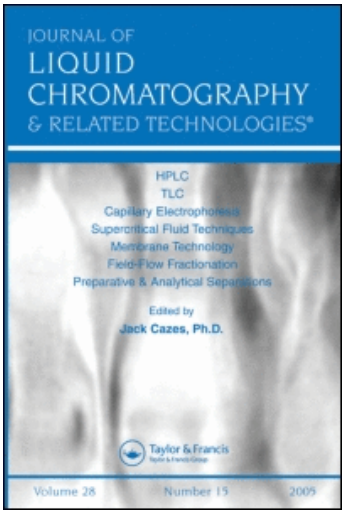

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Simultaneous Determination of Five Volatile and Non-Volatile N-Nitrosamines in Biological Fluids and Cosmetic Products by Liquid Chromatography with Photodiode Array Detection

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Abstract: An HPLC method has been developed for the determination of nitrosamines. Five nitrosamines were separated simultaneously on a 250 mm × 4.6 mm i.d. Phenomenex Luna CN (particle size 5 μm) column with methanol-1.0 m M K_2HPO_4 (pH 4.0) solvent, programmed as mobile phase and with a photodiode array detector. The limits of detection were 0.02, 0.02, 0.02, 0.03, and 0.03 mg L^{-1} for N-nitrosodiethanolamine, N-nitroso-bis-(2-hydroxypropyl)amine, N-nitrosodimethyamine, N-nitrosodi-n-propylamine and N-nitrosodiphenylamine, respectively. The method is applied for the simultaneous quantitative determination of nitrosamine in cosmetics and biological fluids.

Keywords: RP-LC, Photodiode array detector, Volatile and non-volatile N-nitrosamines, Cosmetics and biological fluid

INTRODUCTION

The N-Nitrosamines are classified into four classes, which are as follows: (I) volatile N-nitroso compounds such as N-nitrosodimethylamine and N-nitrosodi-n-propylamine; (II) low polarity, non-volatile N-nitroso compounds such as N-nitrosodiphenylamine; (III) high polarity, non-ionic,

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non-volatile N-nitroso compounds such as N-nitrosodiethanolamine; (IV) high polarity, ionic, non-volatile N-nitroso compounds such as N-nitrosoproline. Di-and triethanolamine or certain derivatives there of, such as diethanolamines fatty acids are used extensively as cosmetic ingredients.[1–3] These cosmetic ingredients functions as a surfactant foam booster and an aqueous viscosity increasing agent. The high reactivity of secondary amines, such as diethanolamine (DEA) with nitrosating agents, such as nitrite, can result in the formation of N-nitrosodiethanolamine (NDELA), a potent carcinogen. Diethanolamine, the major amine precursor of NDELA in cosmetics and found in a number of consumer products, reportedly induces liver tumors in mice.[4] N-Nitrosamines, also found in a wide variety of cosmetic products, are either formed within the product itself by reaction of precursors or introduced as a result of the use of contaminated raw materials.[5] The European Economic Community (EC) Cosmetics Directive states that fatty acids diethanolamines raw materials should contain less than 5% diethanolamine and less than 50 ppb NDELA, and that cosmetic products should contain less than 0.5% dialkanolamine.[6,7] The fatty acids in coconut oil and its derivatives (cocamide), monoethanolamine (MEA), and DEA (diethanolamine), were included in this safety assessment. These data suggest little acute, short term, or chronic toxicity associated with dermal application.[8] MEA vapor is highly toxic. It is has been reported that cigarette smoke contains many mutagenic, carcinogenic, and cocarcinogenic substances, such as N-nitrosamines.[9] Methods for determining N-Nitroso compounds in cosmetic products include a gas chromatography method coupled either with a electron capture detector,[10] thermal energy analyzer (TEA),[11] or with a mass spectrometer (MS),[12] a high performance liquid chromatography method combined with either a UV detector,[13] or, chemiluminescence detection after reduction of nitrite to nitric oxide by suitable reductants,[14,15] MS,[16] TEA,[17] and a polarographic method.[18–20] However, the sample preparation procedure of GC with derivatization and chemiluminesence detection before GC-TEA analysis was very time consuming. The LC-MS, which has now become a routine application technique, permits the determination of N-nitrosamines. Drawbacks are that the equipment is expensive and complex to operate. There are, however, no reports of the simultaneous determination of N-nitrosamines, which are presence in cosmetic products. Diode array detection (DAD) presents three major advantages to the HPLC analyst: (a) multiple signal detection, (b) peak identification, (c) peak purity determination. The use of the DAD has allowed improved qualitative work due to rapid spectra acquisition. Since spectra can be obtained simultaneously with multiwavelength data, identifying, as well as quantifying the compound is accomplished from a single injection, thus greatly improving productivity. The purpose of the present work was to develop a screening method with DAD for the simultaneous detection of N-Nitrosamines in cosmetic products and pharmacokinetics studies in rabbit urine and serum.

