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Selective photo-reduction of *N*-nitroamines combined with micellar electrokinetic chromatography and laser fluorimetric detection

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Abstract

N-nitroso compounds (NOC) are potent carcinogens. Reliable methods for the analysis of volatile carcinogenic NOC are well established; however selective and sensitive methods for routine analysis of thermally unstable, ionic or non-volatile NOC are still needed. For this purpose, a method based on micellar electrokinetic chromatography (MEKC) with laser induced fluorescence (LIF) detection is described for the simultaneous determination of a broad range of *N*-nitroso compounds. In this procedure, the nitroso group is photolytically cleaved from the NOC to yield the corresponding amine. The amines are then derivatized with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), identified and quantified using MEKC-LIF. For the standard mixture of NOC, this method has good sensitivity and a large dynamic range. The detection limit provided by the method is 9 ppb for *N*-nitrosopyrrolidine.

Keywords: Nitrisamines; Laser fluorimeric detection; Carcinogen

1. Introduction

N-nitroso compounds (NOC) are potent carcinogens. More than three hundred nitrosamines have been tested for animal carcinogenicity and of these, 90% were found to be animal carcinogens and by extrapolation human carcinogens as well. [1] The ease of formation of *N*-nitroso compounds and the ubiquity of the precursors give *N*-nitroso compounds a potentially wide range of occurrences. Reducing human exposure to these carcinogenic compounds depends on the ability to accurately detect their presence in the environment and in food products. Development of reliable methods for the analysis of volatile *N*-nitroso compounds and methods for the analysis of low molecular weight NOC has aided in understanding the sources of NOC formation and has lead to the discovery of these compounds in the environment and food products. However, there remains a wide variety of NOC, specifically large nonvolatile, polar, ionic and thermally unstable NOC that are not monitored due to lack of appropriate analytical methodologies. Until suitable methods for the determination of total NOC content in diets are developed, their presence cannot be correctly ascertained. This limits the reliability of epidemiological studies that associate the total NOC content in diets with occurrence of some human cancers. Without suitable methods to determine the presence of non-volatile NOC, the ability to assess the total human exposure to NOC remains severely limited [2].

For the analysis of non-volatile nitrosamines, several methods for high performance liquid chromatographic separations of nitrosamines have been reported. Pre-column and post column derivatization has been used in the past to enable the selectivity and sensitivity needed for trace analysis in environmental samples and foodstuffs. High-pressure liquid chromatography with a thermal energy analyzer (HPLC–TEA) [3] and high-pressure liquid chromatography with a laser induced fluorescence detector (HPLC–LIF) are the most predominate of these techniques [4]. HPLC separations of nitrosamines have been reported using fluorescence detection after chemically cleaving the *N*-nitroso group forming the corresponding amine [5].

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This work demonstrates that micellar electrokinetic capillary chromatography (MEKC) with laser-induced fluorescence detection (LIFD) is a viable means for the analysis of NOC. A two-step process is described that involves photolysis of the nitrosamine to form the less hazardous corresponding amine followed by derivatizing the amine with 4-chloro-7-nitrobenzofuran (NBD-Cl) to yield a fluorescent product. The method is been demonstrated on a certified, commercially available volatile NOC standard. Since the method described does not require significant heating and permits separation and detection in the condensed phase, it is amenable to both volatile and non-volatile NOC. In addition, for those classes of *N*-nitroso compounds that can be analyzed using current techniques, this method may offer significant advantages over existing methods. One advantage of the method is a greatly simplified sample preparation. Currently, the most commonly used detector for NOC determination is the thermal energy analyzer (TEA). The TEA requires extensive sample conditioning prior to injection, consisting of distillations, solvent extraction and preconcentration. These steps are necessary to remove polar and inorganic salts that foul the detector. The method introduced here does not use extensive sample preparation procedures, which eliminates the loss of volatile nitrosamines and artificial formation of nitrosamines in the analytical procedure. The stability of the MEKC–LIFD system circumvents the need for sample purification and the sensitivity of the fluorescence detection eliminates the need for preconcentration for most samples. The method also possesses a high degree of selectivity, to which MEKC and fluorimetric detection both contribute.

Fluorescence detection contributes to selectivity, since only those molecules that both absorb at the chosen excitation wavelength and fluoresce at the chosen emission wavelength yield a signal. However, the main advantage of LIFD results from its unsurpassed sensitivity. The directionality of laser emission is well suited to capillary techniques. However, commercial electropherographs with laser fluorimetric detection are costly. A fluorescence cell has been designed and constructed that is inexpensive, simple and does not require the use of micro-positioners or expensive optics. Another advantage of the method is the ability to develop the electrophoretic separation without the use of the actual *N*-nitroso compounds, making the development safer for the experimenter. The first step in the analysis involves selective cleavage of the *N*-nitroso group to the corresponding amine. From that point on, the experimenter works with non-carcinogenic material. Optimization of the separation conditions was developed and tested without the use of hazardous standards. The presence of a new NOC may be identified by using the corresponding amine standard. Another advantage of MEKC–LIFD is that analysis may be performed on very small volumes of sample. Thus, research involving living organisms may require fewer subjects and be less traumatic to the subjects employed. Small sample volumes are an important aspect necessary to make in vivo sampling possible. A method applicable to endogenously formed non-volatile and thermally labile NOC such as *N*-nitroso proteins and peptides would be of great importance to the understanding of biogenic NOC production as well as aiding in the development of strategies for blocking it. Due to the derivitization step, quantitation of NOCs in "real samples" would best be performed using the method of standard additions [6].

