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DETERMINATION OF NON-VOLATILE N-NITROSAMINES IN BABY BOTTLE RUBBER NIPPLES AND PACIFIERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–THERMAL ENERGY ANALYSIS

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SUMMARY

A method is described for the determination of non-volatile N-nitrosamines in baby bottle rubber nipples and pacifiers. It consists of extraction of the sample with dichloromethane in the presence of ascorbyl palmitate (an inhibitor of artifactual formation of nitrosamines), clean-up on silica or basic alumina, and final analysis by high-performance liquid chromatography–thermal energy analysis, a technique which is highly specific for N-nitroso compounds. The method worked well for the determination of four rubber-related non-volatile nitrosamines, namely, N-nitrosomethylphenylamine, N-nitrosoethylphenylamine, N-nitrosodicyclohexylamine, and N-nitrosodibenzylamine (recoveries from spiked samples >80%; detection limit, *ca.* 5 μg/kg for each). Eighteen out of twenty four samples analyzed were found to contain varying levels (mean, 41 μg/kg; range, 8–146 μg/kg) of N-nitrosodibenzylamine. The identity of the compound was confirmed by gas chromatography–thermal energy analysis as well as by gas chromatography–mass spectrometry analyses.

INTRODUCTION

Since the initial report of the finding of volatile N-nitrosamines in elastomers and elastomer-based products[1], considerable data have been published on the occurrence of these compounds in various rubber-based consumer products[1,2], especially baby bottle rubber nipples and pacifiers[3–6]. The nitrosamines in these products mainly originate or form from various amine derivatives which are commonly used as accelerators or stabilizers during the rubber vulcanization process. The following volatile nitrosamines have been detected in rubber chemicals and rubber products: N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodi-*n*-butylamine (NDBA), N-nitrosodi-*sec.*-butylamine (NDSBA), N-nitroso-

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piperidine (NPIP), N-nitrosopyrrolidine (NPYR), and N-nitrosomorpholine (NMOR)[1–6]. All of these nitrosamine are potent carcinogens in laboratory animals[7]. Further details regarding the chemistry of formation of these nitrosamines in rubber, their concentration in rubber factory atmosphere and in various consumer products, and about the significance of the findings can be obtained from several publications[4–6,8,9].

The occurrence of nitrosamines in baby bottle rubber nipples and pacifiers is of special concern because traces of these chemicals may migrate to infant saliva during sucking, and then be ingested. Furthermore, the nitrosamines can also migrate to liquid infant foods which come in contact with rubber nipples during home sterilization or during storage[2–5]. Because of these concerns, various governments in different countries have passed regulations limiting the total volatile nitrosamine content of such products[10,11]. The rubber industry also responded well to this concern, and reformulated the rubber curing formulations which resulted in a significant decline in the levels of volatile nitrosamines in such products. Recent surveys by both the Canadian and U.S.A. government agencies have confirmed this trend[10,11].

Although considerable data are available on the volatile nitrosamine contents of various rubber nipples and pacifiers, corresponding data for the non-volatile N-nitrosamines, which may also occur in such products[2,12], have been lacking. This has been mainly attributed to lack of suitable analytical methodologies for these compounds. Such data are, however, urgently needed for a full assessment of the health hazard to infants that may arise from the use of such products. In this paper, we report a high-performance liquid chromatography–thermal energy analysis (HPLC–TEA) method for the determination of non-volatile N-nitrosamines in baby bottle rubber nipples and pacifers. Some data on the non-volatile nitrosamine contents of such products are also reported.

EXPERIMENTAL*

Materials

Glass-distilled dichloromethane (DCM) was purchased from Burdick & Jackson, (Muskegon, MI, U.S.A.) and was redistilled from an all-glass apparatus and tested for its suitability for nitrosamine analysis as reported previously[13]. Glass-distilled *n*-pentane, *n*-hexane, and acetone were obtained from Caledon Labs. (Georgetown, Canada). The last three solvents were passed through highly activated (<0.1% water content) basic alumina before use. Basic alumina for column chromatography and ascorbyl palmitate were purchased from ICN Biomedicals, K&K Laboratories (Plainview, NY, U.S.A.) and Kieselgel 60 (Si-60) (70–230 mesh) was obtained from Merck (Darmstadt, F.R.G.). Aliquots of alumina and Si-60 were heated in an oven and then deactivated by adding appropriate amounts of water (1.5 and 6%, respectively) as described previously[14]. All other reagents used were of analytical grade.

