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Fast microwave-assisted dansylation of *N*-nitrosamines Analysis by high-performance liquid chromatography with fluorescence detection

Lourdes Cárdenes, Juan H. Ayala, Venerando González, Ana M. Afonso*

Department of Analytical Chemistry, Nutrition and Food Science, University of La Laguna, Campus de Anchieta, Astrofísico Francisco Sánchez s/n, E-38205 La Laguna, Spain

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Abstract

A fast microwave-assisted dansylation procedure has been developed for the derivatization of *N*-nitrosamines prior to high-performance liquid chromatography determination. *N*-Nitrosomorpholine, *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosopyrrolidine and *N*-nitrosopiperidine are first denitrosated by hydrobromic acid–acetic acid to produce secondary amines, which are then quantitatively dansylated in 5 min using radiation power of 378 W and a maximum pressure of 1.4 bar inside the reactor. The reaction mixture is separated on a C_{18} column with acetonitrile–water (55:45, v/v) as mobile phase with fluorimetric detection at 531 nm (excitation at 339 nm). The detection limits range from 8 to 75 pg for *N*-nitrosomorpholine and *N*-nitrosodiethylamine, respectively. The method was applied to study the recoveries of *N*-nitrosamines in beer and their determination in cigarette smoke.

Keywords: Microwave-assisted derivatization; Derivatization, LC; Beer; Food analysis; Cigarette smoke; Nitrosamines; Amines

1. Introduction

N-Nitrosamines are commonly present in the environment and in food. Most of them are known to be potent carcinogens in animals [1,2]. Volatile *N*-nitrosamines are found in foodstuffs [3–5], drinking water [6,7], rubber products [8,9], drug formulations [10], tobacco and tobacco smoke [11–13]. *N*-Nitrosamines are formed by the reaction of secondary amines with nitrosating agents such as nitrite or nitrate in the human diet [14], the environment [15] and in vivo in the stomach or small intestine of experimental animals [16]. The toxicity of *N*-nitrosamines is manifested even at μg/kg levels [17]. For this reason, a sensitive and selective method for the determination of these *N*-nitrosamines at trace levels is essential.

High-performance liquid chromatography is a powerful technique for the analysis of these types of compounds. *N*-Nitrosamines do not show absorption in the UV region, so that to increase detection sensitivity and improve selectivity, generally methods using derivatization agents are employed. High-performance liquid chromatographic separations of *N*-nitrosamines with pre-column derivatization or post-column derivatization [18,19] have been de-

*Corresponding author. Tel.: +34-922-318-039; fax: +34-922-318-090.

E-mail address: aafonso@ull.es (A.M. Afonso).

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scribed. However, the sensitivities of these methods for the determination of *N*-nitroso compounds are unsatisfactory. A more sensitive method is the denitrosation of *N*-nitroso compounds and the subsequent detection of liberated secondary amines via fluorescent derivatization [20,21].

In this sense, dansyl chloride has been used for the determination of some *N*-nitrosamines by pre-column derivatization [22]. However, the time required to carry out dansylation (30 min at 40 °C) is not particularly suited to automation. The application of microwave ovens in chemistry offers a good way for supplying high temperatures in a short time. On the other hand, a microwave-assisted procedure can also be employed to accelerate organic reactions [23].

In this work, a rapid microwave-assisted method for dansylation of *N*-nitrosamines has been developed to accelerate this step of *N*-nitrosamines analysis. The use of a factorial experimental design to optimize three experimental variables, pressure, reaction time, and radiation power, allows us to find an optimal set of operational conditions. The dansyl derivatives were analyzed by HPLC with fluorimetric detection. The developed method was applied to study the recoveries of *N*-nitrosamines in beer and the determination of *N*-nitrosamines in cigarette smoke of different commercial brands.

2. Experimental

2.1. Chemicals and reagents

N-Nitrosomorpholine (NMOR), *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosopiperidine (NPIP) were supplied by Sigma (St. Louis, MO, USA) and dansyl chloride was supplied by Aldrich (Beerse, Belgium). For chromatographic analysis, acetonitrile of HPLC grade (Merck, Darmstadt, Germany) and water purified with a Milli-Q system (Millipore, Bedford, MA, USA) were used throughout.

