added and the mixture was vortex mixed for 1 min. with an S/P Deluxe vortex Mixer. The mixture was then centrifuged in a Clay Adams Dynac Centrifuge at approx. 2790 rpm for 10 minutes. After conditioning a C18 solid phase extraction cartridge with 3mL methanol followed by 3mL of water; 5mL of the supernatant from the centrifuged cosmetic solution was gravity filtered through the SPE cartridge. The first 3mL of filtrate was discarded and the following 2mL was collected in a vial for chromatographic analysis.

Two additional samples of Germall® 115 preservative were prepared as above and spiked with NDELA to achieve a final concentration of 10 µg/mL NDELA. Triplicate samples of 100µL each were analyzed prior to addition of the cosmetic. After addition of NDELA to a 2.0g sample of cosmetic, the sample was mixed and separated as detailed above. Samples were analyzed post-mixing and extraction to gain a measure of recovery rate.

Results and Discussion

NDELA elutes as 2 early peaks at 3.86 and 5.05minutes when the photolysis unit was on and a single peak at 3.86min. when the photolysis unit was off (Figure 3). We were suspicious of the first peak, in that NDELA should theoretically not have a peak without photolysis. We analyzed nitrous acid standard both with and without photolysis and with and without the Griess reagent. Nitrite ions elute at 3.86min. with and without photolysis. No nitrite peak is present without Griess reagent. We also added nitrous acid to the NDELA standard and achieved only 2 peaks as was observed with the NDELA peak alone. Through comparison of elution times, addition of nitrous acid to the standard for simultaneous determination and discovering only 2 peaks, and the observation that nitrous acid does not require photolysis to be detectable, we determined that the early peak (3.86min.) is attributable to nitrous acid present as a contaminant in the NDELA standard.

NDELA and nitrous acid each exhibit a linear correlation of peak areas with concentration (Figures 4, and 5 respectively). The lower limit of detection of NDELA (measured as 3 times the background noise) was 0.97ppb.

To study the suitability of this method for the determination of other *N*-nitrosamine compounds, we also analyzed *N*-nitrosopyrrolidine and *N*-nitrosodimethylamine both of which have known health and regulatory implications. *N*-nitrosopyrrolidine induces liver cancer, lung cancer, and mesothelioma in mice and rats and has been found in cutting oils and tobacco products among other products (7). Acute and chronic exposure to *N*-nitrosodimethylamine damages the liver in humans and may increase liver tumors. *N*-nitrosodimethylamine is found in occupational settings, cured meat products, smoked fish, and cigarette smoke (8). Samples were run separately to determine individual elution times and then run simultaneously with NDELA (Figure 6). When the photolysis unit is turned on, 4 peaks are present. The first peak at 3.82min. is consistent with the nitrous acid contaminant peak. There is excellent peak separation of the three different *N*-nitrosamine compounds. When the PHRED™ is turned off, no corresponding peaks are observed indicating that post-column photolysis is required to release nitrous acid from both of the additional *N*-nitrosamine compounds. A small amount of nitrous acid is present in all three standards.

The slopes of each *N*-nitrosamine standard differed greatly from each other (Figure 7). To test whether the respective *N*-nitrosamine compounds received adequate exposure to UV light for consistent photolytic cleavage and resultant release of nitrous acid, we tested the simultaneous response of the 3 *N*-nitrosamine compounds (250ppb NDELA, 2500ppb *N*-nitrosodimethylamine, and 1250ppb *N*-nitrosopyrrolidine) measured as area under the curve as a function of UV exposure time for 3 different exposure periods (Figure 8). Three different exposure periods were accomplished by changing the size of the knitted reactor coil in the PHRED™. Exposure period was measure as the void volume of the knitted reactor coil divided by the flow rate of 0.5mL/min. A KRC 5-25 has a void volume of 0.25mL and an analyte would therefore be exposed to 0.5 minutes of UV irradiation as it travels through a PHRED with KRC 5-25. A KRC 10-25 with a void volume of 0.50mL has a 1.0minute exposure time, and a KRC 15-25 has a void volume of 0.75mL and an exposure time of 1.5minutes. A single-classification ANOVA for each compound was performed to determine whether there was a statistical difference among the responses attained from the three different exposure times. The p-values for *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine were 0.998 and 0.972, all much great than 0.1, and thus we can conclude that no significant increase in response was achieved with increased UV exposure. The p-value for NDELA however was 0.021, which is somewhat significant. Increasing the KRC length from 5 to 15meters may increase the amount of photolysis thus increasing the response, however we were able to achieve a linear response curve with the KRC 5-25 (Figure 4) and the absolute difference in added response as measured by area under the curve was slight. We therefore chose to keep the KRC length consistent with the ISO methodology (5). Shorter exposure times were not tested.

