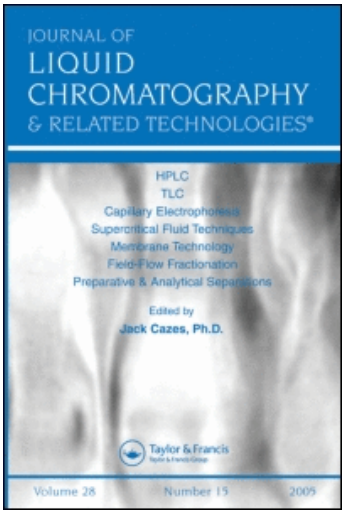

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

DETERMINATION OF N-NITROSODIMETHYLAMINE BY HPLC, WITH FLUORESCENCE DETECTION. A SURVEY OF N-NITROSODIMETHYLAMINE IN COMMERCIAL BEERS

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Online publication date: 05 September 2002

J. LIQ. CHROM. & REL. TECHNOL., 25(6), 977–984 (2002)

DETERMINATION OF N-NITROSODIMETHYLAMINE BY HPLC, WITH FLUORESCENCE DETECTION. A SURVEY OF N-NITROSODIMETHYLAMINE IN COMMERCIAL BEERS

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ABSTRACT

A new method has been developed for determination of N-nitrosodimethylamine (NDMA) in beers. Beer samples, previously degassed, were extracted with diethyl ether containing 25% 2-propanol and the extract was denitrosated by hydrobromic acid-acetic acid to produce secondary amines. These amines were then subjected to microwave-assisted reaction with dansyl chloride to form dansyl derivatives in 5 min, using radiation power of 378 W and a maximum pressure of 1.4 bar inside the reactor. The reaction mixture was analyzed by HPLC on a

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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 limit of method is 0.04 μg/L. N-nitrosodimethylamine was found in 20% of the beer samples at levels ranging from 0.04 to 0.50 μg/L. The mean NDMA level for whole beers (44 samples) was 0.16 ± 0.25 μg/L with a range from ND to 1.05 μg/L.

INTRODUCTION

N-Nitrosodimethylamine (NDMA) is one of the few proven potent carcinogens (1,2). To date, research on NDMA has been focussed mainly on its toxicity effects on humans and animals (3,4).

Food is an external source of N-nitrosamines for humans. Although the daily amount of total volatile N-nitrosamines to which we are exposed is less 1 μg, there is a big concern about its presence in food (5). Attending to the causes of the presence of N-nitrosamines in food products, they can be classified in four groups (6): those that contain nitrates or nitrites as preservative additives; those that suffer a smoked procedure during their elaboration; products that include a kilning process in their manufacturing, like the malt used for the fabrication of beer; and finally, pickles and products in salting (7,8).

N-Nitrosodimethylamine is the most abundant and frequent nitrosamine in malt and beer. It is formed during malt kilning by reactions involving amines formed in germinating barley and active forms of nitrogen oxides (9). Precursor amines and nitrosating agents, such as nitrogen oxides and nitrites, are involved in the formation of NDMA.

Dimethylamine (DMA) is the direct precursor of NDMA. The nitrosation of DMA to give NDMA does not require high temperatures, so, all the NDMA coming from the free DMA is formed at the first stages of toasting in presence of nitrosating agents (10).

Hordenine is considered as the main precursor of NDMA in malt due to the lability of the dimethylamine group of its molecule. The degradation of the hordenine by toasting in presence of nitrogen oxides consists of a set of reactions, in which some nitrosated intermediates are formed, releasing finally DMA, that will give NDMA by nitrosation (9).

Nowadays, due to acquired knowledge and adopted solutions to decrease and control the NDMA levels in beer, there isn't any fear that the consumption of beer can pose a risk for the health. NDMA concentrations in beer are so low that more sensitive analytical techniques are required for their detection.

In this work, a new method has been developed for determination of N-nitrosodimethylamine in beers. This method is based on a fast microwave-

assisted dansylation procedure, reported by us, for the derivatization of N-nitrosamines prior to HPLC determination (11). Forty-four samples of different kinds of beer were analyzed to provide current information on the levels of NDMA in commercial beers.

