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Determination of N-nitrosodiethanolamine as nitrite in ethanolamine derivative raw materials by high-performance liquid chromatography with fluorescence detection after alkaline denitrosation☆

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Abstract

A precise and sensitive method is described for the determination of N-nitrosodiethanolamine (NDELA) at the 1 $\mu g\ l^{-1}$ level. NDELA was found to be extracted from aqueous solution by ethyl acetate at pH 1.0. The technique is based on the alkaline denitrosation of NDELA and reaction of the nitrite liberated with Coumarin 120 (4-methyl-7-aminocoumarin) in sulphuric acid medium to give the corresponding 7-diazo compound. After hydrolysis at 100°C, the corresponding 7-hydroxycoumarin (4-methylumbelliferone) is detected fluorimetrically at 380 nm after excitation at 325 nm. HPLC is essential to separate excess Coumarin 120 from 4-methylumbelliferone. Linearity was obtained from 1 to 30 $\mu g\ l^{-1}$ ($r = 0.997$). The limit of detection was 0.8 $\mu g\ l^{-1}$ for a signal-to-noise ratio of 3. The repeatability expressed as R.S.D. was 6.8% at 1 $\mu g\ l^{-1}$ and 5.5% at 30 $\mu g\ l^{-1}$. The reproducibility, established by two operators on three consecutive days at 5 $\mu g\ l^{-1}$ ($n = 6$), was 9.2% (R.S.D.). Under the conditions adopted, detection of trace levels of NDELA in ethanolamine derivatives is possible.

Keywords: Derivatization, LC; N-Nitrosodiethanolamine

1. Introduction

Mutagenic [1] and carcinogenic [2,3] properties of aliphatic N-nitrosamines require precise and sensitive methods for their determination; among them, nitrosodiethanolamine (NDELA) is certainly one of the most common species encountered [4]. Several methods based on different principles have been described for the determination of NDELA, including HPLC combined with thermal energy analysis (TEA), gas chromatography coupled with TEA [5], high-resolution mass spectrometry [6] and electron-capture detection [7]. The reference method is based on chemiluminescence [8,9]. This last technique is based on the reaction between ozone and nitric oxide produced by homolytic scission of nitrosamines to give an excited nitrogen dioxide mole-

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cule and its return to the ground state produces energy by chemiluminescence. This method is interesting owing to the specific response for nitroso- and some nitro compounds, but has the disadvantage of requiring expensive equipment unusable for other quantitative analyses. With such a method, trace levels of about 1 ppb of nitrosamines can be determined [5]. Another method used the spectrophotometric determination of coloured diazo compounds obtained from heterolytic scission of N-nitrosamines [10]. This approach is easily affordable in most laboratories but the limit of detection is insufficient for the determination of nitrosamines.

Owing to the above, our laboratory has developed two spectrofluorimetric methods for nitro- and N-nitroso compounds. One of them allows the chromatographic separation and determination of amines liberated in an acidic medium from selectively extracted N-nitrosamines [11]. The other allows the determination of traces of nitrites in water at the 1 $\mu g\ l^{-1}$ level [12] (Fig. 1). The goal of this work is to extend the method described for nitrites to N-nitrosamines after a selective extraction and denitrosation in alkaline medium. Based on this denitrosation method, only nitrites were produced in this medium.

For the development of the proposed method, we were especially interested in NDELA, which is difficult to separate from cosmetic products and determine owing to its non-volatile and polar character. Ethanolamines are largely used as auxiliary substances for fatty acids to give ethanolamides, which have foam-boosting and moisturizing properties [13,14]. As an example, the synthesis of diethanolamide can lead to by-products such as NDELA. Diethanolamine (DELA) can also appear during the treatment of diethanolamide under unfavourable conditions [13,14]. In both cases, DELA can undergo further nitrosation to NDELA. Hence the determination of NDELA at low levels is of importance owing to its well established carcinogenic characteristics [15,16].