The *N*-nitrosamines were dissolved in dichloromethane (Merck) and the stock solutions (20 mg/ml) were diluted before being used and stored at 4 °C.

The denitrosation reagent (0.88 *M*) was prepared by diluting 1 ml of 48% aqueous hydrobromic acid (Aldrich) to give a final volume of 10 ml with acetic acid glacial (Panreac, Barcelona, Spain).

Dansyl chloride solution (1.2×10^{-3} *M*) was prepared by weighing 3.3 mg of dansyl chloride and diluting to 10 ml with acetone (Merck).

All other reagents were of an analytical-reagent grade and used as received.

2.2. Equipment

All measurements were made with a Waters (Milford, MA, USA) model 600 Multisolvent Delivery System equipped with a Waters U6K sample injector and a Waters 474 Scanning fluorescence detector. Autoanalysis 2.4 (Sciware, vcerda@p01.uib.es) software was used for acquisition data. The analytical column was a NovaPak C_{18}, 4 μm, 60 Å (150×3.9 mm I.D.) supplied by Waters with a Pelliguard LC_{18} guard column, supplied by Supelco (Bellefonte, PA, USA). Acetonitrile–water (55:45, v/v) was used as mobile phase at a flow-rate of 1.2 ml/min. The injection volume was 25 μl and the fluorescence excitation and emission wavelengths were 339 and 531 nm, respectively.

The microwave extraction system was a CEM (Matthews, NC, USA) MDS 2000 microwave digestion system. The system delivers approximately 630 W (100%) of microwave energy at a frequency of 2450 MHz at full power and provides constant feedback control of reaction conditions through continuous monitoring of pressure data on a control vessel. The system is provided with method and data storage capabilities and a printer.

The microwave vessels used for sample dansylation were CEM teflon PFA®-lined advanced composite digestion vessels (Matthews, NC, USA). The vessels are constructed with a PTFE liner and a cap for high-purity analysis that are capable of sustaining temperatures up to 200 °C and pressures of 13.9 bar.

For the statistical treatment the Statgraphics Plus for Windows V 4.2 package (Statiscal Graphics, Rockville, USA) was used.

2.3. Dansylation methods

A 75-μl volume of a standard solution containing 5 μg/ml of each of the five *N*-nitrosamines was mixed with 9 μl of denitrosation reagent in a test-

tube with stopper. After denitrosation reaction for 10 min at 100 °C, the stopper was removed and dichloromethane solvent was evaporated. Then, 1 *M* NaOH solution was added to adjust the pH to about 9, followed by the addition of 1 ml of 0.5 *M* $NaHCO_3$ buffer and 2.5 ml of dansyl chloride solution (0.33 mg/ml). The reaction mixture was either allowed to stand for 30 min at 40 °C (conventional dansylation) or transferred quantitatively to a microwave vessel (microwave-assisted dansylation). The microwave reaction was performed at 60% (378 W), keeping a maximum pressure of 1.4 bar inside the reactor for 5 min. The derivatives were diluted with water to 5 ml for HPLC analysis.

2.4. Determination of N-nitrosamines in beer and cigarette smoke

A 20-ml aliquot of beer was spiked with suitable amounts of each *N*-nitrosamine. The ionic strength was fitted to 1 *M* with NaCl, and then the beer was extracted twice with 5 ml of dichloromethane for 5 min. After centrifuging (300 rpm, 5 min), the organic layer was transferred into another tube and derivatized and analyzed in accordance with the methods previously described.

Cigarette smoke was collected by suction with a laboratory-made system by bubbling in a trapping bottle containing 10 ml of 5% (v/v) hydrochloric acid. An aliquot of 5 ml was extracted twice with 2.5 ml dichloromethane for 5 min. The organic layer was transferred into another tube and derivatized and analyzed in accordance with the methods previously described.

3. Results and discussion

3.1. Conventional dansylation method

The conventional dansylation is based on the direct reaction of secondary amines with dansyl chloride. The reaction conditions for denitrosation and subsequent dansylation were investigated to establish the optimum derivatization method for NMOR, NDMA, NDEA, NPYR and NPIP.