2. Experimental

2.1. Reagents and materials

All materials were obtained from commercial suppliers and used without further purification, unless specifically noted. Analytical reagent grade chemicals were used along with deionized water to prepare solutions. Standard nitrosamines and amines were purchased from Aldrich (Milwaukee, WI, USA). The Nitrosamine mixture was an Environmental Calibration Standard used for EPA method 8270. This standard solution, originally 2000 mg/L of each NOC in methylene chloride, was diluted with an appropriate amount of 30% methanol solution to obtain target concentrations. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was supplied by Pfaltz and Bauer (Stanford, CT, USA). Buffer solutions were prepared using sodium dodecyl sulfate (SDS) from Sigma Chemical Co. (St. Louis, MO, USA) and boric acid from J.T. Baker (Phillipsburg, NJ, USA). The pH was adjusted using 1.0 M sodium hydroxide.

Diethylamine (DEA) was purified by fractional distillation using a Vigreux column and a cow collection flask. 10 mL of DEA was slowly heated in an oil bath to 70 °C. The initially yellow DEA, was distilled to give a clear colorless liquid. NBD-Cl was by re-crystallized from ethanol and water. NBD-Cl in the amount of 0.9 g was added to a 250 mL Erlenmeyer flask containing approximately 80 mL of water. The flask was then heated to 80 °C with stirring. The solution turned yellow, but less than half of the material dissolved. Approximately 50 mL of ethanol was added to complete the dissolution. The solution was then hot-filtered to remove undissolved solids. Upon cooling to room temperature, needle-shaped crystals slowly began to form. To speed the crystallization, the flask was put in an ice bath. On cooling, crystal plates also began to form. The crystals were cold-filtered and rinsed with ethanol. The recrystallization yielded about 50% of the original material.

2.2. Instrumentation

MEKC experiments were performed using an Isco Capillary Electropherograph Model 3850 (Lincoln, NE), equipped with a UV absorbance detector and a vacuum/electromigration injection accessory. The electropherograph employs a fan for temperature control. The configuration employed is diagrammed in Fig. 1. The 488 nm line from a 20 mW American Model 60× argon-ion laser (MWK Industries, Corona, CA, USA) operating at approximately 5 mW was chopped at 240 Hz with a locally fabricated chopper and focused into a 125 μm optical fiber for fluorescence excitation. The 125 μm excitation fiber and the 400 μm collection fiber were positioned flush to the separation capillary at a 90° angle. The 400 μm collection fiber terminated into a 540 nm interference filter (Esco Products, Oak Ridge, NJ, USA) in line with a Model HNF-488-1.0 holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI, USA) and finally,

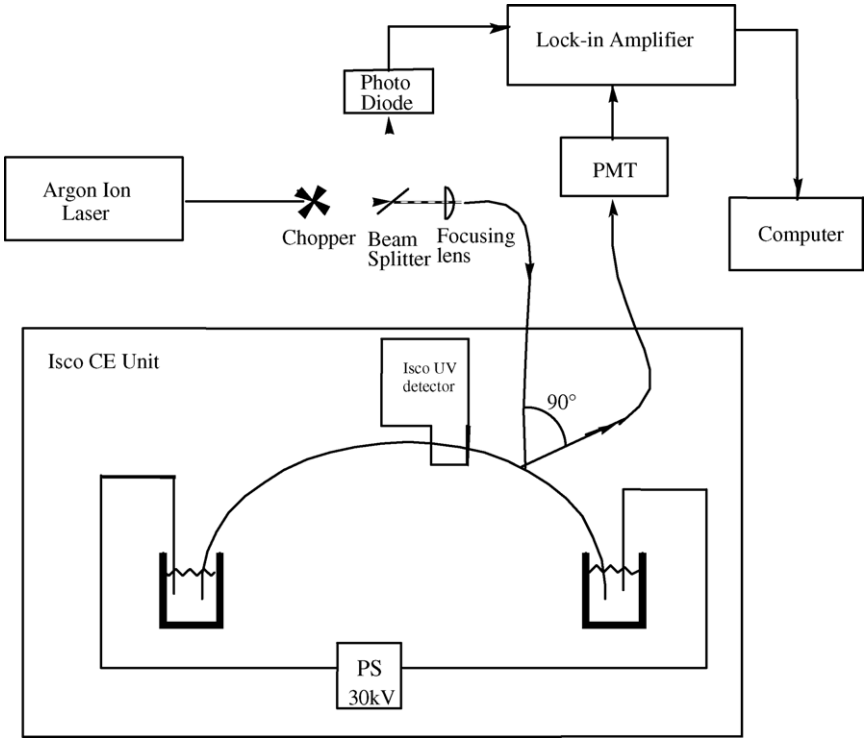

Fig. 1. A schematic diagram of the detection appartus.

a Model R-928 photomultiplier tube (Hamamatsu Corporation, Bridgewater, NJ, USA). No focusing lenses were used for collection of fluorescence; rather the collection fiber was placed flush against the first filter. The signal from the PMT was monitored using a Model 5101 lock-in amplifier (Princeton Applied Research, Princeton, NJ, USA) and processed using a National Instruments Lab-NB computer board and Lab View software (Austin, TX, USA) installed on a Macintosh computer. The use of a chopper and a lock-in amplifier helped reduce the baseline drift due to variations is the laser power. Data were analyzed using an Igor Pro software package from WaveMetrics, Inc. (Lake Oswego, OR, USA).

The fluorescence cell was built in-house using an HPLC cross and a tee, both purchased from Upchurch Scientific (Oak Harbor, WA, USA). The tee was attached, using epoxy, to the cross that had a central opening. The polished ends of the collection and excitation fibers were fitted into sleeves with the appropriate inner diameter and inserted into the cross or tee and held in place by Micro Fingertight fittings (also purchased from Upchurch Scientific). The capillary, with a 2 cm window, passed through the cross and was also held in place by sleeves and a Micro Fingertight fitting on both sides of the cross.

Fluorescence emission and excitation spectra were acquired using an SLM 8000C Spectrophosphorimeter equipped with SLM 48000S software (SLM Aminco Instruments, Urbana, IL, USA). NMR was performed using a Model EFT 90 MHz spectrometer (Anasazi Instruments, Indianapolis, IN). Infrared spectra (IR) were recorded on a Bomem MB-122 FT-IR Spectrophotometer (Bomem, Quebec, Canada). The purity of volatile organics was established using a Model QP 5050A GC/MS (Shimadzu, Columbia, MD).