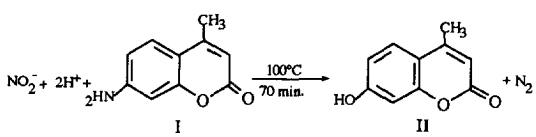


Fig. 1. Mechanism of the derivatization reaction.

2. Experimental

2.1. Reagents and standards

All chemicals were of analytical-reagent grade, except hexane and 2-propanol used in the chromatographic separation, which were of HPLC grade (Merck, Darmstadt, Germany). Nitrosodiethanolamine and diethanolamine were purchased from Sigma (St. Louis, MO, USA) and 4-methyl-7-aminocoumarin (Coumarin 120) and 4-methyl-7-hydroxycoumarin (methylumbelliferone) from Kodak (New York, USA). High-purity water obtained with a Milli Q system (Millipore, St. Quentin, France) was used throughout.

2.2. Apparatus

Direct absorbance measurements were performed with a Shimadzu (Kyoto, Japan) UV 2100 absorbance spectrometer at 20 ± 2°C using 1-cm optical path quartz cuvettes.

2.3. Solutions

Solution 1 (S_1) was buffer solution (pH 12.4) prepared by dissolving 3.8 g of trisodium phosphate dodecahydrate in 100 ml of water previously boiled ana adjusted to pH 12.4 with 0.1 HCl. Solution 2 (S_2) was a $2 \cdot 10^{-4}$ *M* methanolic solution of Coumarin 120 obtained by appropriate dilution of 10^{-3} *M* Coumarin 120 kept protected from light at 4°C for no more than 2 days. Solution 3 (S_3) was 0.5 *M* H_2SO_4. Solution 4 (S_4) was an acidic methanolic solution of Coumarin 120 (50 μl of S_2 in 950 μl of S_3).

2.4. Extraction

To 20.0 ml of aqueous solution to be tested, 200 μl of freshly prepared 0.01 *M* ascorbic acid were added to prevent artefactual nitrosation [7,8,17,18]. The solution obtained was adjusted to pH 1.0 with 1 *M* HCl and vortex mixed. This solution was extracted with 3×50 ml of ethyl acetate. The organic layer was dried by passing it through a column (250 mm $\times$ 10 mm I.D.) packed with a 1.0-cm layer of anhydrous disodium sulphate. The organic solution was evaporated to almost dryness by a rotary evaporator at room temperature. The remaining organic phase was then removed under a gentle stream of nitrogen (Air Liquide, Puteaux, France).

2.5. Denitrosation and precolumn derivatization

A 0.2-ml volume of S_1 was added to dissolve the residue, vortex mixed and kept protected from light for 15 min, then 1 ml of S_4 was added. The sealed tubes were heated at 100°C for 70 min, cooled to room temperature and the fluorescent products were extracted with ethyl acetate (2×2 ml). The organic layer was carefully transferred into an appropriate vial and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in anhydrous ethanol (0.4 ml) and 20 μl were injected into the liquid chromatograph. As the derivatization spontaneously yields (in the absence of nitrites ions) a small amount of 4-methylumbelliferone, a blank must be performed.

2.6. Liquid chromatography (LC)

Normal-phase LC was performed with a Shimadzu LC 9A metering pump equipped with a Rheodyne (Cotati, CA) Model 7125 injector with a 20-μL loop. Isocratic elution was performed with a mobile phase of hexane–2-propanol (95:5, v/v), which was first degassed by ultrasonication for 30 min. The flow-rate was set at 1.8 ml min^{-1}. The column was a 100 mm $\times$ 4.6 mm I.D. Spheri-5 cyano-bonded column from Brownlee thermostated at 20 ± 0.5°C with a Crococil (Ste. Foy, France) oven. Detection was performed with a Shimadzu RF 551 fluorimetric detector with excitation and emission wavelengths set at 325 and 380 nm, respectively. The chromatograms were recorded with a Shimadzu LC 5A integrator.