The nitrosamine standards were purchased or obtained as gifts from various sources as indicated below: N-nitrosodibenzylamine (NDBzA) from W. Fiddler of

* Reference to brand or firm name does not constitute endorsement by the Consumer and Corporate Affairs of Canada or by Health and Welfare Canada over others of a similar nature not mentioned.

USDA (Philadelphia, PA, U.S.A.); N-nitrosoethylphenylamine (NEPhA), N-nitrosomethylphenylamine (NMPhA), N-nitrosodicyclohexylamine (NDChA), from ISCONLAB (Heidelberg, F.R.G.); N-nitroso-4-hydroxybutyl-*n*-butylamine (NHBBA) from M. Okada of Tokyo Biochemical Research Institute (Tokyo, Japan); diphenylnitrosamine (NDPhA) and N-nitrosodiethanolamine (NDELA) from Thermedics, and dinitrosopentamethylenetetramine (NDPMTA) from W. Lijinsky of NCI Frederick Cancer Research Facility (Frederick, MA, U.S.A.).

Apparatus

The HPLC system consisted of two solvent delivery systems (pump Model 6000A, Waters Assoc., Milford, MA, U.S.A.) connected to a solvent programmer (Model 660, Waters Assoc.) and a Rheodyne injector (Model 7125; sample loop 20 or 50 μl). The chromatographic separation was carried out using a Alltech 250 mm × 4.6 mm I.D. stainless-steel column packed with Lichrosorb Si 100 (5 μm) (column 1) or Econosil silica (5 μm) (column 2). The mobile phases and programming conditions were as follows: 1% acetone in *n*-hexane initially, then programmed to 40% acetone in *n*-hexane in 15 min using curve 10 of the solvent programmer. The flow-rate was 2 ml/min. Prior to use, the solvents were passed through basic alumina (to remove nitrosamine contamination) as described under *Materials*.

The tubing from the exit end of the HPLC column was connected via a three-way switching valve to a Model 502 thermal energy analyzer (Thermedics). This switching valve was used to divert the column flow to a waste reservoir, when TEA was not in use. This was also done every time the analyzer was switched to the vent mode or purge mode. (Caution: failure to divert the flow as above would build-up pressure inside the analyzer furnace and also may promote carbon deposition on the pyrolyzer tube causing noise problems with decreased sensitivity). Other TEA operating conditions were as follows: vacuum, 0.8 mm; furnace temperature, 600°C; cold trap-dry ice and ethanol.

In some cases, HPLC–TEA results were confirmed by gas chromatography (GC)–TEA. A Varian gas chromatograph (Model Vista 6000) equipped with a 10 ft. × $\frac{1}{4}$ in. coiled glass column (2 mm I.D.) packed with 10% Carbowax 20M on Chromosorb W, HMDS-treated, 80–100 mesh, or with 3% OV-225 on Chromosorb W-HP, 80–100 mesh, was used for this purpose. The temperature programming mode for both columns was as follows: 2 min at 120°C, then 6°C/min to 150°C with 5 min hold at 150°C, followed by further heating to 220°C at 10°C/min (held at 220°C for 10 min). Injector and transfer line temperatures were 200 and 380°C, respectively.

A VG analytical hybrid mass spectrometer system (Model 7070EQ) was used for mass spectrometric (MS) confirmation. Both selected-ion monitoring at a resolution of 2000 (10% valley definition) and repetitive exponential scanning were used for MS confirmation. The instrument was operated as described before[12,15].

Extraction and clean-up procedure

A 3–5 g aliquot of the sample (cut into small pieces) was mixed with 100 mg ascorbyl palmitate (nitrosation inhibitor), the mixture extracted with DCM by overnight shaking at room temperature and then again by an ambient temperature column extraction procedure as described previously[4,13] for the determination of volatile nitrosamines. The final extract was then carefully concentrated to 1 ml using

a Kuderna–Danish concentrator[13]. A 6–8 μl aliquot of this extract, if desired, could be analyzed by GC–TEA for the presence of volatile nitrosamines[4]. The remaining extract was cleaned-up as described below for the determination of non-volatile nitrosamines.

