Collaboration

Prospective study of N-nitrosamines and N-nitrosatable substances in rubber and silicone teats and soothers

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Abstract

Teats and soothers for infants are usually made of rubber and silicone rubber. During the vulcanization process of rubber, nitrosatable substances can be formed which in contact with nitrates present in the saliva may lead to the formation of N-nitrosamines, chemicals considered to pose a health risk due to their carcinogenic and/or mutagenic properties. These substances are regulated at European level by Directive 93/11/EEC, transposed into the Spanish Royal Decree 1184/1994. Taking into account the lack of data available at national level, and also the frequent use of this type or articles intended for the infant population, we carried out a prospective study on the release of N-nitrosamines and N-nitrosatable substances into artificial saliva solution, on 24 samples of teats and soothers marketed in Spain. Gas chromatography coupled to mass spectrometry (GC-MS) was used as the analytical method, along with UNE-EN 12868 as standard for the release test. The analytical method was internally validated with satisfactory results for the criteria set in *Guidelines for performance criteria and validation procedures of analytical methods used in controls of food contact materials*.

N-nitrosamines and N-nitrosatable substances determined in this study were below the regulated limits of 0.01 $mg.kg^{-1}$ and 0.1 $mg.kg^{-1}$, respectively, in all the 24 samples. These results are in agreement with data published by other authors.

Key words

N-nitrosamines, N-nitrosatable substances, teats, soothers, rubber, silicone, GC/MS.

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1. Introduction

N-nitrosamines are a broad group of chemical compounds containing N-nitroso groups (>N-N=O) considered hazardous for health due to their carcinogenic and/o mutagenic nature. The International Agency for Research on Cancer (IARC) has classified the majority of these compounds in the groups 2A or 2B (IARC, 2016).

It is well-known that N-nitrosamines are normally formed as a result of the interaction of a nitrosating agent (nitrite salts or nitrogen oxides) with secondary amines (Challis, 1985). They are usually present (normally as trace contaminants) in the environment (tobacco smoke), certain foods (smoked fish and meat), cosmetics and products manufactured from natural or synthetic rubber (Sen et al., 1997) (Andrade et al., 2005) (Feng et al., 2011) (Jones et al., 2016).

The dithiocarbamates and thiourames used as accelerators in rubber vulcanisation (to provide greater elasticity and resistance) may break down in the process and form secondary amines. When any of these reacts with some type of nitrogen oxide specie from the environment or from the manufacturing process, nitrosamines and/or their precursors (nitrosatable substances) may be formed. If a nitrosatable substance (for example dibenzyalmine) reacts with the nitrites present in foods or with the nitrates in saliva, nitrosamines may also be formed (Fishbein, 1983).

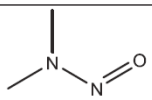
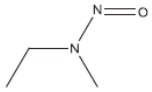
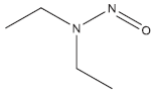
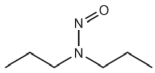
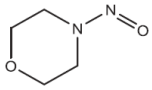
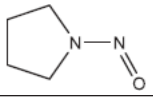
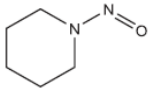
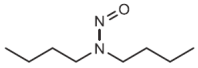
The restrictions of these substances are regulated by the European Commission in Directive 93/11/CE, transposed into Royal Decree 1184/1994 (BOE, 1994), which establishes the criteria applicable to the method of determination, which must be able to detect the following quantities:

- 0.01 mg of the total N-nitrosamines released/kg (from the elastomer or rubber teat or soother).
- 0.1 mg of the total N-nitrosatable substances /kg (from the elastomer or rubber teat or soother).

These release limits are based on the analytical tolerance and not on a toxicological assessment. Since teats and soothers used by babies and infants, a vulnerable population group, are normally manufactured using rubber or silicone rubber, the control of these products is important for guaranteeing the protection of their health.