Of the 4 cosmetics tested: Stepanol® WAT-K, Dowicil™ 200 preservative, Glydant® XL-1000 preservative, and Germall® 115 preservative; none had detectable levels of NDELA. Glydant® XL-1000 and Germall® 115 both had peaks of nitrous acid. Using a nitrous acid calibration curve established from duplicate samples of 3 concentrations (5, 10 and 50ppb), we calculated the concentration of nitrous acid in each cosmetic sample. Glydant® XL-1000 has a concentration of 11.5ppb (±1.29ppb) nitrous acid and Germall® 115 has a concentration of 13.7ppb (±0.35ppb) nitrous acid. We spiked a sample of Germall® 115 with 10ppm NDELA to obtain an estimate of % recovery (Table 1). A recovery rate of 105.08% (±1.85%) was achieved with the SPE method for water-soluble cosmetics (5).

Our findings suggest that this HPLC method coupled with Aura's PHRED™ and EPOCOD™ offers a sensitive, robust and accurate determination of NDELA in cosmetics. The method may also be extended to the determination of other *N*-nitrosamine compounds and we suspect that it is applicable to other products beyond cosmetics where potentially hazardous *N*-nitrosamine compounds are found.

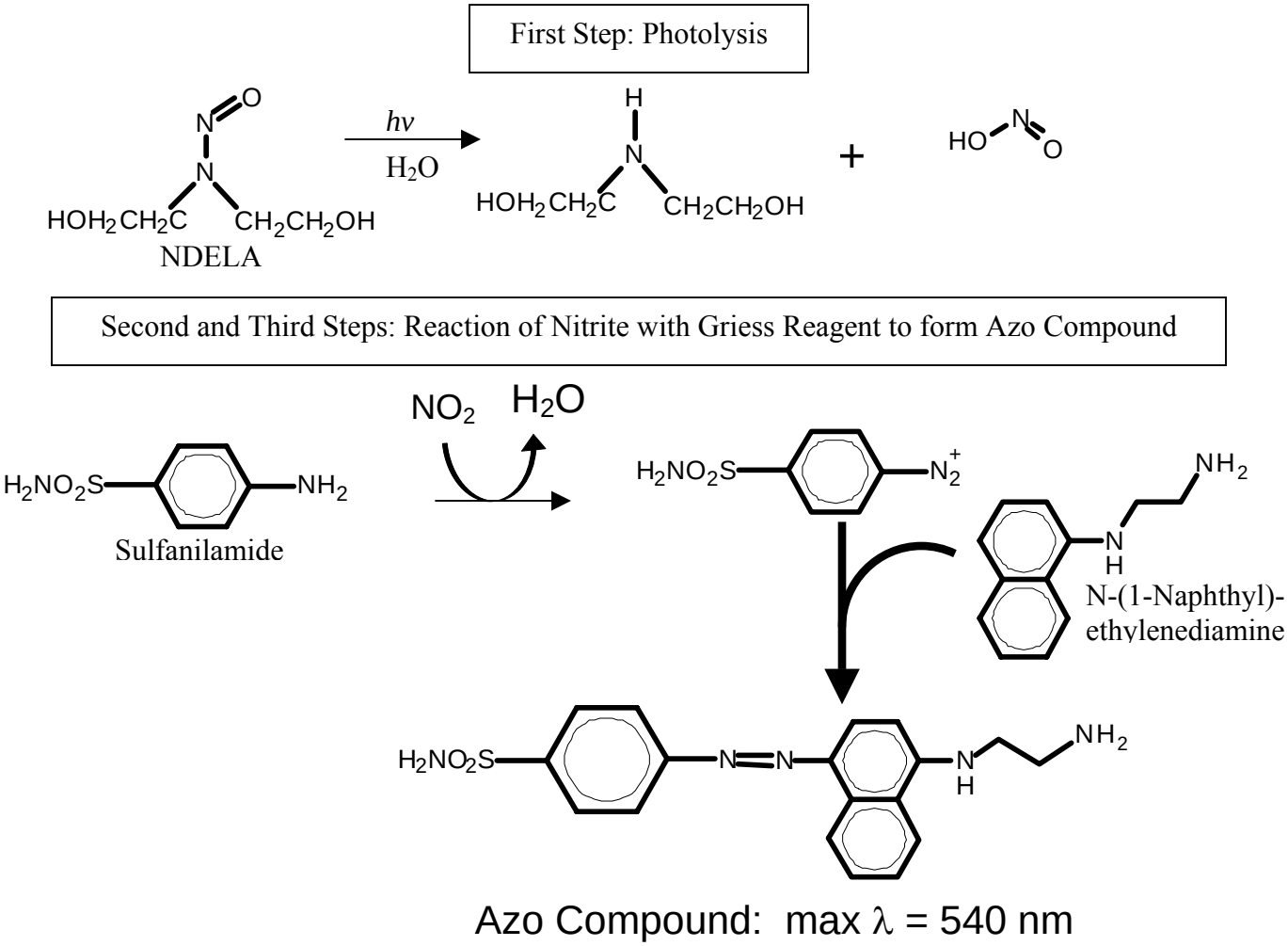

Figure 1. Photolysis of NDELA and the reaction of nitrite with Griess reagent to form an azo dye.