EXPERIMENTAL

Apparatus and Materials

HPLC was performed with a Hitachi model L-7100 pump and model 7125 injector equipped with a 20 μL sample loop and model L-7455 photodiode array detector. Chromatograms were acquired and peak areas calculated by means of D-7000 chromatograms Data Integrator. The tested nitrosamines were N-nitrosodiethanolamine (NDELA), N-nitroso-bis-(2-hydroxypropyl) amine (NBHPA), N-nitrosodimethylamine) (NDMEA), N-nitrosodi-n-propylamine (NDPLA), and N-nitrosodiphenylamine (NDPHLA) from TCI (Tokyo Kasei, Kogyo Co. JP). Control serums were purchased from Sigma Chemical Co. (St. Louis. MO) and Chemicon (International Temecula). Samples of cosmetic products were bought from a number of retail outlets in the south of Taiwan. All solvents and analytes were filtered through 0.45 μm cellulose acetate and polyvinylidene fluoride syringe (PVDF) membrane filters, respectively.

Rabbits

Male and female rabbits, weighing 2836–4200 g, were used. After an overnight fast, NDELA (60 mg) was administered orally with 10 mL of water. The blood and urine were separately collected at prescribed intervals and stored at −30°C until analyzed.

Extraction of NDELA

Sample urine (1.0 mL) and blood (1.0 mL), from rabbits were centrifuged at 6000 g for 30 min, respectively. The serum, 0.5 mL, and 0.9 mL supernatant urine were transferred to another centrifugal tube containing 5 mL ethanol and centrifuged for 30 min to sediment protein aggregates, respectively. The deproteinized samples were then extracted two times with 1 ∼ 3 mL dichloromethane. The organic phase was collected and evaporated under nitrogen at a temperature less than 37°C. Samples were reconstituted with mobile phase(1 mL), vortex mixed and filtered through 0.45 μm PVDF membrane filters before LC analysis.

Cosmetic Sample Preparation

Taking into account the N-nitrosamines content of the hair shampoo, foam bath and shower gels, washing creams, samples (approx. 1.0–3.0 g) of the latter were weighed accurately in a 15 mL beaker, dissolved in 8 mL of

ethyl acetate and mixed by vertex treatment for 15 min. The mixture is placed on a 9 cm length of glass column (1.5 cm i.d.) packed with 3.0 g silica gel 230–400 mesh). The ethyl acetate fraction was discarded, and the column was then washed with 20 mL of acetone-dichloromethane (60:40, v/v) and was subsequently connected to a stream of nitrogen to remove all solvent. After cleanup, the dried extracts were solubilized in 2 mL of methanol-water (1:1, v/v) for HPLC analysis.

Determination of N-Nitrosamines by HPLC-DAD

Reverse-phase LC was on a Phenomenex Luna CN column (particle size 5 μm, 250 mm × 4.6 mm i.d.). The mobile phase and gradient conditions were: gradient I solvent A: methanol; solvent B: 1.0 m M K_2HPO_4, adjusted to pH 4.0 with 0.1 M phosphoric acid. The gradient curve was set at G-three. A-B (10:90) was used as the initial condition. The methanol concentration was increased from 10% to 40% A in 5 min, then to 80% A in 10 min. After 5 min at 80%, the gradient was reversed to the initial condition in 10 min and equilibrated for an additional 5 min before the next sample was injected. The mobile phase flow rate was 1.0 mL min^{-1}. The absorbance was measured as full spectrum (190–400 nm).