About 9 ml anhydrous *n*-pentane were mixed with the extract and the mixture was passed through a basic alumina (10 g; 1.5% water content) column (2 cm I.D.) at a flow-rate of 1–2 ml/min. The column was washed with 40 ml anhydrous *n*-pentane and the rinsing discarded. The nitrosamines adsorbed on the column were eluted with 50 ml DCM, the eluate concentrated to 1.0 ml as described previously[13], and a 20–50 μl aliquot analyzed by HPLC–TEA using the conditions mentioned above.

Prior to GC–MS confirmation, the alumina (10 g) clean-up step was repeated using a narrower column (1 cm I.D.) and collecting five successive 10-ml DCM eluates. Each eluate was separately concentrated and analyzed by HPLC–TEA as above. The fraction containing bulk of the nitrosamine (NDBzA) was used for GC–MS confirmation. This procedure provided a cleaner extract because of finer separation of NDBzA from the impurities.

For plastic-based products, a different clean-up step was used. After the overnight extraction of the sample with DCM and the subsequent ambient temperature column extraction, the combined DCM extract was concentrated to *ca.* 10 ml (instead of 1 ml). One-half of the extract was quantitatively transferred into a 250-ml Erlenmeyer flask and mixed with 50 ml *n*-pentane. The flask containing the mixture along with white precipitate, which often formed upon mixing with *n*-pentane, was cooled for 30–45 min inside an insulated box containing dry ice. The mixture was then quickly filtered through a coarse sintered glass funnel containing *ca.* 1 cm layer of granular anhydrous sodium sulfate. Any remaining precipitate was washed with two 10-ml portions of cold *n*-pentane and filtered as above. The combined filtrate was cleaned-up by passing (1–2 ml/min) through a column (2 cm I.D.) of Si-60 (10 g; 6% water content) and washing the column with two 5 ml portions of *n*-pentane. Finally, the column was successively eluted with 50-ml portions of (a) 20% DCM in *n*-pentane, (b) 50% DCM in *n*-pentane, and (c) DCM. The last fraction was concentrated to 1 ml and then analyzed by HPLC–TEA as above.

RESULTS AND DISCUSSION

As previously stated, the method described above is an extention of our earlier method developed for the determination of volatile nitrosamines in baby bottle rubber nipples and pacifiers[4,13]. Since the extraction used in the two methods is the same, aliquots of the same DCM extract can be used for the determination of non-volatile nitrosamines without any additional work except the minor clean-ups on basic alumina or silica as described. The method worked well with all the rubber nipples and pacifiers tested. Since TEA is highly specific for N-nitroso compounds, very little interference was observed (Figs. 1 and 2).

It should be noted, however, that both the resolution and retention times of various nitrosamine peaks may vary depending on the column used for HPLC separation. For example, good resolution of NMPhA and NDChA was obtained with column 1 in Fig. 1 but not with column 2 used to obtain Fig. 2, even though both columns were packed with similar packing materials. Failure to separate the above two