2.3. Separation

The capillary diameter was 75 μm and the total capillary length was 70 cm with 46.5 cm from the inlet to the absorbance window and 63.5 cm from the inlet to the fluorescence window. The fluorescence cell was assembled on the capillary as close to the outlet as possible in order to achieve maximum theoretical plates. The running buffer consisted of 50 mM SDS and 10 mM borate solution at pH 9.3, a voltage of 20 kV and a 74 μA of current resulted. The voltage was established by an Ohm's Law plot. The capillary was conditioned periodically with 0.1 M sodium hydroxide and re-equilibrated with the run buffer prior to each set of injections. Samples were injected for two seconds at a nominal pressure of 12.8 kPa.

2.4. Sample preparation

Amines were derivatized by adding equal volumes of the sample, methanol saturated with sodium acetate, and a solution of 10 mg/mL NBD-Cl in methanol. [6] The mixture was then heated at 60 °C for 2 h in a sealed container. The derivatized material was diluted 1:4 prior to injection. Derivatization of NOC was carried out in the same manner as that of the amines, with the inclusion of an irradiation step prior to reaction with NBD-Cl. Irradiation was performed to decompose the carcinogenic *N*-nitrosamines to much less hazardous corresponding amines. The irradiation was performed in one of two setups. The first set up utilized a Hg pen lamp to irradiate the sample in the low UV while the second setup used miniature fluorescent lamps to irradiate the sample at 365 nm.

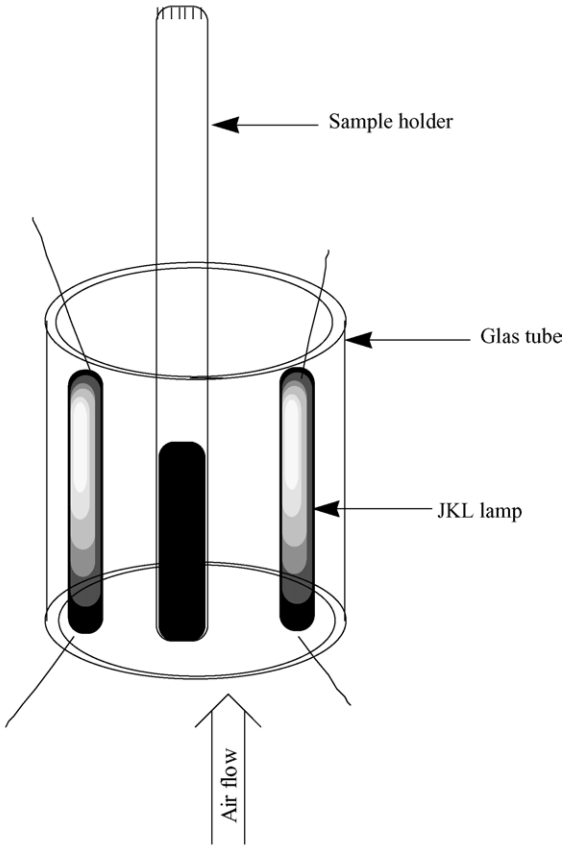

Fig. 2. Diagram of photo-reduction device.

Irradiation in the low UV used a low pressure Hg pen lamp (VWR, West Chester, PA), which delivered approximately 0.074 μW at 25 cm. The sample to be irradiated was placed in either a quartz or a glass cuvette, and irradiated for one hour while stirring. Irradiation at 365 nm utilized JKL miniature fluorescent lamps, BF325-UV1, (JKL Components Corporation, Pacoima, CA, USA). The JKL lamps provided 0.30 μW/cm2 from 2.5 cm away and were 25 mm long and 3.2 mm in diameter. Lamp emission is at 353 nm with a bandwidth of about 15 nm at half intensity. The lamps were connected to a JKL power inverter BXA12529 that required a 12 V DC power supply. A sample irradiation assembly was constructed of an NMR tube, which housed the sample and two JKL UV lamps, Fig. 2. The lamps and the sample were held in position by a glass cylinder as shown in Fig. 2. The glass was lined on the inside with foil to enhance the irradiation of the sample by internal reflection. Some of the heat generated by the lamps was lost by convection of air flow through the glass cylinder. Some sample heating did occur with irradiation temperature was approximately 57°.

3. Results and discussion

Laser induced fluorescence is an extremely sensitive method of detection and adapts readily to capillary separation methods. In order to utilize this method of detection for nitrosamines, they must be made to fluoresce. While no fluorescent derivatives of NOC are readily made, a variety of fluorescent labels are available for amines. Thus, sensitive detection for NOC can be achieved by reducing the NOC to the amine and using amine derivatives. Due to the complexity of real samples, extensive sample preparation would be required for many reduction schemes. A mild and selective reduction process would mitigate these concerns.

3.1. Reduction of nitrosamines

Electrochemical cleavage of the N–N bond in *N*-nitroso compounds to yield the corresponding amine has been described by Lunn and coworkers [7,8]. In acidic media, a nitrosamine can be reduced to an amine by a 2-electron reduction or to a hydrazine by a 4-electron reduction. The general reduction pathway of a nitrosamine to the 1,1-disubstituted hydrazine and the secondary amine is governed by the substituents, R1 and R2. With R1 and R2 both aliphatic, approximately 80% of the product is in a hydrazine form; when R1 or R2 is aromatic, the hydrazine output is reduced to about 40%; and only when R1 and R2 are both aromatic, conversion results in a 100% amine product [9]. The amine-to-hydrazine ratio also depends on the pH of the reducing solution [10].