The migration of N-nitrosamines from different materials in contact with food has been studied by several authors (Sen, 1988) (Zou and Yu, 2010) (Fenget al., 2011) (Küne et al., 2018), with special emphasis on teats and soothers in light of the population for whom these articles are intended (Senet al., 1987) (Bouma et al., 2003) (Vieira et al., 2006) (Sung et al., 2010) (Mutsuga et al., 2013). Based on a literature review, and considering the most frequently found N-nitrosamines, the substances listed in table 1, together with their chemical structure, CAS No, molecular weight and IARC classification were selected for this study.

Table 1. N-nitrosamines included in this study

Name (abbreviation)	Structure	CAS No	Molecular weight ($g.mol^{-1}$)	IARC Classification
N-nitrosodimethylamine (NDMA)		62-75-9	74	2A
N-nitrosomethylethylamine (NMEA)		10595-95-6	88	2B
N-nitrosodiethylamine (NDEA)		55-18-5	102	2A
N-nitrosodipropylamine (NDPA)		621-64-7	130	2B
N-nitrosomorpholine (NMOR)		59-89-2	116	2B
N-nitrosopyrrolidine (NPYR)		930-55-2	100	2B
N-nitrosopiperidine (NPIP)		100-75-4	114	2B
N-nitrosodibutylamine (NDBA)		924-16-3	158	2B

Different analytical techniques have been applied in the determination of these compounds. Gas Chromatography coupled to Thermal Energy Analysis (GC-TEA) or to a Nitrogen Chemiluminescence Detector (GC-TEA) are considered to be the ideal techniques due to their high level of selectivity and sensitivity. However, as the use of these techniques is limited to the analysis of N-nitroso components or compounds containing nitrogen, they are not considered as universal detection techniques (Sen et al., 1997) (Byun et al., 2004), and are not frequently found in laboratories. Other detectors, such as the nitrogen-phosphorus detector (NPD) and the flame ionisation detector (FID) are neither sufficiently sensitive nor specific (Aygun et al., 2004) (Grebel et al., 2007). Gas chromatography coupled to mass spectrometry (GC-MS) is suitable for the detection of N-nitrosamines, in particular, operating in chemical ionisation mode. Feng et al. (2011) compared the sensitivity of these detectors and found that GC-MS revealed lower limits of detection, followed by GC-TEA and GC-NCD. More recently, liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) has been applied to the analysis of N-nitrosamines in food simulants (Kühne et al., 2018).

In this study, the GC-MS technique has been used. In our case in electron impact mode (EI), together with the UNE-EN 12868 standard as the release test method, as it is widely used in the lite-

rature and is in line with the indications given in the European Union Directive. The purpose of the study, motivated by the scarcity of national data and the vulnerability of the infant population, was to provide data on the levels of N-nitrosamines and N-nitrosatable substances released into an artificial saliva solution from rubber and silicone rubber teats and soothers sold in the Spanish market.

2. Material and methods

2.1 Samples analysed

Between March and August 2017, 24 samples of teats and soothers were obtained from retailers (large and medium-sized) in the Community of Madrid, distributed as follows: 12 teats and 6 soothers made of natural rubber latex, 5 silicone teats and 1 silicone soother, corresponding to 16 different brands, and for which the countries of origin were in the European Union, except for 7 samples which came from Asian countries.

The samples were commercially packaged including two units for the teats and one or two units for the soothers. As the size, (and consequently the weight) varied according to the age for whom they were intended (new born, 4 m+, 6 m+, 6-12 m, +18 m) and their intended use (milk, infant formula…), in some cases it was necessary to buy several packages (of the same batch) in order to conduct duplicate tests.

The samples were stored at ambient temperature in their original packaging until the start of the tests. Once the package had been opened, all non-rubber or non-elastomer parts were removed and the teats/soothers were boiled in water. They were placed in a flask with boiling water (the minimum amount of water needed to cover the samples) and boiled for 10 minutes. They were then left to cool at ambient temperature, cut into two parts longitudinally and left to air dry. The samples prepared in this way were wrapped in aluminium paper to protect them from the light (N-nitrosamines are susceptible to degradation by ultraviolet light) and stored at temperatures of below 5 ºC until the start of the release test.