RESULTS AND DISCUSSION

Optimization of HPLC Separation

Several methanol-water mixtures (5:95, 10:90, 20:80, *v*/*v*) were used as mobile phases. We found that 10:90 (*v*/*v*) methanol-water was the most suitable mobile phase for NDELA, NBHPA, and NDMEA. However, the retention times of NDPLA and NDPHLA were still the same and not well separated. Acetonitrile and methanol gave similar selectively. A satisfactory separation could not be obtained when we used an isocratic mobile phase with methanol-water or aetonitrile–water. Since the retention time of NBHPA and NDMEA increased with increasing proportions of methanol, we concluded that methanol acts as a kind of controller for suitable separation. Therefore, we tried to separate the five N-nitrosamines using a linear gradient elution. Separation improvement during chromatography is achieved by increasing the proportion of methanol in the eluent. The effect of the proportions of methanol and aqueous solution was investigated. We chose a suitable solvent programme as follows: methanol-water (10:90, *v*/*v*) for 5 min, then ramping the solvent composition to methanol-water (80:20, *v*/*v*) up to 15 min. This type of programme would greatly increase the retention time of the NDMEA, and was considered satisfactory with respect to separation and total retention time and was used for further studies. Therefore,

the gradient was developed for these five components from the mixture and was shown in Figure 1.

Linearity and Limit of Detection

The standard curves for all five compounds were determined at 234 nm. Their slopes, intercepts, correlation coefficient and limit of detection are shown in Table 1. The UV intensity showed a linear relationship with the concentration of N-Nitrosamines over a wide range up to 0.2 m g L^{-1}. The limit of detection (LOD) was given by the equation LOD = K So/S, where K was a numerical factor chosen according to the confidence level desired. The standard deviation of the blank measurement (n = 6) and S was the sensitivity of the calibration graph. Here, a value of 3 for K was used, and the LOD was shown in Table 1.

Recovery and Limit of Quantification

N-Nitrosamines mixtures for fortification were prepared by mixing the stock solution and diluting with deionized water, respectively. A 500 μL aliquot of the mixture was added to 0.5 mL of delipidated serum and urine samples, respectively, and to serum and urine samples that contained known amounts of endogenous N-nitrosamines, and extraction was carried out as

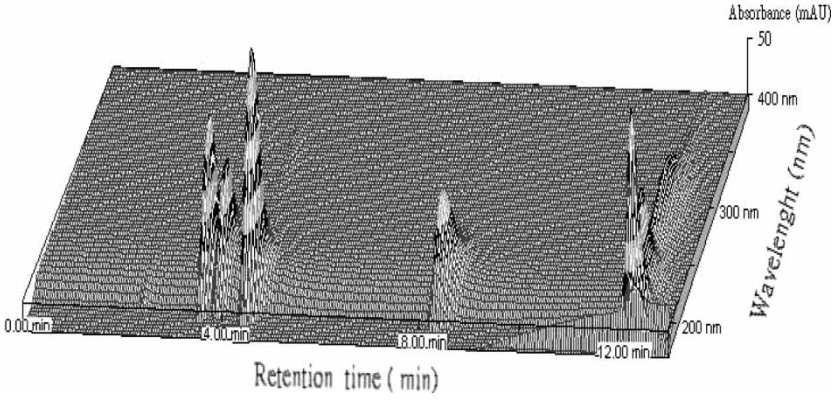

Figure 1. Chromatogram and three-dimensional spectrochromatogram of N-nitrosamines (20 mg L^{-1}) mixtures calibration standards. Column on Phenomenex Luna CN column (particle size 5 μm, 250 mm × 4.6 mm i.d.). Gradient: 10% CH_3OH/90% H_2O (K_2HPO_4, pH = 4) to 40% CH_3OH/60% H_2O to 80% CH_3OH/20% H_2O. Flow rate was 1.0 mL min^{-1}. Detection: UV-DAD. Peak identification: (a) N-nitrosodiethanolamine (NDELA); (b) N-nitroso-bis-(2-hydroxypropyl)amine (NBHP; (c) N-nitrosodimethylamine (NDMEA); (d) N-nitrosodi-n-propylamine (NDPLA) and (e) N-nitrosodiphenylamine (NDPHLA).