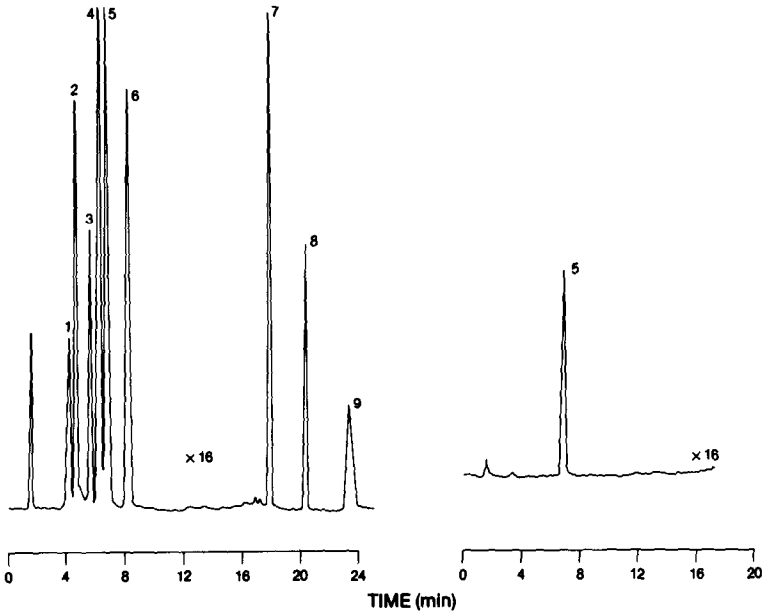

Fig. 1. HPLC–TEA chromatograms of several nitrosamine standards (left) and of NDBzA isolated from a rubber-based pacifier (right). Details of column and mobile phase are given in the Experimental section. Peak identification: 1 = NDPhA, 2 = NEPhA, 3 = NDChA, 4 = NMPhA, 5 = NDBzA, 6 = N-nitrosodi-*n*-propylamine, 7 = NHBBA, 8 = NDPMTA, 9 = NDELA. About 20 ng of each was injected using column 1 for analysis.

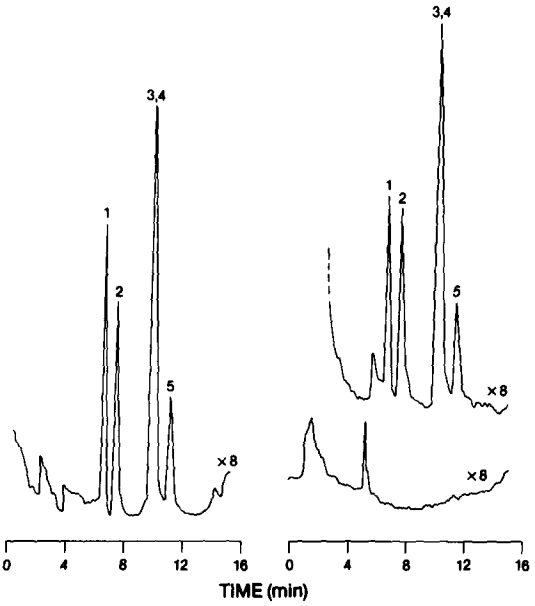

Fig. 2. HPLC–TEA chromatograms of five nitrosamine standards, *ca.* 8 ng each (left); of a rubber nipple, unspiked (bottom-right); and of the same nipple spiked with *ca.* 40 ppb each of the five nitrosamines (top-right). HPLC separation was carried out using column 2. Peak identification: same as in Fig. 1.

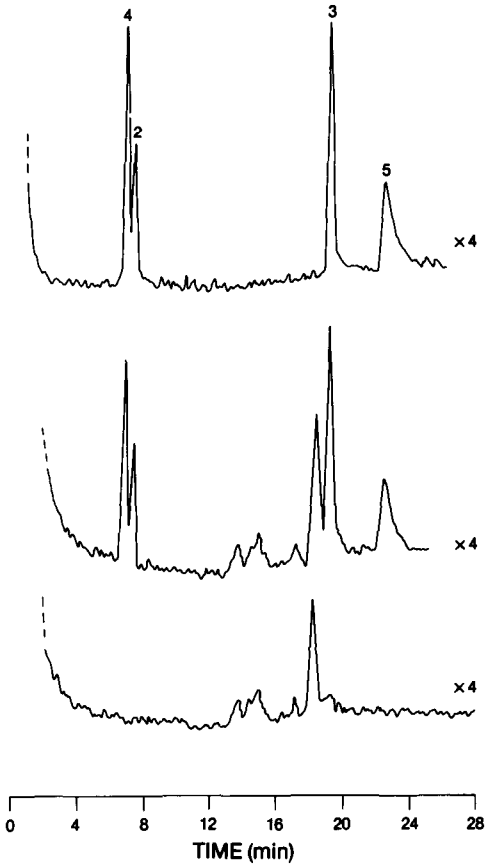

Fig. 3. GC–TEA chromatograms using OV-225 column. Bottom, 10 μl injection of the unspiked rubber nipple extract (from Fig. 2); middle, 10 μl injection of the spiked sample (from Fig. 2); and top, 2 ng each of five standards. GC conditions are described in the Experimental section. Peak identification is the same as in Fig. 1. The sample showed an unidentified peak eluting at *ca.* 18 min.

nitrosamines by HPLC–TEA using column 2 was, however, a minor drawback because these two compounds could easily be separated by GC (Fig. 3). On the other hand, NDPhA could not be measured by GLC–TEA because of its thermal instability[16]; the HPLC–TEA technique had to be used for this purpose. The two techniques were, therefore, complementary to each other.