3. Results and discussion

3.1. Denitrosation process

Denitrosation in acidic medium

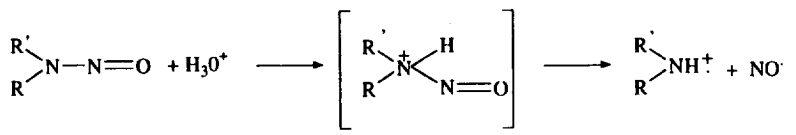
The occurrence of the emergence of nitrosonium ion (NO^+) photolytically induced from nitrosamine in acidic aqueous solution was deminstrated [10,19,20]. The NO^+ ions were then diazo coupled with the solvent or with a primary amine in order to be quantified. In the first case some side-reactions contributed to diminish the nitrous acid yield according to reactions 1 and 2 displayed in Fig. 2.

Reaction 3 (Fig. 2) occurred when the pH was lower than the p*K* of nitrous acid (i.e., <3.4). The photolytic reaction in El Assaf's method was applied in a solution with a low concentration of NDELA in order to perform spectrofluorimetric detection. Under these conditions the signal obtained was not correlated with the initial amount of nitrosamine involved; especially for the lowest concentration, this amount was greater than that corresponding to stoichiometry. This was attributed to the previously cited side-reactions (reactions 1 and 2, Fig. 2).

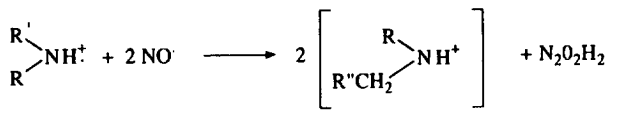
Another possibility reported in the literature [21–23] consists in denitrosation of the nitrosamine by hydrobromic acid in anhydrous acetic acid. The results cannot be exploited quantitatively; the presence of bromide ion gives 4-methyl-7-bromocoumarin after reaction with diazonium salt at 100°C; this last product is not fluorescent (reaction 4, Fig. 2) and consequently rules out the possibility of analytical use.

Denitrosation in alkaline medium

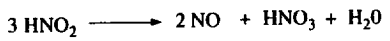
According to the previously reported unsuccessful attempts and the instability of nitrosamines in alkaline medium, degradation at high



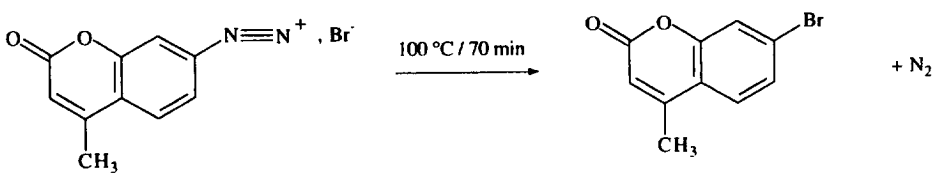
REACTION 1



REACTION 2



REACTION 3



REACTION 4

Fig. 2. Degradation pathway of N-nitrosamines in acidic medium.

pH could certainly be ascribed to denitrosation. The idea developed here is to exploit this degradation process in order to determine nitrosamines indirectly. To our knowledge, this approach has never been reported for the determination of nitrosamines. A preliminary spectrophotometric study of DELA and NDELA aqueous solutions at different pH values was performed to evaluate the possible interest of the proposed approach. Whatever the conditions, the DELA spectrum shows an important transition ($\log \varepsilon = 4$) at $\lambda = 223 \pm 5$ nm. In contrast, the UV spectrum of NDELA is greatly affected by the pH.

In alkaline medium (pH > 9), the spectrum NDELA became similar to that of DELA with two bands, one centred at 203 nm and the other at 223 ± 2 nm (absorbance maximum). Moreover, as shown in Table 1, for the same concentration of DELA and NDELA the absorbance at 223 nm is similar. This strongly suggests the formation of the corresponding amine by the elimination of the nitroso group in alkaline medium. Then, nitrosonium ions and the hydroxide ions react according to the following reactions: $NO^+ + OH^- \rightarrow HNO_2$ and $HNO_2 \rightarrow NO_{2-} + H^+$ (with $[NO_{2-}]/[HNO_2] = 10^9$ at pH 12.4). This alkaline solution shows a band centred at 203