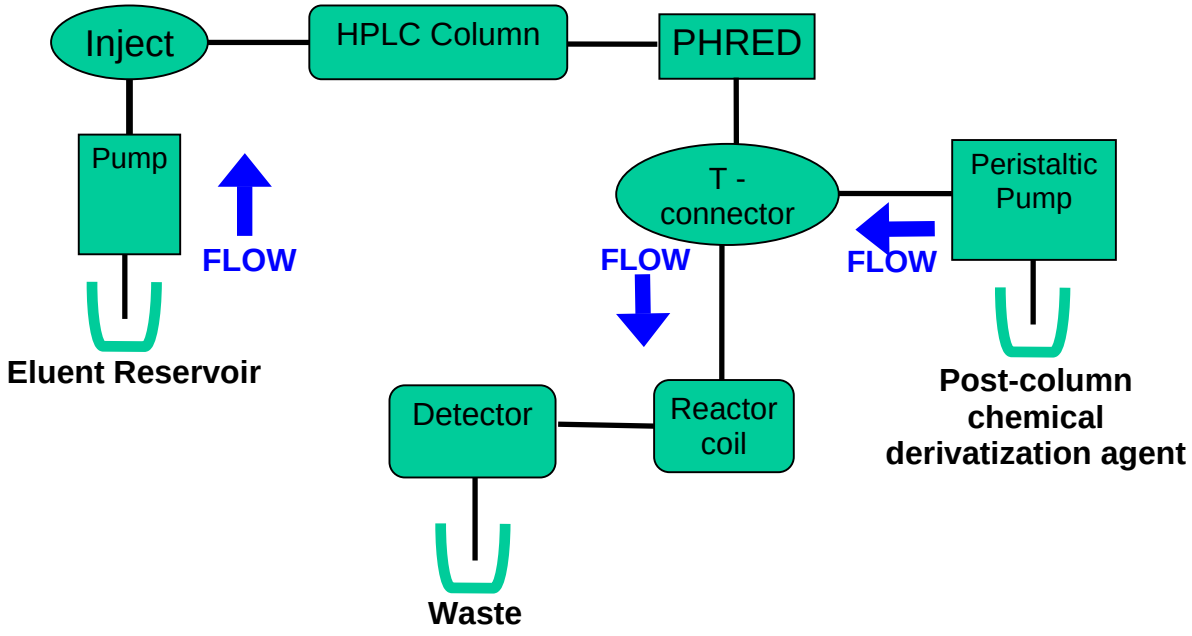

Figure 2. Schematic of post-column photochemical and chemical derivatization set-up for nitrosamine determination.

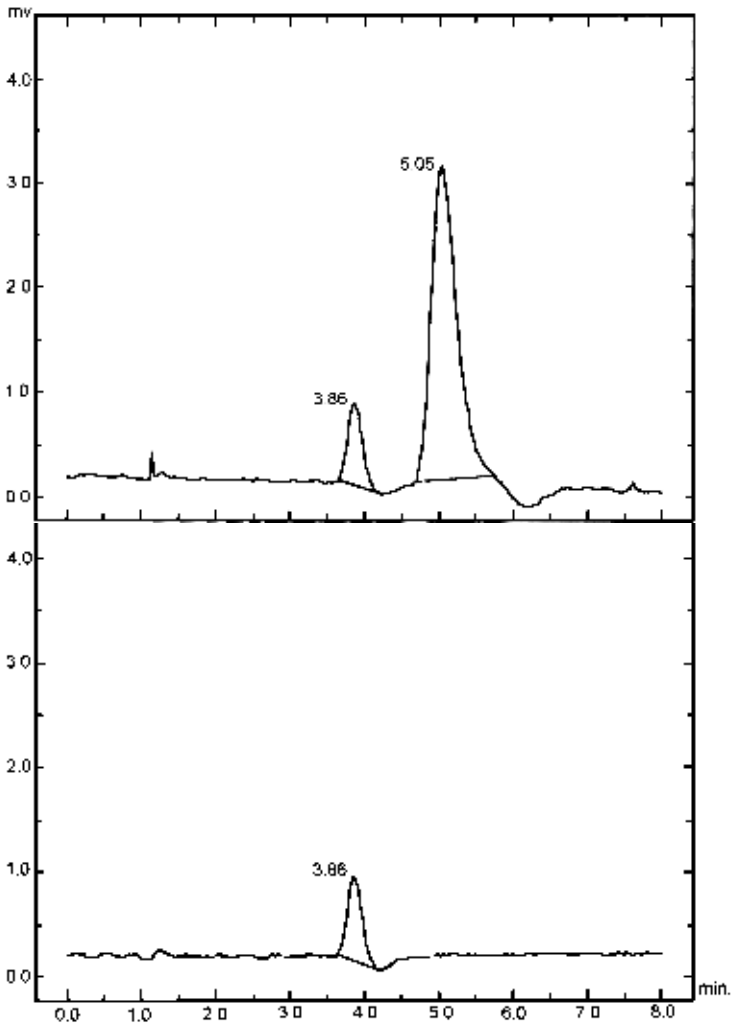

Figure 3. Chromatogram of NDELA standard solution at 500ppb a. with photolysis and b. without photolysis. The peak at 3.86 was determined to be nitrous acid, which is present in all NDELA standards. Nitrous acid is derivatized by the Griess reagent to form a fluorophore without the aid of UV derivatization.