Our attempts to achieve electrochemical reduction of the nitrosamines failed to produce adequate quantities of derivatized amines. Various pH values were tried, and a broad range of electrochemical potentials were applied, 0.8–5.0 V. The best derivatization effort following an electrochemical reduction was achieved with *N*-nitrosopyrrolidine (NPYR), as shown in Fig. 3. The solution was reduced by applying 1.0 V over 10 min across two platinum electrodes immersed in a sealed vial containing 10 mM NPYR and 1.0 mM KCl with the pH adjusted to 4.0 with HCl. Derivatized 10 mM NPYR without attempted reduction, Fig. 3C, does not exhibit a significant amount of derivatized PYR, whereas derivatized 10 mM NPYR after attempted reduction, Fig. 3B, shows a small peak appearing at 8.2 min, the migration time associated with derivatized pyrrolidine (NBD-PYR). Reduction of 10 mM NPYR solution followed by derivatization produced only less than 3% the peak area of the derivatized 10 mM PYR, as seen in Fig. 3A and B. The peak at 6.4 min in all electropherograms results from the excess NBD, apparently in the NBD-OCH3 form.

Photolytic decomposition of nitrosamines into amines and nitric oxides was investigated. This pathway is environmentally important as nitrosamines may tend to persist in the environment. Some NOC are extremely resistant toward acid/base hydrolysis but prove to be photo-chemically labile. Photolytic cleavage is the most promising reduction mechanism due to the ease of implementation and reduced probability of contamination. Photolytic reduction allows cleavage of the N–N bond without the need to introduce additional reagents. The three advantages of this venue are thus: (1) a reduced chance of sample contamination; (2) the ionic strength of the solution is not increased and (3) additional dilution of analyte is avoided. The disadvantage of photolytic reduction is that there must be devised a way to discriminate against the pre-existing amines in the sample, and to minimize the possibility of generating new amines via

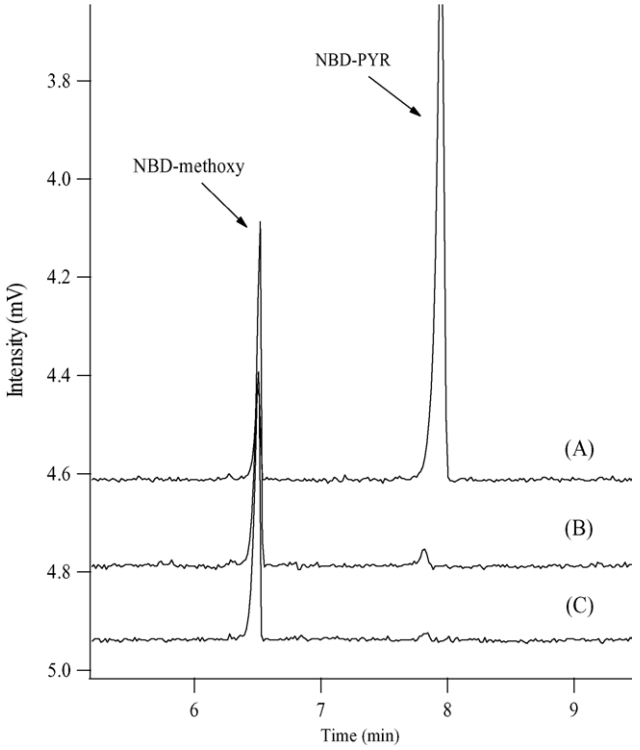

Fig. 3. Derivatization of *N*-nitrosopyrrolidine before and after its electrochemical reduction: (A) derivatized pyrrolidine (for comparison purposes); (B) derivatized pyrrolidine generated via electrochemical reduction of *N*-nitrosopyrrolidine; and (C) derivatization of *N*-nitrosopyrrolidine in absentia of electrochemical reduction.

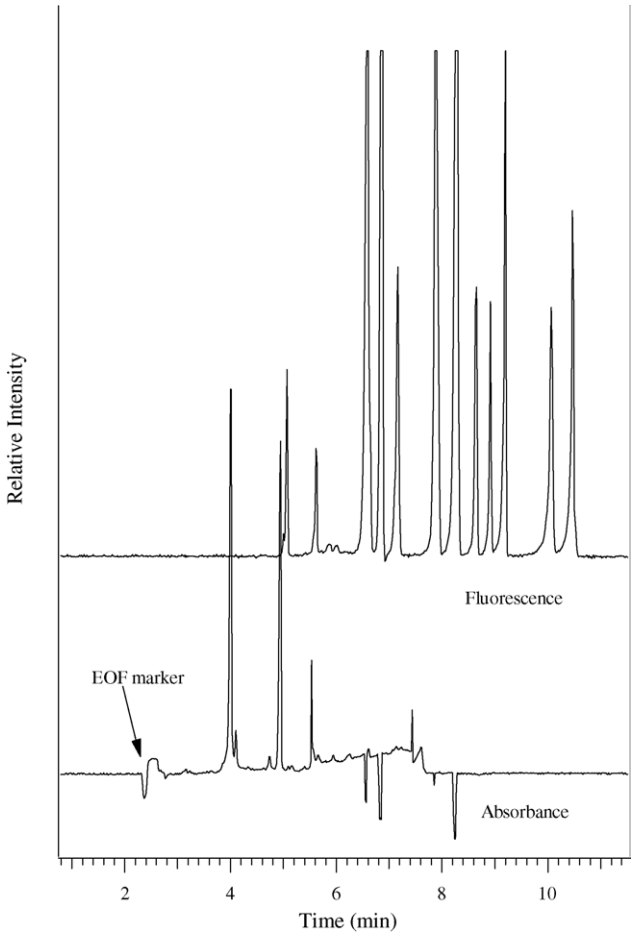

Fig. 4. Electropherogram of a mixture of NOC irradiated with a low pressure Hg pen lamp (maximum output @ 254 nm), followed by derivatization.

decomposition of the non-nitrosamine components, e.g., diazo compounds. Failure to recognize these analytical challenges will result in misidentification and misinterpretation of the results, as well as open a door to many false positive analyses. Irradiation of the NOC was attempted initially with a UV light source emitting at 254 nm. Problems occurred in trying to identify the amine peaks resulting from NOC. Then a longer wavelength light source was tried, emitting at 365 nm. This light source proved to be more effective in generating the corresponding amines without undesirable by products.