nm, which corresponds to the $\pi \rightarrow \pi^*$ transition of the nitro compounds (nitrite or/and nitrate) [24]). In alkaline medium, the –N–N=O bond is cleaved to give nitrite. Indeed, for a pure nitrite solution one band is located at 210 nm and for a pure nitrate solution one band is located at 200 nm. Hence it can be suggested that the nitroso group is eliminated in alkaline medium to give nitrite according to the reactions previously cited, whereas the UV spectrum of an equimolar mixture of nitrite and nitrate shows a band centred at 203.5 nm. Hence this succinct spectrophotometric study confirmed in part the oxidation of nitrite to nitrate.

3.2. Optimization of the nitrite emergence reaction

Surprisingly, the yield of the reaction (calculated by nitrite determination according to Zhou et al. [11]) was only 40%. Since Table 1 suggests complete denitrosation, oxidation of nitrite to nitrate could be envisaged at high pH. In order to improve this yield, first we operated with a jet stream of nitrogen to control the catalytic effect of oxygen during the denitrosation procedure. Probably owing to traces of nitrite in solution and the incomplete elimination of oxygen in ambient air, no increase in yield was observed. Second, 10^{-8} and $5 \cdot 10^{-8}$ M EDTA were added in order to chelate the traces of metal capable of catalysing the redox reactions. The reaction yield remained low (only 45%) and this was re-examined in the light of the NO_3^-/NO_2^- potential evolution as a function of pH according to the reaction

$$NO_2^- + 2OH^- \rightleftharpoons NO_3^- + H_2O + 2e^-$$

The potential of the system follows the Nernst equation with $E_0 = 0.01$ V and, after replacement of the OH^- concentration value by $10^{-14}/[H^+]$ the apparent standard potential E_0' was a function of pH according to $E_0' = 0.84 - 0.059$pH. The higher the pH, the lower is the apparent standard potential, and consequently the easier the oxidation of nitrite to nitrate. Nevertheless, a sufficient concentration of hydroxide ion is essential for the denitrosation. Hence it was necessary to determine the optimum conditions for both pH and the reaction time.

First, we operated at pH 11 to limit the nitrite oxidation, and a reproducible yield ($70.1 \pm 8.7\%$, $n = 6$) with a 50-min reaction time was achieved (Fig. 3). However, a reaction time for denitrosation seemed too long, so we used a higher pH (12.2) to speed up the reaction. In order to maintain the pH stable during the determination, we used phosphate buffer solution (pH 12.2); the results showed a 15% increase in yield compared with NaOH solution at the same pH (Table 2). The optimum pH was fixed at 12.4 and the optimum volume of the sample at 0.2 ml. Under these conditions we obtained reaction yield of $69.7 \pm 4.7\%$ with a denitrosation time of 15 min.

Table 1
Effect of alkaline pH on NDELA spectrum

λ (nm)	Log ε[a]			
	pH 8.90	pH 12.50	pH 13.00	pH 13.80
203	3.5	–[b]	–	–
223 ± 2	–	4.1	4.2	4.1
235 ± 5	4	–	–	–

NDELA concentration = 5 $\mu g\ l^{-1}$.
[a] ε = Molar absorptivity (1 mol^{-1} cm^{-1}).
[b] Dashes indicate absence of a peak.

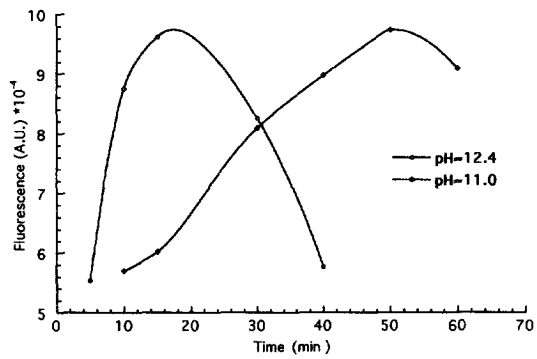


Fig. 3. Effect of time on nitrite emergence after denitrosation with (1) NaOH (pH 11) and (2) phosphate buffer (pH 12.2). NDELA concentration = 5 $\mu g^{-1}\ l^{-1}$. Each point is the mean of three measurements.