EXPERIMENTAL

Chemicals and Reagents

N-Nitrosodimethylamine (NDMA) was supplied by Sigma (St. Louis, MO, USA) and dansyl chloride was supplied by Aldrich (Beerse, Belgium). Acetonitrile of HPLC grade (Merck, Darmstadt, Germany) and water purified by means of a Milli-Q system (Millipore, Bedford, MA, USA) were used throughout for chromatographic analysis.

The N-nitrosodimethylamine was dissolved in dichloromethane (Merck) and the stock solution (20 mg/mL) was diluted before used and stored at 4°C.

The denitrosation reagent was prepared by diluting 100 μL of 48% aqueous hydrobromic acid (Aldrich) to give a final volume of 10 mL with acetic acid glacial (Panreac, Barcelona, Spain).

Dansyl chloride was dissolved in acetone (Merck) to give a $1.2 \cdot 10^{-4}$ M reagent solution.

All the chemicals used were of an analytical-reagent grade and used as received.

Apparatus

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).

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 by 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 were CEM PTFE-lined advanced composite digestion vessels used for sample dansylation. The vessels are constructed with

a PTFE liner and a cap for high-purity analysis and are capable of sustaining temperatures up to 200°C and pressures of 13.9 bar.

Microwave-Assisted Dansylation Method

0–500 μL of standard N-nitrosodimethylamine solution (0.20 μg/mL) was mixed with 20 μL of denitrosation reagent in a test-tube with stopper. After denitrosation reaction for 10 min at 40°C, the stopper was removed and dichloromethane solvent was evaporated. 1 M NaOH solution was added to adjust the pH to about 9, followed by addition of 0.2 mL of saturated sodium bicarbonate solution, and 2.5 mL of dansyl chloride solution. The solution was transferred quantitatively to a microwave vessel. The vessel was closed and introduced into the microwave cavity. The reaction was performed at 60% (378 W), keeping a maximum pressure of 1.4 bar inside the reactor during 5 min. When the vessel was cold, the mixture was taken to 5 mL with water for HPLC analysis.

Chromatographic Conditions

The HPLC method used for the determination of N-nitrosodimethylamine consisted of an isocratic elution procedure, using acetonitrile-water (55/45, v/v) at a flow-rate of 1.2 $mL \cdot min^{-1}$. The injection volume was 25 μL and the fluorescence excitation and emission wavelengths were 339 and 531 nm, respectively.

Determination of N-Nitrosodimethylamine in Beer

A 20 mL aliquot of beer, previously degassed and saturated with NaCl, was extracted twice with 5 mL of diethyl ether containing 25% 2-propanol for 2 min. After centrifugation (speed 300 rpm, 5 min), the organic layer was transferred into another tube and derivatized and analyzed in accordance with the methods previously described.

RESULTS AND DISCUSSION

To determine the N-nitrosodimethylamine, 44 samples were acquired from different commercial establishments of the island of Tenerife. The samples were chosen at random, without regard to their origin, place of packaging, the raw materials used, or the industrial process employed.

The samples were analyzed for N-nitrosodimethylamine by the HPLC procedure described by Cardenes et al. (11) which involves previous microwave-assisted derivatization with dansyl chloride. The dansyl derivative of N-nitrosodimethylamine was separated by HPLC with acetonitrile-water (55/45, v/v), as mobile phase, with fluorescent detection at 531 nm (excitation at 339 nm). Figure 1 shows chromatograms obtained from N-nitrosodimethylamine in a standard solution and in a sample of beer.

The calibration function (relationship between peak area and the amount of N-nitrosodimethylmine) was tested over the range 0–20 ng/mL. The slope of the least-squares linear regression fit of the calibration curve is 0.069 ± 0.001, the intercept is 0.171 ± 0.012, and the correlation coefficient is 0.997. The detection limit, expressed as the amount of N-nitrosodimethylamine required to produce a signal three times the background noise, is 4.0 pg.