Table 1. Statistical evaluation of N-nitrosamines the calibration data obtained by HPLC with DAD

N-nitrosamines	$y = a + bx$[a]	r[b]	Range linearity[c] ($mg\,L^{-1}$)	of LOD[d] ($mg\,L^{-1}$)
NDELA	$y = 78.6 + 2397x$	0.9999	0.2–100	0.02
NBHPA	$y = 32.9 + 2028x$	0.9999	0.2–100	0.02
NDMEA	$y = 53.9 + 4862x$	0.9998	0.2–100	0.02
NDPLA	$y = 69.6 + 3953x$	0.9997	0.2–100	0.03
NDPHLA	$y = 113.9 + 1332x$	0.9999	0.2–100	0.03

[a] a = intercept on the ordinate; b = slope.
[b] r = correlation coefficient.
[c] $mg\,L^{-1}$, which corresponds to amounts injected from 4 to 2000 ng in 20 μL.
[d] Limit of detection ($mg\,L^{-1}$) at a signal-to-noise ratio of 3.

described above. To calculate percentage recovery, the amount of endogenous N-nitrosamines was subtracted from the measured total amount, divided by the added amount, and multiplied by 100. Table 2 and 3 show the LC-DAD traces obtained for rabbit serum and urine sample spiked with

Table 2. Recovery of N-nitrosamines from rabbit serum measured by LC-DAD

	Concentration ($ng\,mL^{-1}$)[a]		
N-nitrosamines	Added ($ng\,mL^{-1}$)	Found ($ng\,mL^{-1}$)	Recovery (%)
NDELA	400	396	99% (1.4%)[b]
	800	768	96% (1.9%)
	1600	1552	97% (1.8%)
NBHPA	400	380	98% (1.6%)
	800	744	98% (0.8%)
	1600	1488	97% (1.0%)
NDMEA	400	388	97% (1.5%)
	800	784	98% (1.7%)
	1600	1560	98% (1.2%)
NDPLA	400	396	99% (0.6%)
	800	784	98% (0.8%)
	1600	1536	96% (1.1%)
NDPHLA	400	400	100% (0.80%)
	800	768	96% (0.92%)
	1600	1568	98.% (1.12%)

[a] Number of determination (n = 3).
[b] Relative standard deviation.

Table 3. Recovery of N-nitrosamines from rabbit urine measured by LC-DAD

N-nitrosamines	Concentration ($ng\,mL^{-1}$)[a]		
	Added ($ng\,mL^{-1}$)	Found ($ng\,mL^{-1}$)	Recovery (%)
NDELA	400	396	99% (0.5%)[b]
	800	792	99% (0.7%)
	1600	1616	101% (1.0%)
NBHPA	400	388	97% (1.0%)
	800	776	97% (0.6%)
	1600	1552	97% (0.4%)
NDMEA	400	388	97% (0.3%)
	800	776	97% (0.8%)
	1600	1568	98% (0.2%)
NDPLA	400	392	98% (1.0%)
	800	784	98% (1.6%)
	1600	1584	99% (1.3%)
NDPHLA	400	392	98% (1.78%)
	800	776	97% (1.42%)
	1600	1568	98% (1.25%)

[a]Number of determination (n = 3).
[b]Relative standard deviation.

N-nitrosodiethanolamine, N-nitroso-bis-(2-hydroxypropyl)amine; N-nitrosodimethylamine, N-nitrosodi-n-propylamine, and N-nitrosodiphenylamine, respectively. Excellent recoveries and precision were observed (recoveries ranging from 96 $\pm$ 0.92% to 101 $\pm$ 1.1%). The limit of quantification 0.4 μg, 0.4 μg, 0.6 μg, 0.6 μg, and 0.6 μg, for N-nitrosodiethanolamine, N-nitroso-bis-(2-hydroxypropyl)amine, N-nitrosodimethylamine, N-nitrosodi-n-propylamine, and N-nitrosodiphenyl-amine, respectively.

Application to Rabbit Serum and Urine

The proposed LC-DAD method was applied to the determination of N-nitrosodiethanolamine in rabbit serum and urine. The representative LC-DAD chromatograms for the N-nitrosodiethanolamine in a rabbit serum and urine extract before and after drug intake are shown in Figure 2A, 2B, and Figure 3A, 3B, respectively. Figures 2A, 2B, and 3A, 3B compare with a chromatogram of pure standard (Figure 2C and 3C). Sample constituents with retention characteristics identical to this of N-nitrosodiethanolamine was