Table 2
Denitrosation yields (%) for two levels of NDELA at pH 12.2 adjusted with NaOH and phosphate buffer

NDELA concentration ($\mu g\ l^{-1}$)	Denitrosating agent	
	NaOH	Phosphate buffer
5	41 ± 2	69 ± 4
25	43 ± 9	68 ± 7

Results are means ± S.D. for three measurements.

3.3. Analytical parameters and application to real samples

Experiments have shown that the separation of the solvent peak from the 4-methylumbelliferone peak is possible only under strictly controlled conditions. Hence the composition of the hexane–2-propanol mobile phase was maintained at 95 (±1) to 5 (±1) v/v with a selected resolution factor criterion of $R_s = 2 \pm 0.2$. Under these conditions, linearity was obtained from 1 to 30 $\mu g\ l^{-1}$ ($r = 0.997$). As the derivatization process yields 4-methylumbelliferone, we first determined the limit of detection (LOD) and limit of quantification (LOQ) for this compound in standard solution. The LOD (for $S/N = 3$) and LOQ were found to be 0.3 and 0.9 $\mu g\ l^{-1}$, respectively. Second, the LOD and LOQ were determined under the derivatization conditions (see Experimental), and were 0.8 and 1 $\mu g\ l^{-1}$, respectively. The repeatability, expressed as R.S.D., was 6.8% at 1 $\mu g\ l^{-1}$ ($n = 6$) and 5.5% at 30 $\mu g\ l^{-1}$ ($n = 6$). The reproducibility was established by two operators on three consecutive days and the R.S.D. was 9.2% at 5 $\mu g\ l^{-1}$ ($n = 6$).

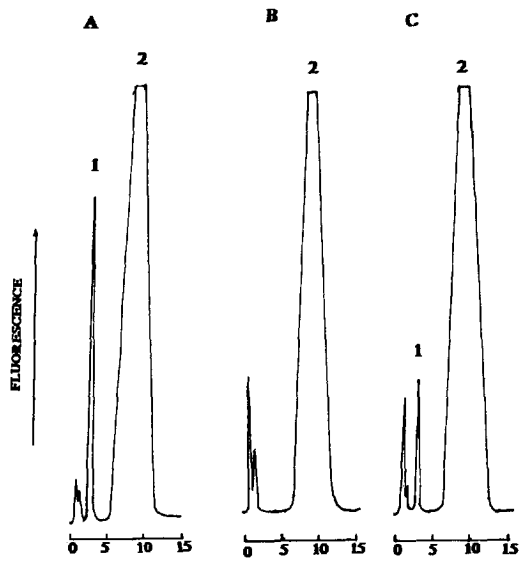


Fig. 4. Typical chromatograms of aqueous solutions of (A) NDELA standard (8 $\mu g\ l^{-1}$), (B) DELA solution blank and (C) DELA spiked with NDELA (5 $\mu g\ l^{-1}$). Peaks: 1 = 4-methylumbelliferone; 2 = excess of Coumarin 120. For derivatization and chromatographic conditions, see text.

Fig. 4 shows the chromatogram of NDELA obtained after performing the proposed procedure. The selectivity of the method can be seen; moreover, for real samples (DELA) the resolution remains satisfactory with a convenient separation between 4-methylumbelliferone and the excess of unreacted Coumarin 120 ($R_s = 6.5$). Hence the selected mobile phase previously optimized for the determination of nitrite [11,23] seems well adapted for our purpose. In our experience, extracted impurities do not interfere in the chromatogram, which could have indirectly perturbed the selectivity of the chromatographic procedure used here.

In conclusion, these first results obtained with NDELA in ethanolamine derivatives (DELA) indicate that the present method should be widely applicable to non-volatile or volatile N-nitrosamines (N-nitrosodimethylamine, N-nitrosodiethylamine, etc.) after performing an appropriate extraction of the nitrosamines from cosmetic products and toiletry articles.

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