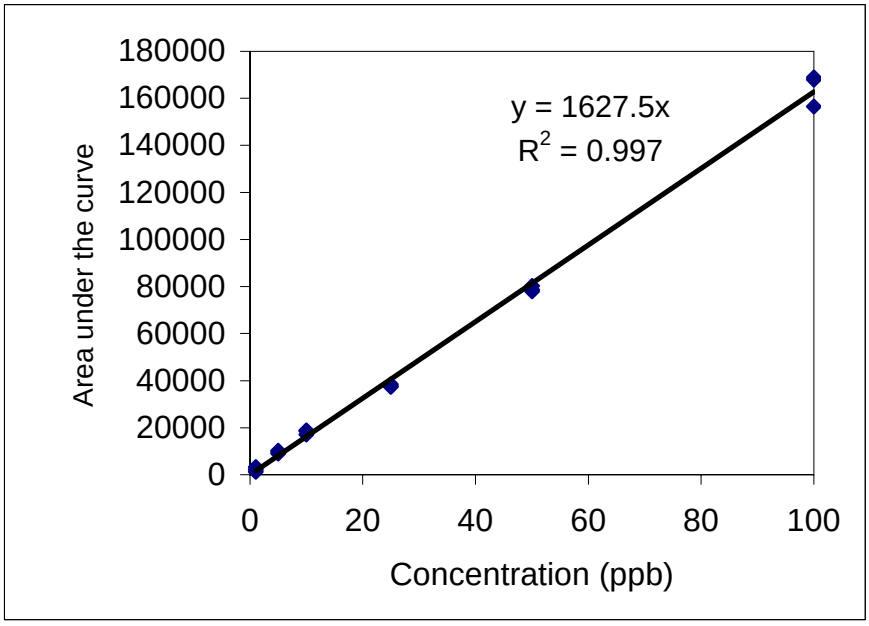

Figure 4. Calibration curve of NDELA with sample concentrations ranging from 1 – 100ppb. (n=3). R^2 value and equation for linear regression line are displayed on the graph.

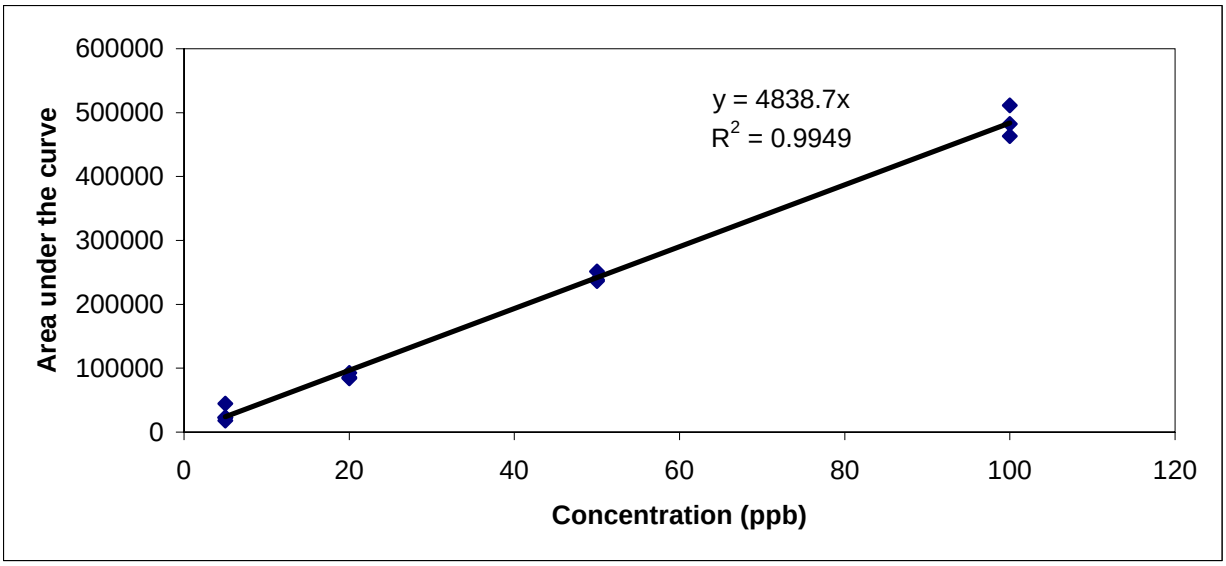

Figure 5. Calibration curve of Nitrous Acid with sample concentrations ranging from 5 – 100ppb. (n=3) Equation for regression line and R^2 value are displayed on the graph.