2.2 Reagents and standards

Dichloromethane trace analysis grade for GC (Scharlab); n-hexane and methanol HPLC grade (Scharlab); sodium sulphate anhydrous, sodium carbonate acid, sodium chloride, potassium carbonate and sodium nitrite (Scharlab, reagent grade); sodium hydroxide, hydrochloric acid and nitric acid (Merck, reagent grade) and ammonium hydroxide p.a. (Fluka, reagent grade).

The artificial saliva saline solution used in the release tests was prepared as indicated in the UNE-EN-12868 standard (section. 5.8).

The standard used was a MIX of N-nitrosamines of purity ≥ 99.8 % (Sigma-Aldrich) (EPA 8270), of 2 000 $\mu g.ml^{-1}$ in methanol. An intermediate solution of 10 $\mu g.ml^{-1}$ in methanol was prepared from the concentrated solution, and this was used to prepare the calibration solutions in the range of 15 $\mu g.l^{-1}$ to 300 $\mu g.l^{-1}$ (equivalent to 2 $\mu g.kg^{-1}$-40 $\mu g.kg^{-1}$), in hexane. In addition, a solution of 1 $\mu g.ml^{-1}$ in methanol was prepared for the testing of samples fortified with N-nitrosamines.

N-nitroso-di-iso-propylamine (NDiPA), 99.5 % purity (Dr. Ehrenstorfer), of 100 $ng.\mu l^{-1}$ in methanol was used as the internal standard. The concentration of the internal standard in the calibration solutions was 0.2 $\mu g.ml^{-1}$.

All these solutions were kept in darkness at a temperature <5 ºC (maximum 2 weeks).

All the glass materials were previously treated with 10 % nitric acid and a 0.1 M ammonia solution.

2.3 Instruments

Equipment from Agilent Technologies was used for the chromatographic analysis: CG 6890N coupled to a mass spectrometry detector 5973. The column used was a DB-624 25 m x 0.2 mm and 1.12 µm (Agilent), under the following conditions: oven temperature: 60 ºC (5 min) – 20 ºC/min – 120 ºC (5 min) – 3 ºC/min – 140 ºC (3 min) – 20 ºC/min – 160 ºC (5 min) – 40 ºC/min – 240 ºC (8 min). *Postrun* at 250 ºC (2 min). Temperature of 120 ºC for the transfer line, 120 ºC at the injection port and 230 ºC on the MS source. Helium was used as the carrier gas at a constant flow of 1 ml/min. Injection volume of 1 µl in splitless mode. Ionization voltage 70 eV (electron impact mode). Solvent delay: 6.5 min. A SIM acquisition mode was used, monitoring the masses given in table 2.

Table 2. Retention times and masses monitored in GC-MS

N-nitrosamine	TR (min)	Target *m/z*	Q1 *m/z*	Q2 *m/z*
NDMA	7.57	74.1	42.2	-
NMEA	8.97	88.2	42.2	56.2
NDEA	10.42	102.2	44.2	57.2
NDiPA (P.I)	14.11	130.2	70.2	-
NDPA	16.23	70.2	130.2	113.2
NMOR	16.6	116.1	56.2	86.2
NPYR	17.1	100.1	41.2	68.2
NPIP	18.4	114.2	42.2	55.2
NDBA	24.6	84.2	116.2	158.2

Equipment from Agilent Technologies was used for the CG/MS-MS confirmation analysis: CG 7890A coupled to a triple quadrupole mass spectrometry detector 7693. The column used was an HP-5 MS of 30 m x 0.25 mm x 0.25 µm (Agilent). The remaining chromatographic conditions used were the same as indicated previously. The mass spectrometry detection was in MRM mode (with segments). Solvent delay: 1.5 min. The transitions listed in table 3 were monitored.