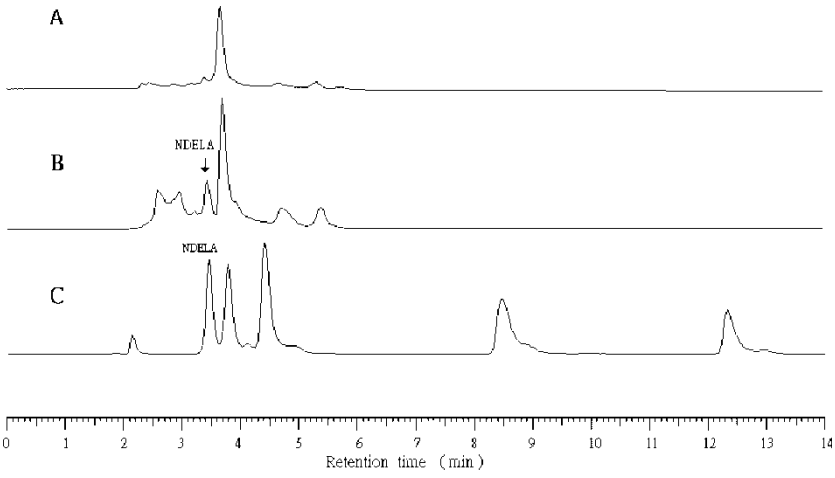

Figure 2. Chromatograms obtained by LC-DAD in rabbit serum from (A) before (blank serum) (B) after oral administration of NDELA (60 mg), and (C) standards of N-nitrosamines (10 $\mu g\,mL^{-1}$). Analysis conditions are identical to those listed in Figure 1.

identified and measured. For three of the rabbits within our experimental population, (male and female) we performed multiple determinations. Results for the analyses of rabbit serum and urine were shown in Tables 4 and 5. The mean concentration of N-nitrosodiethanolamine drug intake for

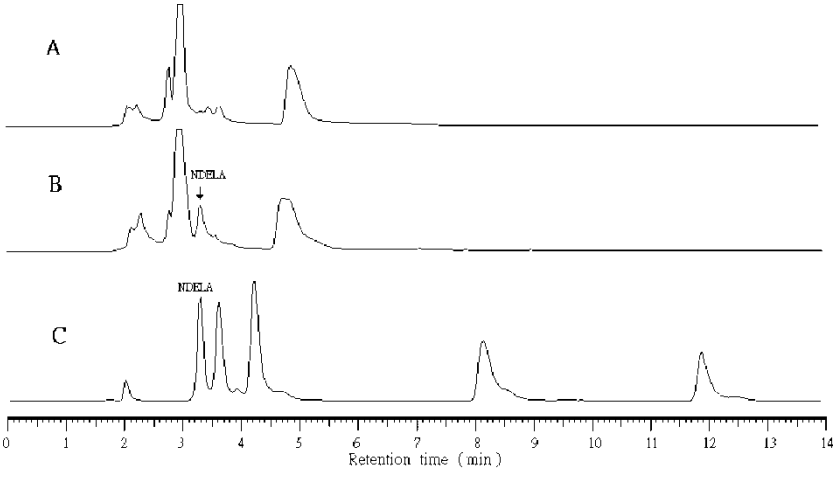

Figure 3. Chromatograms obtained by LC-DAD in rabbit urine from (A) before (blank urine) (B) after oral administration of NDELA (60 mg), and (C) standards of N-nitrosamines (10 $\mu g\,mL^{-1}$). Analysis conditions are identical to those listed in Figure 1.

Table 4. Amount of DNELA in serum after administration of 60 mg to rabbit measured by LC-DAD

	Before oral	After oral hours ($mg\,L^{-1}$)[a]					
Rabbits	0	3	6	9	12	15	24
F1	ND	14.85 (1.1%)[b]	18.03 (0.9%)	5.995 (1.3%)	ND	ND	ND
F2	ND	17.61 (0.9%)	15.53 (1.2%)	11.82 (1.5%)	ND	ND	ND
M1	ND	21.78 (1.2%)	14.42 (1.0%)	9.387 (1.2%)	8.273 (1.0%)	6.640 (0.9%)	ND
Control (Sigma and Chemico)	ND	ND	ND	ND	ND	ND	ND

[a]Number of determination (n = 3).
[b]R.S.D., relative standard deviation.
ND: Not determined.