It is well known that *N*-nitrosamines easily undergo cleavage at the N–NO bond in the presence of a hydrogen bromide–acetic acid mixture, resulting in the formation of the corresponding secondary amines [20,24]. For the *N*-nitrosamines studied, we found that for molar ratios of hydrogen bromide–*N*-nitrosamine of 200:1, the reaction was completed in 10 min at 100 °C. Excess hydrobromic acid in the reaction mixture was neutralized with sodium hydroxide solution before derivatization.

The derivatization of the secondary amines produced from *N*-nitrosamines was carried out by a previously reported method [22], which is based on the direct reaction of secondary amines with dansyl chloride, on presence of $NaHCO_3$ buffer and with reaction time of 30 min at 40 °C.

The effect of the amount of dansyl chloride in the range of 25–100 mol of dansyl chloride per mol of *N*-nitrosamine on the chromatographic peak heights was investigated. The peak height of the derivatives increased markedly as the amount of dansyl chloride was increased, but above the ratio 50:1 it remained almost unchanged. As a result, this amount of dansyl chloride was selected for subsequent experiments.

3.2. Optimization of microwave-assisted dansylation of N-nitrosamines: factorial design

The use of a factorial design to explore the variables that affect the microwave-assisted dansylation allows a consideration of the overall number of experiments and possible interaction effects between the variables. The application of a statistical approach using a factorial design can both reduce the development time and provide less ambiguous data.

Several variables could potentially affect the dansylation efficiency: radiation power supplied, maximum pressure inside the reactor and time at which the maximum pressure is maintained constant (so-called reaction time). The efficiency of the dansylation accomplished in the reactor is established by comparison with the results obtained through conventional dansylation. The amount of each *N*-nitrosamine (75 ng/ml) is kept constant. A two-level factorial design, 2^3, with two central points involving ten runs was used. The upper and lower values given to each factor are shown in Table 1.

Table 2 shows the experimental design matrix and the efficiency of the dansylation obtained in each run. NPIP was chosen as a representative example of

Table 1
Factor levels in the experimental design

Factors	Key	Low	High	Optimum
Pressure (bar)	A	1.4	6.8	1.4
Power (%)	B	40	60	60
Time (min)	C	1	5	5

all compounds, since the five studied *N*-nitrosamines present similar tendencies versus the three variables considered. An analysis of NPIP results, given in Table 2, produced the Pareto chart of main effects shown in Fig. 1. In this chart, bar lengths are proportional to the absolute value of the estimated effects, helping in comparing the relative importance of effects. Both interactions pressure–reaction time and power–reaction time were statistically significant. Effects of main factors in the response show that reaction time and pressure have a negative effect (dansylation efficiency is inversely proportional to both factors), while power has a positive one. Fig. 2 shows the response surface considering the pressure and reaction time, where we can see that low pressure and high reaction time give the best dansylation efficiency.

The relation between the reaction time and efficiency of dansylation for *N*-nitrosamines studied, fixing the other optimum variables, is shown in Fig. 3. As can be seen, except NMOR, the dansylation efficiencies do not increase substantially when the reaction time increases from 1 to 5 min. At higher times the efficiency decreases, possibly due to degradation of dansyl derivatives. Given these findings, we decided to work with the experimental conditions given in Table 1, as optimum values. Under these

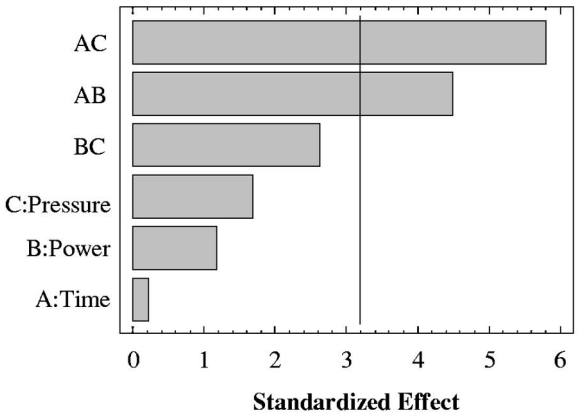

Fig. 1. Standardized Pareto chart for *N*-nitrosopiperidine produced by the factorial design. The vertical line indicates the statistical significance bound for the effects.