3.2. UV irradiation of nitrosamines

Nitrosamines decompose in UV light releasing nitrosyl radicals ($NO^{\bullet}$). This process has been exploited in many methods with the chemiluminescence detection of NOC. (32,64-66) The two major UV-absorbance bands of NOC are from 230–240 nm ($\pi \rightarrow \pi^*, \varepsilon = 7000$) and 350–390 nm ($n \rightarrow \pi^*$, $\varepsilon = 100$). Using a low-pressure Hg pen lamp, a 100 ppm mixture of nine *N*-nitroso compounds were irradiated for one hour in a quartz cuvette while stirring. The lamp was rated to deliver approximately 0.074 μW at 254 nm at a distance of 25 cm. The sample was placed flush against the lamp in a quartz cuvette. Since the intensity of light decreases proportionally to the inverse of the distance squared, it is estimated that the sample received approximately 50 mW of illumination at this close distance. Following irradiation, the sample was derivatized with NBD-Cl and diluted to 8.3 ppm of each component NOC. Fig. 4 shows the resulting electropherogram using absorbance (lower trace) and fluorescence (upper trace) detection. The NOC mixture consisted of the following compounds: *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine, *N*-nitrosomorpholine (NMOR), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosopiperidine (NPIP), *N*-nitrosodi-*n*-butylamine (NDBA), and *N*-nitrosodiphenylamine (NDPhA). Using the two sequential detectors allows evaluation of the relative merits of fluorescence and absorbance detection modes. The most apparent of which is the sensitivity of the fluorescence detector is far superior to the absorbance detector. Most of the nitrosamines could not be detected with the absorbance detector.

The absorbance detector precedes the fluorescence detector so the strong absorbance peaks that precede the fluorescence peaks are synonymous with fluorescence peaks, while much of the undifferentiated absorbance is missing from the fluorescence trace due to improved selectivity. The absorbance trace of the irradiated and derivatized mixture of NOC shows several negative dips. The background buffer solution, 50 mM SDS with 10 mM borate, has appreciable intrinsic absorption in the low UV. The injected original aqueous non-absorbing sample matrix

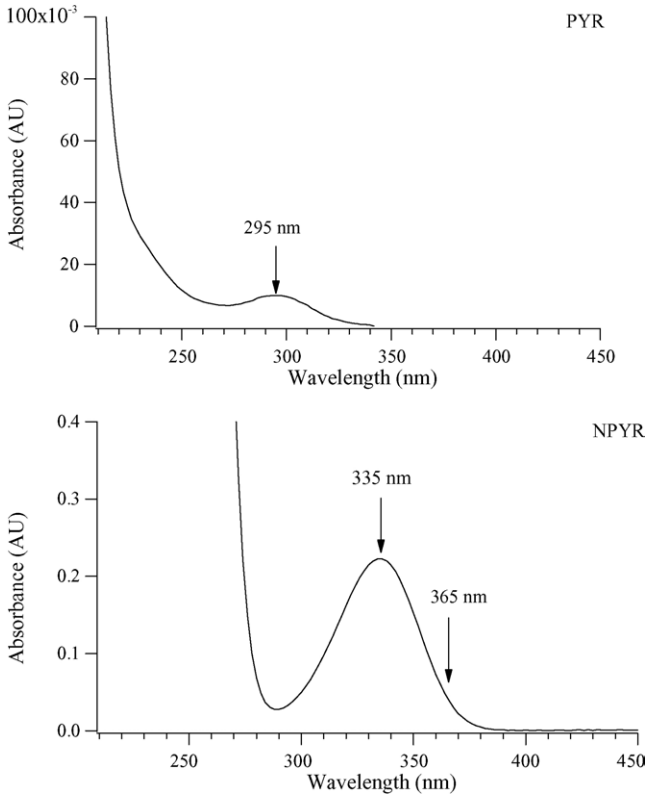

Fig. 5. (Upper) absorbance spectrum of pyrrolidine and (Lower) absorbance spectrum of *N*-nitrosopyrrolidine.

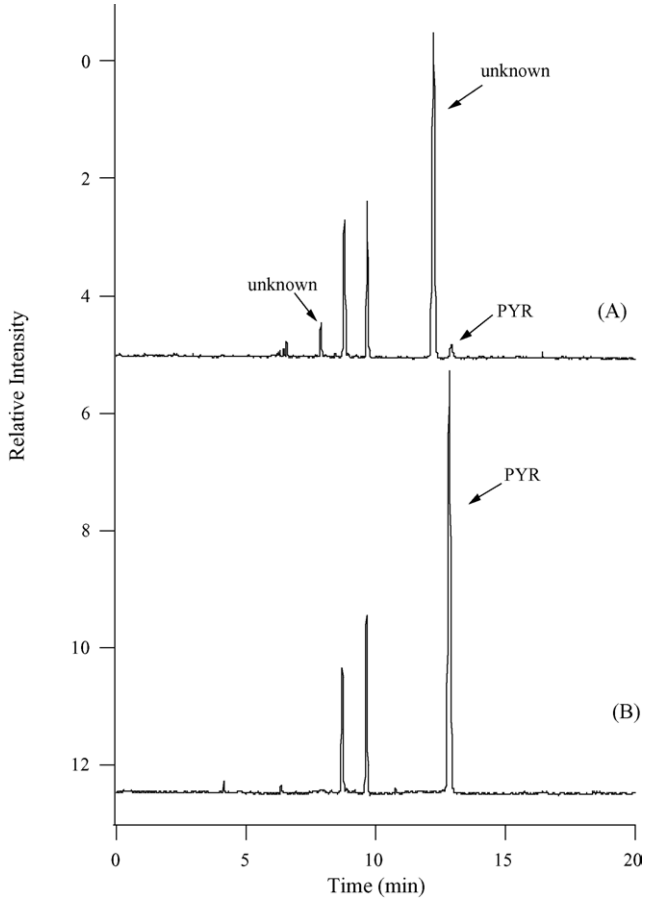

Fig. 6. Comparison of photo-irradiation products of NPYR at: (A) 254 nm and (B) 365 nm.