Table 5. Amount of DNELA in urine after administration of 60 mg to rabbit measured by LC-DAD

	Before oral	After oral hours (mg L^{-1})[a]				
Rabbits	0	3	5	8	17	26
F1	ND	ND	979.7 (1.1%)[b]	5.995 (2 3.%)	424.5 (1.7%)	271.3 (2.3%)
F2	ND	ND	880 (0.21%)	794.9 (3.2%)	750.5 (3.1%)	656.2 (0.90%)
M1	ND	571.4 (1.2%)	190.6 (1.3%)	165.3 (1.4%)	79.80 (1.3%)	ND
Control (Sigma and Chemico)	ND	ND	ND	ND	ND	ND

[a]Number of determination (n = 3).
[b]R.S.D., relative standard deviation.
ND: Not determined.

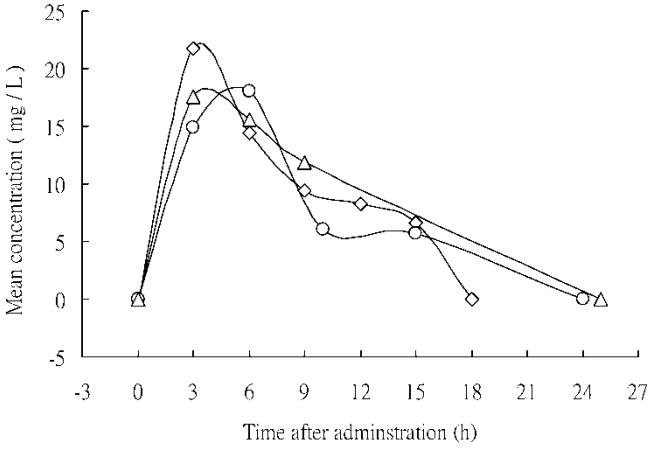

Figure 4. Time of the course of concentrations in serum of rabbits after oral administration of NDELA (60 mg).

3 hours serum and 8 hours urine were 18.08 and 597.2 mg L^{-1}, respectively. These data might be increased after intake and oral administration of N-nitrosodiethanolamine. The intestinal absorption of N-nitrosodiethanolamine was measured from oral administration of N-nitrosodiethanolamine; the time courses of absorption in the rabbit serum and urine were shown in Figures 4 and 5. These data ranged from 6.640 to 17.61 mg L^{-1} for serum, 79.80 to 979.7 mg L^{-1} for urine, respectively. N-nitrosodiethanolamine levels were significantly increased for rabbit oral administration of N-nitrosodiethanolamine at all time points tested up to 30 hours, compared with time zero.

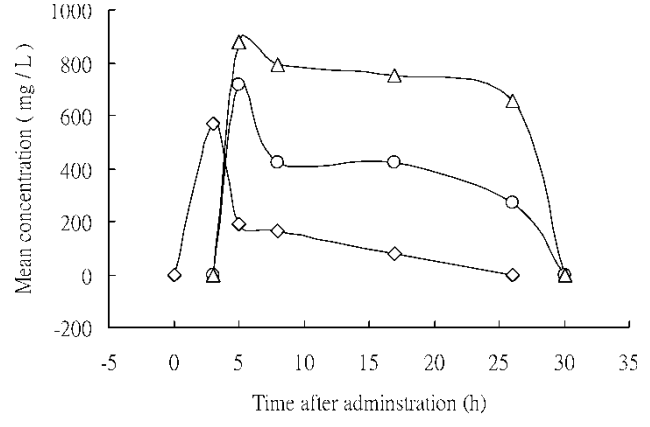

Figure 5. Time of the course of concentrations in serum of rabbits after oral administration of NDELA (60 mg).

Table 6. Recovery of N-nitrosamines from hair shampoo, foam bath, shower gels and skin cream measured by LC-DAD