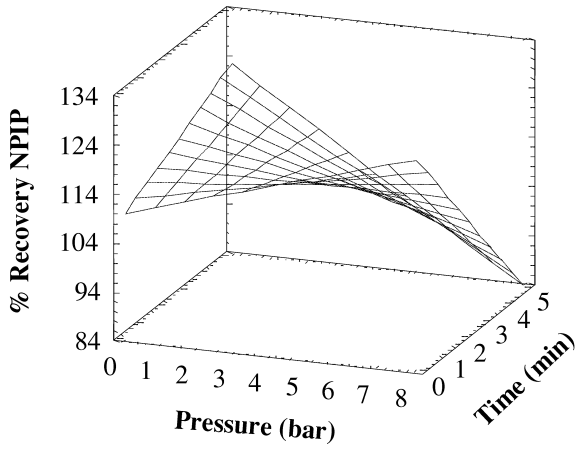

Fig. 2. Estimated response surface plot of the effect of the time and pressure factors on the recovery of *N*-nitrosopiperidine. Radiation power 60%.

Table 2
Design matrix and response values in the factorial design

Run	Pressure (bar)	Power (%)	Time (min)	NPIP (%)
1	1.4	60	5	118
2	6.8	60	1	119
3	1.4	40	1	93
4	4.1	50	3	104
5	6.8	40	5	107
6	6.8	40	1	110
7	4.1	50	3	104
8	6.8	60	5	91
9	1.4	60	1	114
10	1.4	40	5	119

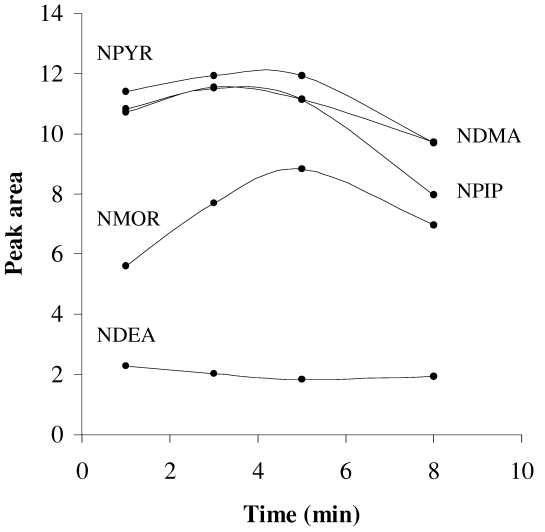

Fig. 3. Effect of the reaction time on the dansylation efficiency, fixing the other optimum variables. Radiation power: 60%; maximum pressure inside reactor: 1.4 bar.

conditions, the dansylation efficiency reach 118% in comparison with the one obtained through conventional dansylation.

To determine the accuracy of the dansylation method, six independent dansylations were carried out keeping the established experimental conditions. Table 3 shows the average results obtained. The obtained SD values show the reproducibility of the proposed dansylation method.

One of the advantages of microwave dansylation devices is the possibility of performing many simultaneous dansylations. When several dansylations are carried out simultaneously, optimum experimental conditions can be maintained. The obtained efficiencies, as shown in Table 3, are similar to the ones obtained with a single reactor. In this case the required time for reaching maximum pressure is about 45 s for six reactors in comparison with 15 s for a single reactor.

3.3. Chromatographic conditions

Several ratios of methanol–water and acetonitrile–water mixtures and different gradient programs were tried to reduce the analysis time while keeping a good resolution of all the *N*-nitrosamine peaks. Good results were obtained using as mobile phase a mixture of acetonitrile–water (55:45, v/v) at a flow-rate of 1.2 ml/min. The different amines gave satisfactory retention times with RSD that oscillate between 0.16 and 0.24%. Fig. 4A shows a typical chromatogram obtained with a mixture of five *N*-nitrosamines.