The recoveries of NMPhA, NEPhA, NDChA, and NDBzA, the four most rubber-associated non-volatile nitrosamines, added in DCM to rubber nipples at 20–50 μg/kg levels at the start of the analysis were highly satisfactory (80–110%). Typical chromatograms from a recovery study experiment are shown in Fig. 2. Similar good recoveries have been observed with the method for the volatile nitrosamines[4,13]. The recovery of a fifth rubber-related non-volatile nitrosamine, NDPhA, was, however, slightly lower (60–70%; also shown in Fig. 2). This was demonstrated to be due to its instability. When standard NDPhA alone (without any rubber nipple) was allowed to stand overnight in DCM and the solution concentrated to 1 ml using

a Kuderna–Danish concentrator, the recovery was still not improved. The N–NO bond in NDPhA is very weak[16] which explains its instability and good transnitrosation property. Because of this, Spiegelhalder and Preussmann[8] recommended discontinuation of its use as rubber curing retarder. Instead, they proposed to replace it with cycohexylthiophthalimide which does not form nitrosamines. Except for NDPhA, the minimum detection limit of the other four nitrosamines was about 5 μg/kg for each compound. For plastic-based products, however, the detection limit was only 30 μg/kg. Such products alway resulted in an oily residue that could not be separated from the above mentioned nitrosamines. Because of this, a less concentrated extract was injected into HPLC–TEA resulting in a lower overall sensitivity.

Fig. 1 also shows HPLC separation of three highly polar N-nitroso compounds, namely, NHBBA (peak 7), NDPMTA (peak 8), and NDELA (peak 9). Although these compounds are not expected to be present in baby bottle rubber nipples and pacifiers, they were included just to demonstrate the usefulness of the technique. If present in the samples, these compounds could be extracted using the described technique but a stronger eluent (*e.g.*, ethyl acetate, ethanol) must be used to elute them from the alumina column[17]. We have developed previously a method for the determination of these compounds in cured meats[17]. Most of the volatile nitrosamines normally detected in such products eluted between peaks 6 and 7 under the HPLC conditions used. Only NDBA eluted between peaks 4 and 5. Therefore, if NDBA is present, as determined by preliminary analysis for volatile nitrosamines[4,13], the result for non-volatile nitrosamines must be confirmed, as was done here, by GC–TEA and/or GC–MS.

The method was used to analyze 24 samples of various rubber nipples and pacifiers. Six were negative for all of the four nitrosamines and the remaining samples contained only NDBzA (Table I). To our knowledge, this is the first reported finding of NDBzA in such products. The same samples were also analyzed for volatile nitrosamines[11]. The data indicated a significant decline in the levels of these compounds in such products when compared to similar monitoring data gathered 3–5 years earlier[4,12]. This reduction was attributed to recent changes in the rubber curing formulations that eliminated or employed only minimal amounts of volatile nitrosamine-forming accelerators. Unfortunately, our older surveys did not include analysis for non-volatile nitrosamines, and one cannot say with certainty whether the older samples also contained NDBzA (in addition to high levels of volatile nitrosamines). However, we have reanalyzed two remaining samples of rubber nipples from our 1985 survey[12]. Both were negative for NDBzA. This suggested that the rubber industry most likely switched to NDBzA-producing accelerators only recently. Similar observations were made by us in the case of elastic rubber nettings used for packaging cured pork products[18].

All the NDBzA-positive samples were confirmed by GC–TEA for confirmation. Although it is a non-volatile nitrosamine, it could be analyzed by GC–TEA provided a high (350–400°C) transfer line (between GC and TEA) temperature and an on-column injection technique was used. Furthermore, the identity of NDBzA in two samples (Table I) was confirmed by GC–MS using the selected-ion monitoring technique as mentioned earlier. Two characteristic fragment ions, namely, *m/z* 226 $M^{+\cdot}$ and *m/z* 181 $[M-HN_2O]^+$ were monitored simultaneously for this purpose. The mass spectrum of NDBzA isolated from the samples also matched quite well with that of the authentic standard (Fig. 4).