		Concentration ($ng\,mL^{-1}$)[a]		
Samples	N-nitrosamines	Added ($ng\,mL^{-1}$)	Found ($ng\,mL^{-1}$)	Recovery (%)
Hair shampoo	NDELA	1600	1552	97% (1.8%)[b]
	NBHPA	1600	1568	98% (1.0%)
	NDMEA	1600	1560	98% (1.2%)
	NDPLA	1600	1536	96% (1.1%)
	NDPHLA	1600	1552	97% (1.1%)
Foam bath	NDELA	1600	1568	98% (1.4%)
	NBHPA	1600	1552	97% (1.0%)
	NDMEA	1600	1584	99% (1.1%)
	NDPLA	1600	1568	98% (1.7%)
	NDPHLA	1600	1552	97% (1.5%)
Skin cream	NDELA	1600	1568	98% (1.1%)
	NBHPA	1600	1560	98% (1.0%)
	NDMEA	1600	1552	97% (0.9%)
	NDPLA	1600	1584	99% (1.0%)
	NDPHLA	1600	1536	96% (1.1%)

[a]Number of determinations (n = 3).
[b]RSD, relative standard deviation.

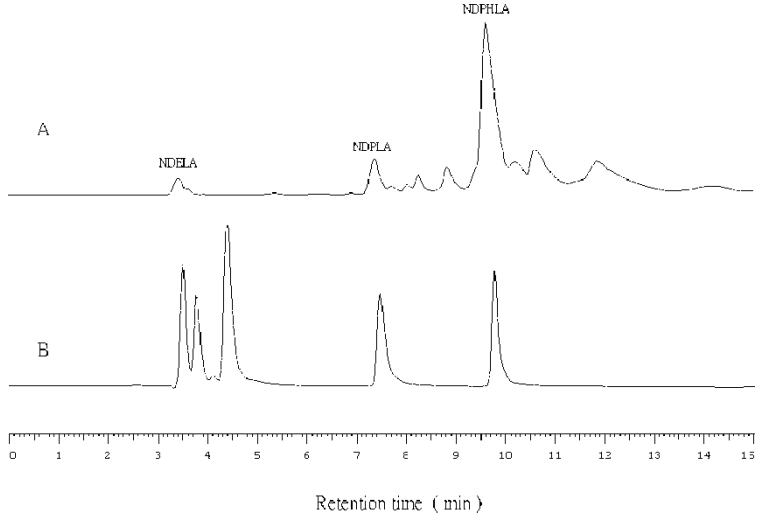

Figure 6. Chromatograms obtained by LC-DAD from (A) skin cream (B) standards of N-nitrosamines ($20\,mg\,L^{-1}$). Analysis conditions are identical to those listed in Figure 1.

Table 7. Analytical Results for the determination of N-nitrosamines in commercial hair shampoo, foam bath and shower gels and skin cream measured by LC-DAD

	Concentration ($ng\,mL^{-1}$)[a]				
	NDELA	NBHPA	NDMEA	NDPLA	NDPHLA
Hair shampoo 1	1.54 (2.5%)[b]	0.761 (0.12%)	ND	ND	ND
Hair shampoo 2	ND[c]	ND	ND	ND	2.49 (2.2%)
Foam bath 1	0.650 (2.4%)	2.76 (2.5%)	ND	ND	ND
Foam bath 2	0.97 (0.13%)	0.943 (1.7%)	ND	ND	ND
Skin cream 1	1.89 (2.4%)	ND	ND	8.51 (0.91%)	26.5 (2.1%)
Skin cream 2	ND	ND	ND	3.43 (2.7%)	ND

[a]Number of determinations (n = 3).
[b]RSD, relative standard deviation.
[c]ND: Not determined.

Application to Cosmetic Products

Recovery tests were carried out on cosmetic products for evaluation of the reproducibility and accuracy of the proposed method. Three commercial products were spiked with the amounts of the agents reported in Table 6 and subjected to full a extraction procedure. As is seen, excellent recoveries and precision were observed. A representative LC-DAD chromatogram of a commercial skin cream was shown in Figure 6A, and compared with a chromatogram of pure standard (Figure 6B). Analytical results were given in Table 7. These results for the NDELA compared with those recently reported elsewhere.[7,16] The mean concentrations of NDELA, NBHPA, NDPLA, and NDPHLA in cosmetic products were 1.26, 1.49, 3.43, and $2.49\,mg\,L^{-1}$, respectively, and the NDELA values compared with reference[7] we found were lower; however, those data were higher than reference.[16]

CONCLUSIONS

Diode array detection after HPLC separation proved to be an easy method for simultaneous determination of N-Nitrosamines in cosmetic products and the urine of workers exposed to various fluids as an indicator of the potential for the presence of N-nitrosamines. The method is rapid and high selectively for N-nitrosamines.

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