Table 3. Retention times and transitions monitored in GC-MS/MS

N-nitrosamine	TR (min)	Quantification transition	Confirmation transition
NDMA	2.2	74.0> 43.0	74.0> 42.1
NMEA	3.2	88.1> 42.1	88.1> 71.1
NDEA	4.7	102.1> 85.1	102.1> 44.1
NDiPA (P.I.)	7.2	130.1> 42.9	130.1> 113.1
NPYR	7.9	100.0> 55.1	100.0> 43.0
NMOR	8.0	116.0> 56.1	116.0> 86.1
NDPA	8.1	130.1> 43.1	130.1> 70.1
NPIP	8.5	114.1> 41.5	114.1> 83.9
NDBA	11.4	158.0> 99.1	158.0> 141.1

2.4 Extraction of the samples

2.4.1 Release test

10 g of the boiled and cut samples were weighed in a migration tube and covered with 40 ml of artificial saliva solution. These were kept in an oven at 40 ± 2 ºC for 24 hours (+30 min). The migration solution was decanted into a 50 ml flask. The samples were rinsed with 4 ml of artificial saliva, transferred to the flask and made to volume with distilled water.

These migration solutions were kept refrigerated and in darkness until their analysis.

2.4.2 Extraction of the released N-nitrosamines

After the migration phase, 40 ml of the solutions were taken, and transferred into a separation funnel. Then, 1 ml of 1 M sodium hydroxide, 1 ml of the internal standard solution of 0.2 µg.ml^{-1} and 20 ml of dichloromethane (DCM) were added. The funnel was shaken vigorously and the phases were left to separate. The lower layer was collected over sodium sulphate anhydrous (previously washed with DCM) and the process repeated with a further 20 ml of DCM.

2 ml of hexane were added and evaporated under a gentle flow of N_2 (T: 35 ºC) to a volume of less than 1 ml (not reaching dryness). The volume was made up to 1 ml with hexane for subsequent injection in the GC-MS.

2.4.3 Extraction of the released N-nitrosatable substances

The remaining 10 ml of the migration solution were transferred into a separation funnel and 1 ml of 1 M hydrochloride acid was added. This was shaken vigorously and left in the dark for 30 min. This resulted in a solution with a pH of approximately 1.4.

Next, 2 ml of 1 M sodium hydroxide (to make alkaline the solution), 1 ml of 0.2 µg.ml^{-1} internal standard and 10 ml of DCM were added, and the same extraction process as used for the N-nitrosamines was followed.

The N-nitrosatable substances after their hydrolysis were quantified as N-nitrosamines, subtracting the value obtained in the above section.

2.5 Validation

The set up method was validated in an artificial saliva solution and in a release solution in saliva from a pool of rubber teats and another of silicone teats.

The following parameters were assessed, taking as a reference the guide *Guidelines for performance criteria and validation procedures of analytical methods used in controls of food contact materials* (Bratinova et al., 2009):

- Selectivity/specificity. It was assessed that in the reagent blank (artificial saliva solution) there were no peaks interfering in the retention time of the different N-nitrosamines, with responses of more than 1/3 of that obtained at the quantification limit. The variation in retention time of the N-nitrosamine peaks in the samples could not exceed the margin of ± 2 %, with respect to the standards. In addition, the ion ratio of the transitions monitored should not vary, with respect to those obtained with the standards, in a percentage greater than ± 20 % - ± 30 %, depending on the N-nitrosamine analysed.
- Calibration function. This was assessed obtaining lineal regression calibration curves in the range 15 $\mu g.l^{-1}$ to 300 $\mu g.l^{-1}$ (2 $\mu g.kg^{-1}$ to 38 $\mu g.kg^{-1}$ sample) in n-hexane, on different days. The linearity should be such that the lineal regression correlation coefficient had to be ≥ 0.99, and the residuals of the calibration levels were less than 15 %, except at the quantification limit where a variation of 20 % was acceptable (if residuals were higher, calibration weighting could be applied).
- Precision/accuracy. This was evaluated by spiking artificial saliva solution with N-nitrosamines at levels of 16 $\mu g.l^{-1}$, 80 $\mu g.l^{-1}$ and 200 $\mu g.l^{-1}$ (equivalent to 2 $\mu g.kg^{-1}$, 10 $\mu g.kg^{-1}$ and 25 $\mu g.kg^{-1}$), in triplicate and on 4 different days. Subsequently, to verify the quantification limit, three series were carried out by fortifying release solutions from rubber and silicone teats at 16 $\mu g.l^{-1}$ level (equivalent to 2 $\mu g.kg^{-1}$).