TABLE I
LEVELS OF N-NITROSODIBENZYLAMINE DETECTED IN DIFFERENT BRANDS OF BABY BOTTLE RUBBER NIPPLES AND PACIFIERS

Type	*Brand*	*Country of origin*	*NDBzA (μg/kg)*
Nipples			
Latex	A	U.S.A.	N*
Latex	B	U.S.A.	86
Latex	C	U.S.A.	20**; 39**
Latex	D	F.R.G.	10
Latex	E	U.S.A.	39
Latex	H	U.S.A.	14
Latex	X	F.R.G.	11
Silicone	II	Thailand	N
Latex	III	F.R.G.	29
Latex	IV	Thailand	N**; 10**
Pacifiers			
Latex	C	U.S.A.	30
Latex	E	U.S.A.	146
Latex	F	F.R.G.	71***
Latex	L	F.R.G.	18
Latex	M	U.K.	N**; N**
Latex	N	F.R.G.	8
Latex	Q	F.R.G.	24
Latex	U	F.R.G.	34
Latex	V	F.R.G.	136***
Plastic	W	U.S.A.	N
Latex	VI	F.R.G.	12

* N = Negative (< 5 μg/kg).
** Two different samples.
*** Confirmed by GC–MS.

Our present investigation suggests that majority of the baby bottle rubber nipples and pacifiers currently available to consumers in Canada contain varying levels (8–146 μg/kg) of NDBzA. The significance of the finding is, however, not clear. NDBzA is reported to be non-carcinogenic in rats[19]. This is one of the reasons that might have prompted the industry to switch from carcinogenic nitrosamine-forming accelerators to one that produces only NDBzA. In a recent study, however, it has been reported that NDBzA can induce DNA single-strand breaks in primary hepatocytes from rat and hamster[20]. Therefore, further research is desirable to fully evaluate the toxicity, if any, of NDBzA.

In summary, the HPLC–TEA method described in this paper offers a simple and rapid method for the determination of non-volatile N-nitrosamines in baby bottle rubber nipples and pacifiers. It is hoped that the method along with the GC–TEA and GC–MS confirmatory techniques will be useful for both regulatory and monitoring purposes.

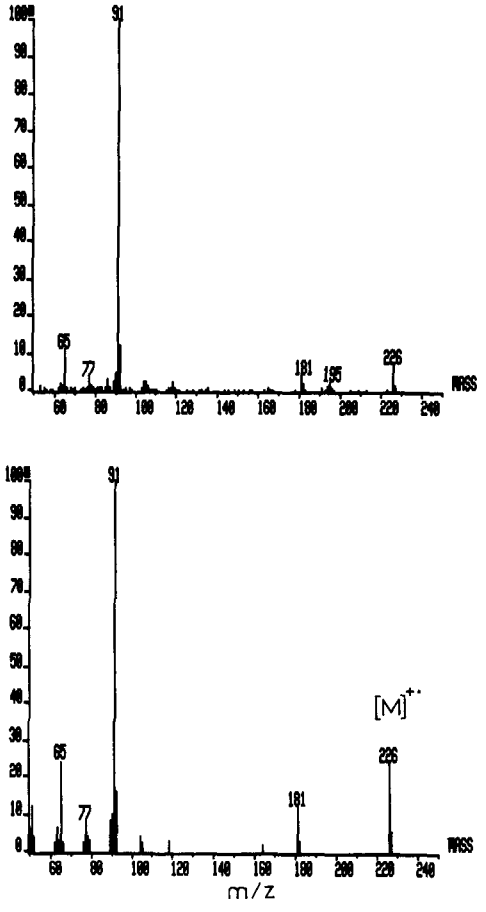

Fig. 4. Electron-impact ionization mass spectra of NDBzA standard (bottom) and that of NDBzA isolated from the pacifier containing 136 μg/kg NDBzA (Table I).

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