In order to verify the linearity of the fluorescence detector response at $\lambda_{em}=531$ nm ($\lambda_{ex}=339$ nm) for the working concentrations of each amine, a portion of the five amines standard solution was derivatized and injected into the HPLC system. Calibration graphs were constructed by plotting the peak-height against the *N*-nitrosamine concentration. A linear relationship with $r^2>0.99$ was always obtained, and detection limits were between 8 and 75 pg. Quality parameters for the chromatographic method are reported in Table 4.

3.4. Applications

To test the applicability of the established method in real samples, we have chosen beer samples because it has been reported that some *N*-nitrosamines are formed during malt kilning by reactions involving amines formed in germinating barley and active forms of nitrogen oxides.

Table 3
Reproducibility of microwave dansylation method for each *N*-nitrosamine (50 ng/ml) using the optimum operation conditions

N-Nitrosamine	Average±SD[a]	
	Individual dansylation	Simultaneous dansylation
NMOR	50.9±2.1	50.4±1.7
NDMA	50.4±2.5	49.8±2.2
NPYR	49.5±2.4	50.0±1.9
NDEA	49.8±2.1	49.1±1.4
NPIP	49.8±2.0	49.6±1.3

[a] Mean of six dansylations.

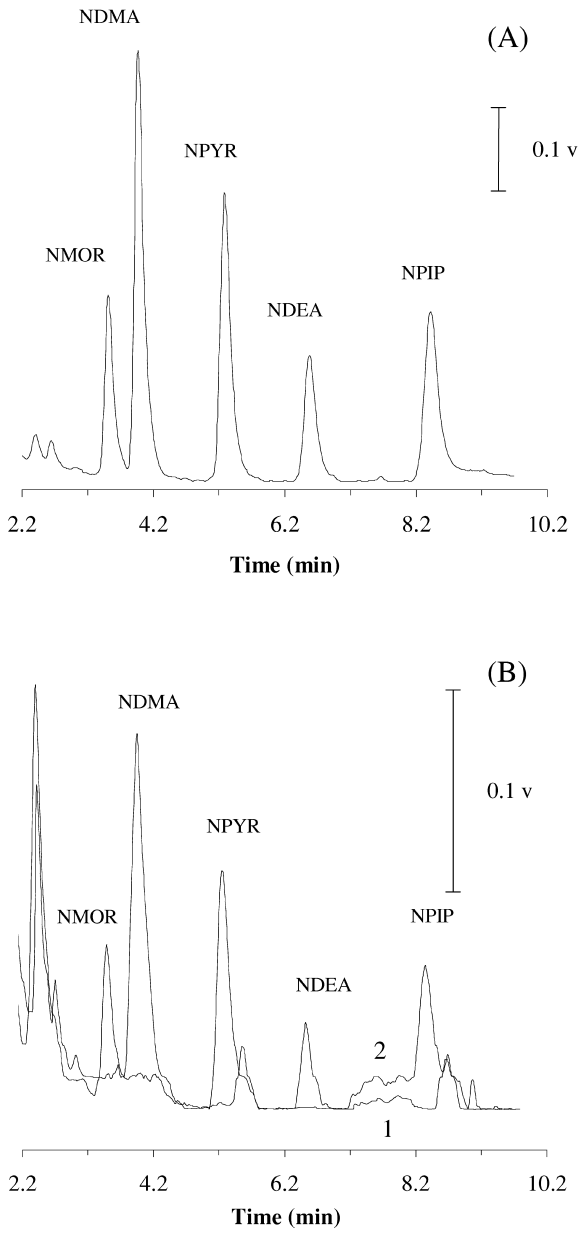

Fig. 4. Optimum chromatographic separation of the dansylated *N*-nitrosamines. Mobile phase, acetonitrile–water (55:45, v/v); flow-rate, 1.2 ml/min; detector wavelength, λ_{em}=531 nm (λ_{ex}= 339 nm); injection volume, 25 μl; response, relative fluorescence intensity. (A) Standard solution 30 ng/l of each *N*-nitrosamine. (B) A beer sample (1) and the same one spiked with 2.5 ng/l of each *N*-nitrosamine (2).