travels through the capillary as a plug. Its passage through the detector, results in a negative dip. This phenomenon is common in MEKC and is often used to determine the EOF, electroosmotic flow (flow of the bulk solution through the capillary). In our separation, the negative dip is observed at 2.3 min. At the capillary length to the detector of 46.5 cm, the velocity of the electroosmotic flow is 20.2 cm/min. The negative dips observed in the absorbance trace at 6.5, 6.8 and 8.2 min coincide with strong fluorescence signals. A probable reason for these peaks is the internal reflection of the strong omni-directional fluorescence signal along the separation, which is registered by the absorbance detector photomultiplier tube. PMT is indiscriminate toward the signal wavelength, and is registering the excess fluorescence-derived photons as negative spikes. This phenomenon is particularly noticeable with the most prominent fluorescence peaks.

To identify the peaks in the electropherogram of Fig. 4, the amine standards were derivatized, ran independently, as well as spiked into the mixture. During the peak identification process it was found that pyrrolidine (PYR) was missing from the UV-irradiated and derivatized NOC mixture. The UV-irradiation of the NOC mixture failed to convert nitrosopyrrolidine to pyrrolidine. The low-pressure Hg lamp emits strongly at nm 254 where both PYR and NPYR absorb. The absorbance spectra of PYR and NPYR are shown in Fig. 5A and B, respectively. The local absorbance maximum of NPYR at 335 nm is due to the nitroso group. It is not clear if the 254 nm irradiation is either not producing PYR from NPYR or if pyrrolidine may have been depleted by photodecomposition. It is clear that irradiation at 254 nm does not yield PYR from NPYR.

Narrow-band irradiation in the long wave UV region was attempted next to provide a more selective light source for photolysis of the nitrosamines. Micro-fluorescent lamps purchased from JKL Components, Inc. provide an output with a maximum at 365 nm with a 20 nm band with at half height. Because fewer compounds absorb at 365 nm, the JKL lamps were expected to provide a better source for the selective photo-cleavage of NOC. To compare the two sources of irradiation, the Hg pen lamp with its maximum output at 254 nm and a 365-nm JKL lamp, each light source was used to irradiate a 10 ppm NPYR sample. Electropherograms obtained upon irradiation and derivatizations of these two samples are shown in Fig. 6. Two distinctly different photoproducts were obtained. The product of the pen lamp irradiation (upper trace) gave rise to a major peak at 12.3 min and minor peak at 12.8 min, whereas JKL lamp (lower trace) generated primarily the peak at 12.8 min. Based on the derivatization of a 10 ppm PYR standard with NBD-Cl, it was concluded that irradiation with the JKL lamp quantitatively decomposed NPYR to PYR, while the pen lamp failed to do so.

The absorption spectrum of a 68-ppm PYR solution, Fig. 6A, shows that PYR does not have any appreciable absorbance above 250 nm so that irradiation of NPYR at 365 should not cause photodecomposition of this compound. Fig. 7A shows the

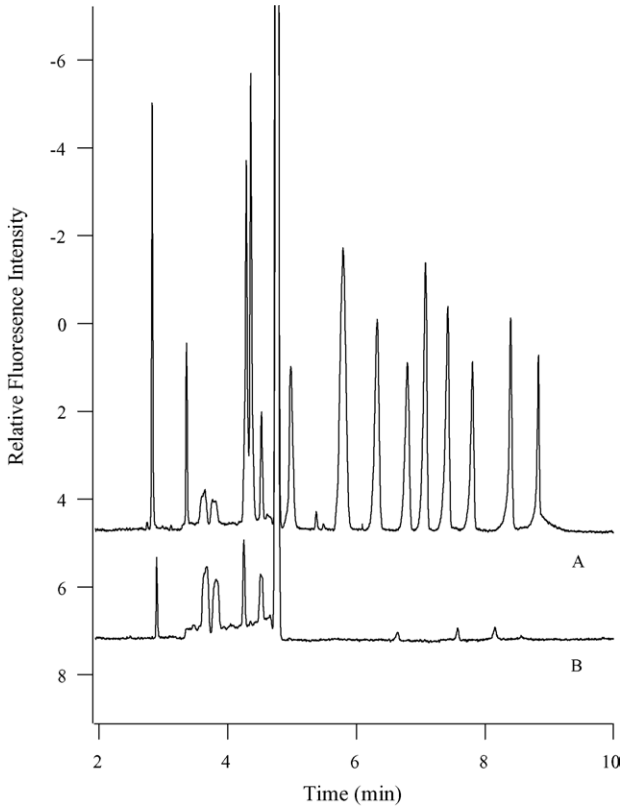

Fig. 7. Separation of *N*-nitrosamine mixture (A) and comparison to a derivatization blank (B). Mixture was irradiated at 365 nm and derivatized with NBD-Cl.

electropherogram of the NOC mixture following irradiation at 365 nm and subsequent derivatization. For comparison the electropherogram for a derivatization blank is also shown, Fig. 7B. The derivatization blank was a water sample subjected to an identical derivatization procedure. The blank reveals some of the many impurities arising from the derivatization process.