The CV % were estimated for the internal repeatability and reproducibility. Accuracy was expressed as % recovery. As reference criteria for the precision, the based on Hortwiz-Thompson equation estimated values were used. Recoveries for the levels studied should be between 60 and 110 % for concentrations in the range (10-100) $\mu g.kg^{-1}$, and between 40 and 120 % for concentrations ≤10 $\mu g.kg^{-1}$, in accordance with that established in the above-mentioned guide.

Uncertainty (U %) was calculated using the following expression:

$$U(\%) = 2 * [U_{Reproducibility}(\%)^2 + U_{recovery}(\%)^2]^{1/2}$$

3. Results and Discussion

The validation of the method for the determination of N-nitrosamines and N-nitrosatable substances, conducted in accordance with the criteria indicated above, was satisfactory.

In relation to linearity, the correlation coefficients (r) obtained in all cases were ≥ 0.992 and the relative standard deviation of the slope (Sb (%)= (Sb/b) x 100) was below 4 %. Results for precision

(expressed as CV % of internal repeatability and reproducibility), accuracy (% recovery), and uncertainty, obtained at the quantification limit of 2 µg.kg^{-1} for each of the nitrosamines, are summarised in table 4. The quantification limit for the N-nitrosatable substances was 8 µg.kg^{-1}.

It should be noted that the test method (UNE-EN 12868) has an analytical correction which must be applied to the results obtained. This correction implies the subtraction of the following values from the analytical result: 0.01 mg.kg^{-1}, from the quantitative result of N-nitrosamines, 0.1 mg.kg^{-1} from the result of N-nitrosatable substances. This implies that for a sample to be considered non-compliant to Directive 93/11/EEC, the result of N-Nitrosamines must be > 0.02 mg.kg^{-1}, or > 0.2 mg.kg^{-1} for N-nitrosatable substances.

Table 4. Validation results at the limit of quantification (LQ= 2 µg.kg^{-1})

N-nitrosamine	Precision		Accuracy (% Recovery)	Uncertainty (U %) (K= 2)
	CVr %	CV_R %		
NDMA	5.1	12.8	83.6	26
NMEA	4.8	5.9	98.7	12
NDEA	3.0	3.2	109.2	7
NDPA	4.5	8.8	101.4	18
NMOR	6.1	16.6	103.0	34
NPYR	6.6	8.1	111.0	16
NPIP	4.5	11.8	108.9	24
NDBA	4.1	14.1	112.0	29

With respect to selectivity, the above criteria were also met; although in the case of the NDEA, sometimes an interference in the artificial saliva solution could be present, and the qualifier ion ratios were not always as expected. In these cases, and whenever the ion ratio criteria in the testing of the samples were not met, these were re-analysed by GC-MS/MS.

Figures 1 and 2 show the chromatograms obtained by GC-MS (SIM) from a standard of N-nitrosamines and a sample of a rubber teat fortified at the level of 3 µg.kg^{-1}.