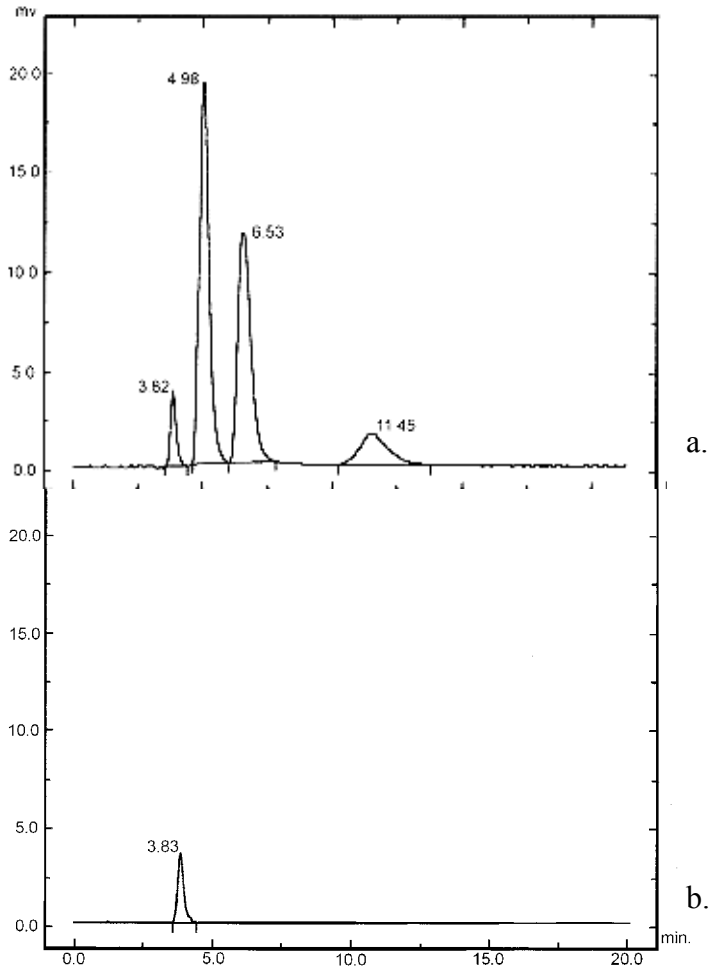

Figure 6. Chromatograms of the simultaneous determination of NDELA (4.98min.), *N*-nitrosodimethylamine (6.53min.) and *N*-nitrosopyrrolidine (11.45min.). a. with PHRED on. b. with PHRED off. The peak at time 3.82min. in a. and 3.83min. in b. is attributed to nitrous acid which is derivatized by the Griess reagent without prior UV derivatization.