The fate of irradiated NPYR was examined using gas chromatography–mass spectrometry (GC–MS) analysis of PYR, NPYR, and NPYR after irradiation with each of the two light sources. The total ion chromatogram of a NPYR in methanol standard showed primarily NPYR but also a small amount of PYR as an impurity. The chromatogram of NPYR after irradiation at 254 nm with the Hg-pen lamp showed a new chromatographic peak. This component has a base peak at $m/z = 61$ and was unidentified by library matching. There were also several small-unidentified peaks indicative of a variety of photo-products. There was no discernable PYR or residual NPYR. Thus, irradiation of NPYR with 254-nm light does not generate PYR, but a variety of unidentified photo-products. The chromatogram of NPYR after irradiation at 365 nm showed primarily a large PYR peak and much fewer unidentified photo-products.

The photolytic formation of amines from NOC is favored in acidic solutions [11,12]. The protonated form of the secondary nitrogen favors cleavage of the N–N bond. Photolysis in acidic, basic and neutral environments was attempted. Irradiation of NPYR in 0.1 M acetic acid and 0.1 M hydrochloric acid followed by derivatization resulted in reduced peak heights

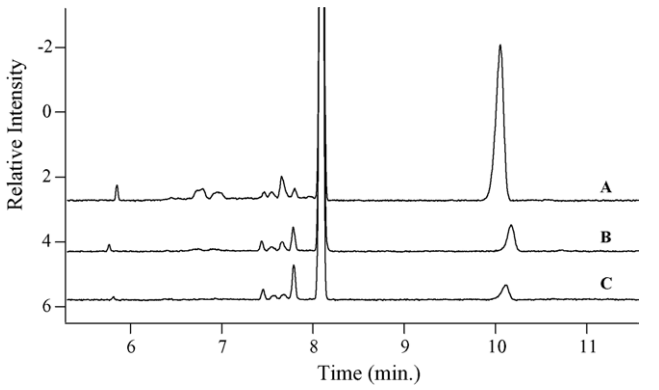

Fig. 8. Separation of NPYR after one hour of UV irradiation at 365 nm and derivatization with NBD-Cl. Irradiation performed in: (A) DI water; (B) 0.1 M acetic acid; and (C) 0.1 M hydrochloric acid.

in the electropherogram. Fig. 8 shows three electropherograms resulting from irradiation in deionized water (A), acetic acid (B) and hydrochloric acid (C) followed by derivatization. In the acidic environments, the size of all of the peaks, including the impurities, was reduced in intensity. The reduction in peak heights suggests that derivatization was less efficient in the presence of acid. Of the three irradiated solutions shown, the irradiation without pH adjustment has the largest NPYR peak height. An acidic environment may promote the photo-reduction of the NOC, but it is also detrimental to the derivatization process. The derivatization reaction is pH-dependent and liberates acid as a product. To maximize the yield of derivatized amines from nitrosamines, the remainder of the photolytic decomposition experiments was conducted in deionized water without pH adjustment.

A study of yield versus irradiation time was performed. An optimal irradiation time of 60 min was chosen as a good compromise between the yield of pyrrolidine and time required to perform experiment. Separation and identification of the EPA mixture of nine *N*-nitrosamines is shown in Fig. 9. The peaks were identified by retention time matching as described above. The migration time of the nitrosamines increased proportionally to the molecular weight increase, with the exception of the cyclic amines, as shown in Table 1. Both of the cyclic amines, pyrrolidine and morpholine, have shorter migration times than could have been predicted based strictly on molecular weights. Not all the peaks that appeared in the electropherogram of the deriva-

Table 1
Correlation between amine retention times and molecular weights

Peak no.	Migration time (min)	Abreviation	Amine	M.W.
1	5.00	AMM	Ammonia	17.03
4	6.44	NDMA	Dimethylamine	45.08
6	7.03	NMOR	Morpholine	87.12
7	7.79	NMEA	Methyl ethylamine	59.11
8	8.28	NPYR	Pyrrolidine	71.12
9	8.69	NDEA	Di ethylamine	73.14
11	9.27	NPIP	Piperdine	85.15
13	10.18	NDPA	Di-*n*-propylamine	101.19
14	10.59	NDBA	Di-*n*-butylamine	129.25

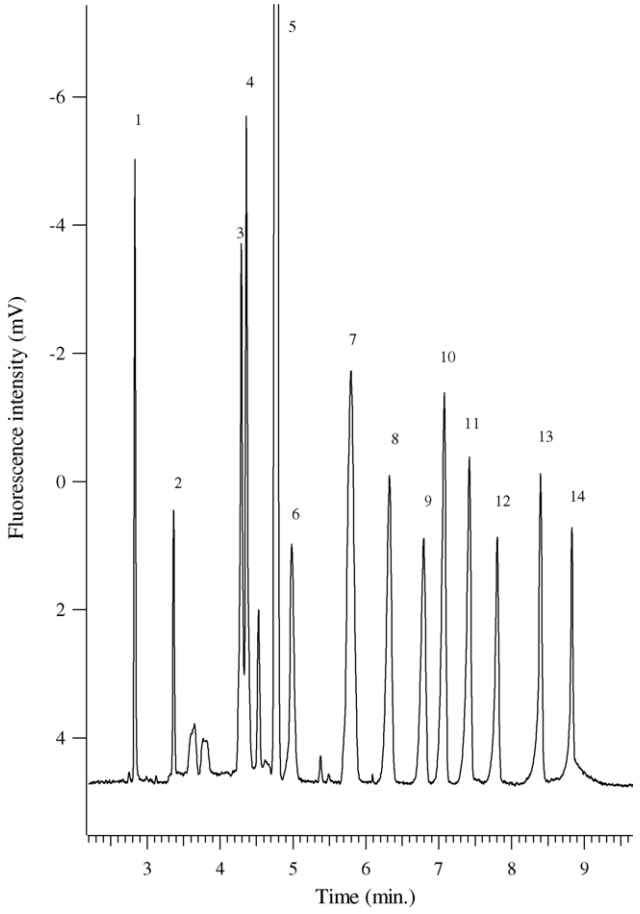

Fig. 9. Separation of nine *N*-nitrosamines, 40 ppm each, after irridation at 365 nm and derivatization with NBD-Cl. Buffer: 50 mM SDS and 10 mM Borate at pH 9.3. Peaks: 1 = ammonia, 2, 4 = NDMA, 5 = NBD-OCH3, 6 = NMOR, 7 = NMEA, 8 = NPYR, 9 = NDEA, 11 = NPIP, 13 = NDPA, 14 = NDBA.

tized NOC mixture were identified. When a standard of DMA was derivatized, two peaks appeared in the electropherogram. These two peaks also appear in the NOC derivatized mixture, Peaks 2 and 4. The origin of these two peaks is unclear. The peaks 0 and 12 could not be unidentified. *N*-nitrosodiphenylamine was not seen in the electropherogram, possibly due to its solubility limitations. Table 2 summarizes the separation parameters for each of the *N*-nitrosamines identified in Fig. 9.