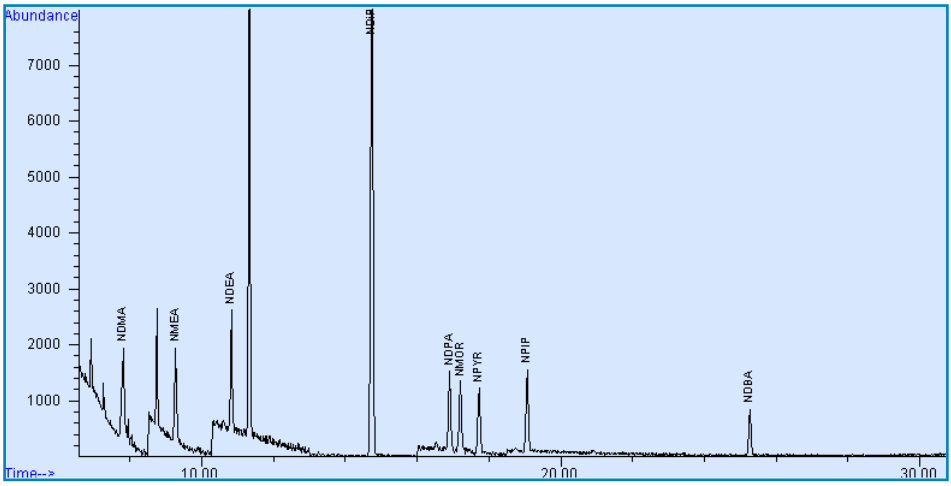


Figure 1. N-nitrosamines standard equivalent to 2 µg.kg^{-1} in sample

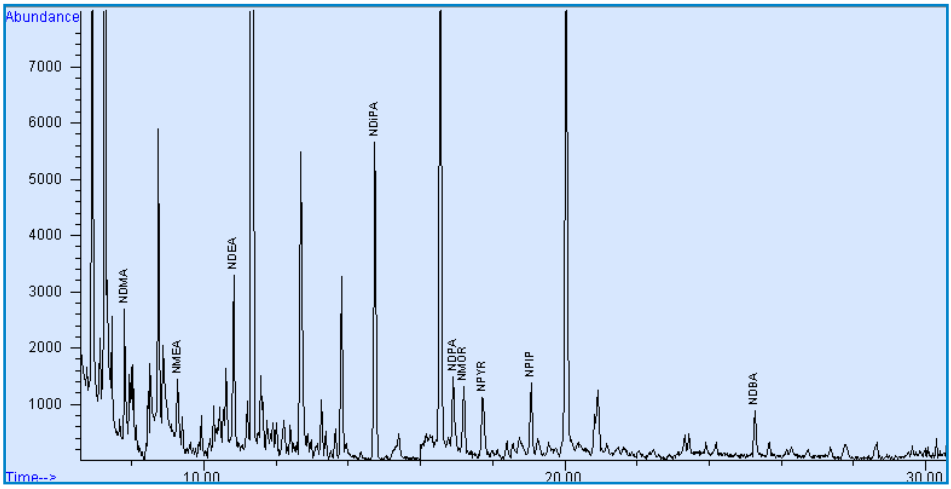


Figure 2. Migration solution of a rubber teat sample, fortified with N-nitrosamines (level 3 µg.kg^{-1})

After verification of the method, this was applied to the determination of N-nitrosamines and N-nitrosatable substances in the samples described above. All of these were analysed in duplicate and parallel recovery tests were conducted on the artificial saliva, as quality control.

The levels found, in the 24 samples analysed, were always below the quantification limit: 2 µg.kg^{-1} for N-nitrosamines and 8 µg.kg^{-1} for N-nitrosatable substances. The controls for recovery were in the mean recovery range of the validation ± 2*CV_R %, and the repeatability, as not quantifiable data was available in the samples, was estimated from the recovery results, and in all cases was ≤2.8*CVr % of validation.

It should be noted that in several samples interferences were found for the target ions of some N-nitrosamines: for example, NDBA in rubber samples or NDMA, NMEA, NDEA and NPYR in rubber and silicone samples. Therefore, a confirmation analysis by CG/MS-MS was necessary. This analysis confirmed that these signals did not correspond to the transitions of these compounds, and their identity was rejected. Furthermore, the presence of certain N-nitrosamines at levels below the quantification limit were detected in some samples, the identity of which was confirmed by GC-MS/MS: NMOR (9 samples), NPYR (1 sample), NPYP (1 sample).

The results of release of N-nitrosamines and N-nitrosatable substances included in this study, for all the samples of rubber and silicone teats and soothers analysed, were below the limits established in Royal Decree 1184/1994 and in agreement with the results published by other authors mentioned in the introduction of this paper.

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