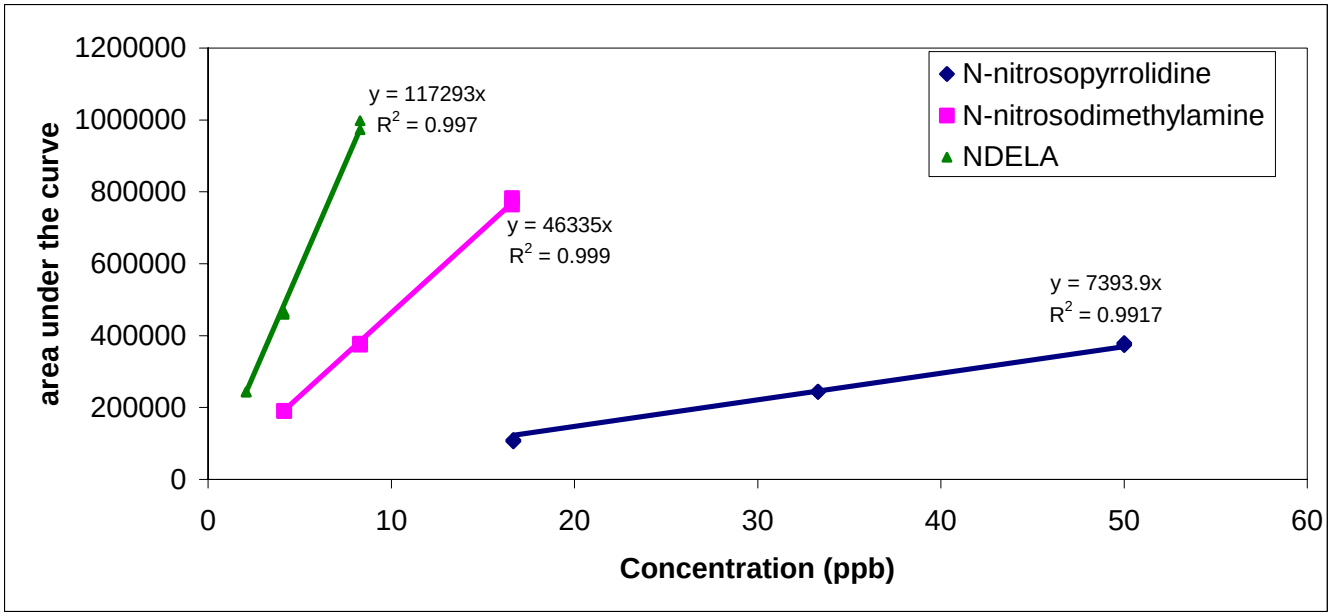

Figure 7. Simultaneous determination of three nitrosamine compounds: *N*-nitrosopyrrolidine, *N*-nitrosodimethylamine and NDELA. (n=2) R^2 values and equations of the linear calibration lines are contained within the figure for each calibration curve respectively.