Table 2
Parameters for the electrophoretic separation of the mixture of *N*-nitrosamines

Peak no.	ID	w_1/2	*N*	*α*	*k*'	*R*_s[a]
1	AMM	0.021	320,000	1.37	0.53	11.61
2	NDMA	0.023	300,000	1.13	0.76	5.05
4	NDMA	0.036	170,000	1.03	1.26	0.81
6	NMOR	0.050	110,000	1.06	1.62	1.36
7	NMEA	0.082	49,000	1.12	2.15	2.24
8	NPYR	0.063	96,000	1.09	2.55	2.34
9	NDEA	0.057	130,000	1.05	2.93	1.58
11	NPIP	0.046	230,000	1.06	3.52	2.18
13	NDPA	0.059	170,000	1.06	4.67	1.96
14	NDBA	0.030	670,000	1.06	5.31	1.96

Calculation and definition of variables provided in text.
[a] Resolution based on $t_0/t_{mn} = 0.22$.

3.3. Quantitation

A calibration curve was generated for NPYR. Each point in the curve was derived from the average peak height of three sequential injections. The dynamic range tested was limited by the electronics of the fluorescence detector. As the concentration increased, the peaks reached the maximum output of the amplifier before deviating from linearity. The R2 value for the NPYR calibration curve was 0.993. The goodness of fit is more accurately described by the standard deviation on the slope, m, and the Y-intercept, b. The coefficients for the PYR calibration curve ± one standard deviation are given as: [Fluorescence signal] = m [PYR conc.] + b, where $b = 112.3 \pm 70.1$ and $m = 51.53 \pm 1.92$. The response was linear from 30 ppb to 5.7 ppm. The detection limit the amount of sample that gives a peak height of three times the baseline noise is considered by convention its detection limit. The noise was evaluated at the baseline by recording the detector response over a period of about 10 times the peak width. Given these conditions, the detection limit of the amine test sample was determined to be as low as 9 ppb. The separation performance also has an impact on the detection limits. Increased separation efficiency will decrease the detection limits because as the peak width decreases, a more concentrated plug of sample will pass through the detector, giving rise to a stronger signal. Both the absorbance and the fluorescence detectors are concentration-dependant, which means that analyte's peak areas and heights are proportional to its concentration in the sample.

4. Conclusions

The ubiquity of the precursors and the ease of formation give *N*-nitrosamines potentially wide range of occurrences. Volatile *N*-nitrosamines have already been reported in malt beverages, foodstuff, biological fluids and rubber products. The high carcinogenicity of these compounds warrants fast reliable methods for detecting their presence at trace levels in complex matrices, such as biological samples. The current methods for *N*-nitrosamine analysis are limited. The most widely used method is thermal energy analyzer, first developed in 1976. [13] It provides fast, sensitive and selective determination of these compounds, but is limited to volatile non-ionic *N*-nitroso compounds. The inapplicability of the TEA to non-volatile, ionic and thermally unstable *N*-nitrosamines has stimulated the discovery of alternative detection needed to accurately evaluate the total *N*-nitrosamine occurrence. As an alternative to the TEA, several groups developed derivatization processes for *N*-nitrosamines. This paper demonstrates the utility of selective photo-reduction of *N*-nitrosamines followed by MEKC-LIF as a means of selective *N*-nitrosamine determination.

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References

[1] R.N. Loeppky, C.J. Michejda, Nitrosamines and Related *N*-Nitroso Compounds: Chemistry and Biochemistry, American Chemical Society, Washington, DC, 1994.
[2] B.C. Challis, J.A. Challis, in: S. Patai (Ed.), The Chemistry of Amino, Nitroso and Nitro Compounds and their Derivatives, Supplement F, John Wiley & Sons Ltd., New York, 1982.
[3] N.P. Sen, P.A. Baddoo, D. Weber, T. Helgason, J. Agric. Food Chem. 38 (1990) 1007.
[4] R.N. Loeppky, in: R.N. Loeppky, C.J. Michejda (Eds.), Nitrosamines and Related *N*-Nitroso Compounds, 553, 1994.
[5] H. Kataoka, S. Shindoh, M. Makita, J. Chromatogr. A 723 (1996) 93.
[6] G.M. Murray, M.J. Sepaniak, J. Liq. Chromatogr. 6 (1983) 931.
[7] G. Lunn, E.B. Sansone, Synthesis 12 (1985) 1104.
[8] G. Lunn, E.B. Sansone, L.K. Keefer, J. Organ. Chem. 49 (1984) 3470.
[9] G. Jacob, C. Moinet, A. Tallec, Electrochim. Acta 27 (1982) 1417.
[10] H. Lund, Acta Chem. Scand. 11 (1957) 990.
[11] Y.L. Chow, Spectr. II (1998) 8.
[12] T.-F. Ho, J.R. Bolton, E. Lepczynska-Kochany, J. Adv. Oxid. Technol. 1 (1996) 170.
[13] D. Fine, D. Ross, D.P. Rounbehler, A. Silvergleid, L. Song, Nature 256 (1977) 268.