The N-nitrosamine must be separated from the coexisting secondary amines in the beer samples before derivatization. Although dichloromethane is usually used for the extraction of N-nitrosamines (12,13), sampling of the organic layer was difficult because this solvent is isolated in the lower layer. In order to

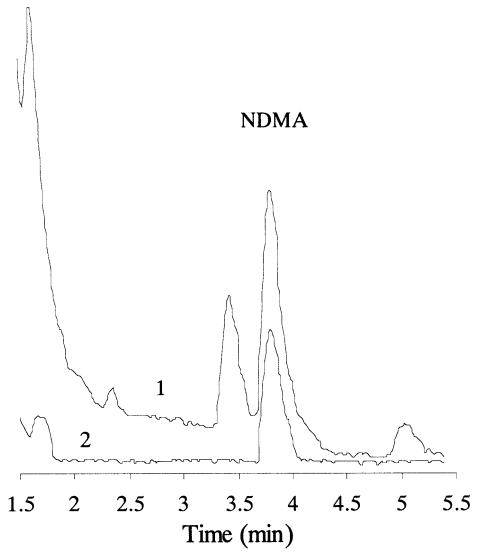

Figure 1. Typical chromatograms obtained from (1) a sample beer and (2) a standard N-nitrosodimethylamine solution (containing 2.5 ppb). Chromatographic conditions: Mobile phase, acetonitrile/water (55/45, v/v); flow rate, 1.2 mL, min^{-1}; detector wavelength, $\lambda_{em} = 531$ nm ($\lambda_{exc} = 339$ nm); injection volume, 25 μL; response, relative fluorescence intensity.

Table 1. N-Nitrosodimethylamine Levels in Beers

		No. of Samples with Levels of					
Type of Beer	No. of Samples	<0.04, μg/L	0.04–0.29 μg/L	0.30–0.50 μg/L	0.50–1.10 μg/L	Range, μg/L	NDMA[a], μg/L
Blonde	26	19	3	3	1	0.04–1.05	0.19 ± 0.30
Dark	13	9	2	1	1	0.04–0.93	0.14 ± 0.25
Wheat	5	5	0	0	0		0.04
Total	44	33	5	4	2		0.16 ± 0.25

[a]Mean ± standard deviation.
0.04 μg/L was used for ND (none detected) NDMA levels.

solve this problem, the extraction was performed twice with diethyl ether containing 25% 2-propanol, the N-nitrosodimethylamine was quantitatively transferred into the organic layer, and the coexisting secondary amines remained completely in the aqueous layer (14).

Recovery studies were conducted with the object of verifying the applicability of the analytical method. 20 mL aliquots of beer sample, previously degassed, were spiked with 50 ng of N-nitrosodimethylamine and extracted twice with 5 mL of diethyl ether containing 25% 2-propanol for 2 min. The organic layers were derivatized and analyzed in accordance with the methods previously described. The obtained mean recovery was $98.6 \pm 3.12\%$. After calculating this recovery, we verified by Student's t-test ($DF = 4$ and $P = 0.05$) that there were no significant statistical differences between the recovery value found and the theoretical value of 100%.

The sample size of beer (20 mL) has allowed us to reduce the limit of detection to 0.04 μg/L, more than 10 times lower than maximum levels permitted generally in Europe, 0.5 ppb and 2.5 ppb in beer and malt, respectively (5).

The levels of NDMA in the different types of beers studied are indicated in the Table 1. NDMA was detected in 20% of the beers at levels ranging from 0.04 to 0.50 μg/L, only 4.5% of the samples analyzed have got levels up to 0.5 μg/L. The mean NDMA level for 44 beers was 0.16 ± 0.25 μg/L and the range was from ND to 1.05 μg/L. No significant difference was observed among blonde and dark beers.

The agreement between the results obtained and those reported by other authors (15,16) shows that methods used effectively separate N-nitrosodimethylamine from possible interferences. The simple preparation of the samples and the rapid microwave-assisted derivatization considerably reduce analysis time.

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Received November 10, 2001
Accepted November 27, 2001
Manuscript 5687