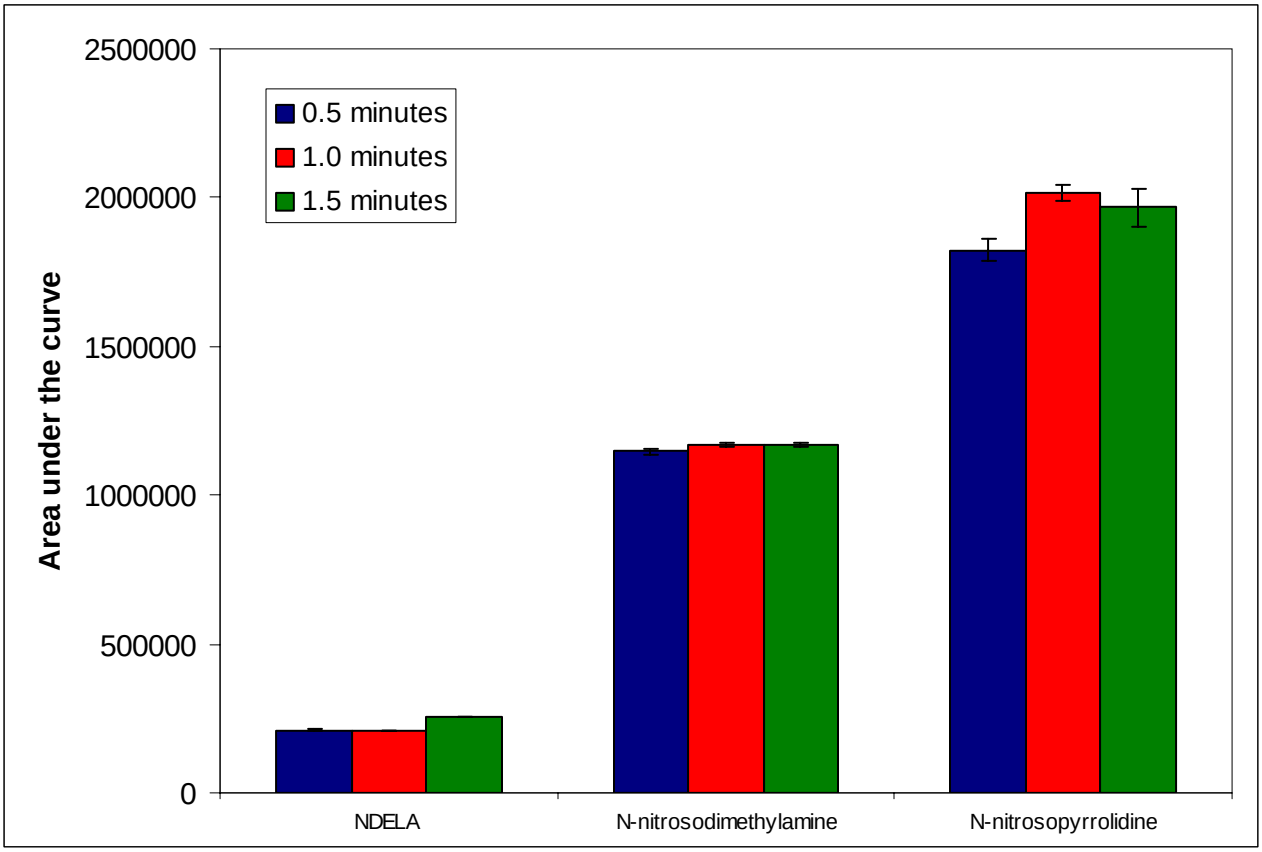

Figure 8. Response of 250ng/mL NDELA, 2500ng/mL *N*-nitrosodimethylamine and 1250ng/mL *N*-nitrosopyrrolidine measured as area under the curve when the sample was exposed in-line to UV irradiation for 0.5min. (void volume of 0.25mL), 1.0min. (void volume of 0.50mL) and 1.5min. (void volume of 0.75mL). One standard deviation displayed on chart. n=2

Cosmetic	Spiked Trial conditions	Concentration of added NDELA	Mean	Coefficient of Variation (%)
Germall® 115	before addition of cosmetic	10 µg/mL	2,036,077	0.79%
	after SPE	10 µg/mL	2,139,468	1.08%
			Recovery (%)	Coefficient of Variation (%)
			105.09%	1.76%

Table 1. NDELA spiked cosmetic test. Triplicate samples were taken and analyzed before the cosmetic was added to create a baseline area under the curve. Triplicate samples were also taken and analyzed after mixing and extraction with a C18 solid phase extraction (SPE) cartridge.

References:

(1) Department of Trade and Industry (UK DTI), 1998. A survey of cosmetic and certain other skin-contact products for *N*-nitrosamines.
(2) Cosmetic Product Manufactures, Feb. 1995. Guide to inspections of cosmetic product manufacturers. US Food and Drug administration, Inspection guides, 8, Nitrosamines. http://www.fda.gov/ICECI/Inspections/InspectionGuides/ucm074952.htm
(3) Brain, K.R., Walters, K.A., Green, D.M., Brain, S., Loretz, L.J., Sharma, R.K., Dressler, W.E., 2005. Percutaneous penetration of diethanolamine through human skin in vitro: Application from cosmetic vehicles. Food and Chemical Toxicology, 43, 681-690.
(4) Ikeda, K and K.G. Migliorese, 1990. Analysis of nitrosamines in cosmetics. Journal of Analytical Chemistry, 41, 283-333.
(5) ISO 10130:2009 Cosmetics- Analytical methods – Nitrosamines: Detection and determination of *N*-nitrosodiethanolamine (NDELA) in cosmetics by HPLC, post-column photolysis and derivatization
(6) Tsikas, D., 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the L-arginine/nitric oxide area of research, Journal of Chromatography B, 851, 51-70.
(7) US Environmental Protection Agency, 1994. Integrated Risk Information System for *N*-Nitrosopyrrolidine (CASRN 930-55-2), http://www.epa.gov/iris/subst/0081.htm
(8) US Environmental Protection Agency, 2000. Technology Transfer Network Air Toxics Web Site. *N*-Nitrosodimethylamine, 62-75-9, Hazard Summary, http://www.epa.gov/ttn/atw/hlthef/nitrosod.html