The *N*-nitrosamines must be separated from the coexisting secondary amines in these samples before derivatization. By extraction twice with dichloromethane the *N*-nitrosamines were quantitatively extracted into the organic layer. Table 5 summarizes the recoveries found when 20 ml aliquots of a beer sample were spiked with 50 and 250 ng of each *N*-nitrosamine. The results obtained show recoveries whose values range from 82.7 to 98.4%, with RSDs between 2.0 and 12.4%. Fig. 4B shows the chromatograms obtained for the spiked and non-spiked beer sample. Sample size of beer has allowed to reduce the detection limits at the range from 8×10^{-2} to 0.75 μg/l for NMOR and NDEA, respectively.

On the other hand, to demonstrate the applicability of the method to environmental samples, the contents of *N*-nitrosamines in cigarette smoke were analyzed. Three commercial cigarette brands were selected on the basis of their tar and nicotine contents. Cigarette smoke was collected by suction with a laboratory-made system by bubbling in 5% hydrochloric acid. This system mainly permits to collect the mainstream smoke.

The recoveries of *N*-nitrosamines added (30 ng/ml) to 5% hydrochloric acid solution and the reproducibility were found to be satisfactory. The limits of detection applying to 1 g of cigarette range from 3.1 to 30 ng/g for NMOR and NDEA, respectively.

Although the presence of NMOR, NDMA, NDEA, NPYR and NPIP have been reported in cigarette smoke samples, we have only detected NDMA and NPYR in the three commercial cigarette brands tested in this study. The contents of these *N*-nitrosamines are shown in Table 6 and their values are similar to those found by other workers [11].

4. Conclusions

The microwave-assisted dansylation of *N*-nitrosamines as a previous step to the separation by HPLC with fluorescence detection allows: shorter analysis time, higher dansylation efficiencies what lead to lower detection limits, in comparison with the conventional dansylation.

The developed methodology is selective and sensitive, permitting the determination of *N*-nitrosamines in beer and cigarette smoke samples without pretreat-

Table 4
Quality parameters for the chromatographic method

	N-nitrosamine				
	NMOR	NDMA	NPYR	NDEA	NPIP
Linear range (ng/ml)	4.5–75	4.5–75	4.5–75	4.5–150	4.5–75
(Intercept±SD)×10^3	10.4±5.5	24.9±9.6	4.6±5.1	−5.3±20.8	0.7±6.9
(Slope±SD)×10^3	14.3±0.2	16.5±0.3	14.7±0.2	4.8±0.03	10.2±0.2
r^2	0.999	0.998	0.999	0.999	0.998
$S_{y/x}$	0.014	0.024	0.013	0.007	0.019
F	0.57	3.78	0.42	2.34	0.2
LOD (pg)	8	27	11	75	34
RSD (%)	3	3.1	2.7	1.1	2.6

r: correlation coefficient ($n=6$); $S_{y/x}$, standard error of the estimate; RSD: relative standard deviation for 30 ng/ml ($n=6$); LOD: limit of detection (calculated as three times the standard deviation for the background noise); $F_{4,12}=4.12$.

Table 5
Mean recoveries and relative standard deviations (RSDs) of *N*-nitrosamines in a spiked beer sample from three replicate measurements

N-Nitrosamine	Spiking level (ng/ml)			
	2.5		12.5	
	Mean	RSD (%)	Mean	RSD (%)
NMOR	94.6	9.5	98.2	7.3
NDMA	96.6	2	97.9	5.7
NPYR	97.3	6.8	97.1	11.8
NDEA	82.7	7.9	89.6	9.8
NPIP	98.4	12.1	97.7	12.4

ment except for separation from secondary amines by solvent extraction and without any interference from other coexisting substances.

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Table 6
Contents of *N*-nitrosamines in the cigarette smoke

Content per cigarette[a]				
Mass (mg)	Tar (mg)	Nicotine (mg)	NDMA (ng)[b]	NPYR (ng)[b]
926	12	1.3	78.4±4.7	3.8±0.3
745	12	0.8	61.0±4.2	5.0±0.4
700	8	0.6	19.5±1.3	ND

[a] The filter is not included in these contents. Contents of tar and nicotine represent the values labelled on the product.
[b] Mean±SD ($n=3$); ND, not detectable.

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