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Research article

# ON THE ISSUE OF DETERMINING TOLERABLE DAILY INTAKE OF TOTAL N-NITROSAMINES FOR TODDLERS

**D.V. Suvorov, P.Z. Shur, S.E. Zelenkin**

Federal Scientific Center for Medical and Preventive Health Risk Management Technologies,
82 Monastyrskaya St., Perm, 614045, Russian Federation

*In Russia, assessment of food products that contain N-nitrosamines (NAs) conventionally focuses on the total N-nitrosodimethylamine and N-nitrosodiethylamine contents. Other NAs are also potential carcinogens and their non-carcinogenic effects require more profound investigation, especially upon low-dose exposures. A child body is more sensitive to NA exposures due to its morphofucntional peculiarities. This involves likely earlier manifestations of non-carcinogenic effects (for example, hepatotoxicity) due to, among other things, their unidirectional effects. More precise health risk assessment requires considering all NAs identified in food products.*

*The present study continues and enlarges previous research works with their focus on experimental determination of N-nitrosamines toxicity in food for babies and toddlers. We assessed hepatotoxicity associated with harmful effects produced by N-nitrosodimethylamine, N-nitrosomethylethylamine, N-nitrosodibutylamine, N-nitrosodipropylamine, N-nitrosopyrrolidine and N-nitrosopiperidine. Lower benchmark dose limit (BMDL) able to cause significantly more considerable hepatotoxic effects (judging from AST, ALT, GGT activity, levels of total bilirubin and alkaline phosphatase) was established through analyzing the total intake of N-nitrosamines identified in meat canned food for toddlers. Establishing BMDL for the total N-nitrosamine contents (0.175 μg/kg of body weight), below which no significant relation existed between likelihood of hepatotoxic effects and NAs dose, made it possible to calculate tolerable daily intake equal to 4.38 ng/kg of body weight considering a potential unidirectional hepatotoxic effects produced by six N-nitrosamines.*

*This established TDI value is considerably lower than a previously established one (15.8 ng/kg of body weight per day) for the sum of four NAs (N-nitrosodimethylamine, N-nitrosomethylethylamine, N-nitrosodibutylamine, and N-nitrosodipropylamine), which is probably due to the estimated unidirectional NAs effect.*

*Combined exposure to a wider range of N-nitrosamines enhances harmful effects, which requires considering the whole range of these substances in health risk assessment.*

*The study findings confirm that it is advisable to develop safe standards for the sum of NDMA and DENA; however, it is also necessary to consider developing safe standards for the analyzed range of six NAs in food products.*

***Keywords:*** *N-nitrosamines, benchmark level, food products, meat canned food, baby food, tolerable daily intake, health risk assessment, safety.*

Infants are highly susceptible to negative health effects produced by N-nitrosamines. This makes it necessary to investigate their contents in food for infants and toddlers, especially in meat-based ones[1] [1]. Presence of these chemicals in food products consumed by this age group creates a substantial health risk. This emphasizes the importance of developing and implementing effective strategies for controlling and decreasing levels of N-nitrosamines (NAs) in meat-based food for infants. Thus, in the Russian Federation, in conformity with the Technical Regulation of the Customs Union, NAs levels are regulated in specialized



**Dmitrii V. Suvorov** – Researcher at the Health Risk Analysis Department (e-mail: suvorov@fcrisk.ru; tel.: +7 (342) 238-33-37; ORCID: https://orcid.org/0000-0002-3594-2650).

**Pavel Z. Shur –** Doctor of Medical Sciences, Chief Researcher-Academic Secretary (e-mail: shur@fcrisk.ru; tel.: +7 (342) 238-33-37; ORCID: https://orcid.org/0000-0001-5171-3105).

**Sergey E. Zelenkin –** Researcher at the Health Risk Analysis Department (e-mail: zelenkin@fcrisk.ru; tel.: +7 (342) 238-33-37; ORCID: https://orcid.org/0000-0002-0259-5509).

[1] Detskoe pitanie: rukovodstvo dlya vrachei [Baby food: guide for physicians], 4th ed., revised and expanded. In: V.A. Tutelyan, I.Ya. Kon eds. Moscow, Meditsinskoe Informatsionnoe Agenstvo LLC Publ., 2017, 784 p. (in Russian).

meat-based and meat- and vegetable-based food products for infants and toddlers where they should not exceed 'more than 0.01 mg/kg of a food product'[2]. However, this level of N-nitrosamines is determined by sensitivity of an employed analytic procedure. The valid Russian safe standards are limited to identifying combined levels of N-nitrosodimethylamine (NDMA) and N-Diethylnitrosamine (DENA). Nevertheless, new techniques applied in the country to quantify N-nitrosamines in food allow identifying up to seven other potentially hazardous chemicals of this class in addition to regulated NDMA and DENA[3], including their identification in food for infants and toddlers [2]. Given the existing safe regulations, this may lead to underestimation of health risks associated with their combined effects on the body[4] [3–5].

Apart from NDMA and DENA, several N-nitrosamines are reported in some studies [6–15] as most frequently detected in food products including N- methylethylnitrosamine (NMEA), N-nitrosodipropylamine (NDPA), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosodiphenylamine (NDPHA), N-nitrosomorpholine (NMOR) and N-nitrosopyrrolidine (NPYR). However, these NAs do not always occur together in all food products. Thus, some studies report only NDMA and DENA identification, whereas simultaneously up to 10 various NAs are reported in other publications [2, 10–15]. Still, only NDMA and DENA should be identified within control and surveillance activities performed in the EAEU countries. At the same time, the European Food Safety Agency (EFSA) points out that risk assessment limited to only NDMA and DENA in food products is insufficient since other NAs can also cause serious health risks [2, 15–18]. Although most identifiable N-nitrosamines are considered either possible or probable carcinogens, their non-carcinogenic effects, particularly hepatotoxic ones, can manifest earlier than carcinogenic ones. It is especially true for infants and toddlers due to specific features of a child body[4] [1, 3–5] and a relatively short period during which specific meat-based infant food is consumed, from the age of 6 months to 3 years. Potential synergy between N-nitrosamines can result in greater combined toxicity of all NAs in food products [17].

In addition, regulation of N-nitrosamine levels in food products within the risk-based approach directly depends on results obtained by assessing carcinogenic and non-carcinogenic health risks. At the same time, there are no available parameters for assessing non-carcinogenic health risks caused by most

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[2] TR TS 021/2011. O bezopasnosti pishchevoi produktsii: Tekhnicheskii reglament Tamozhennogo soyuza (s izmeneniyami na 22 aprelya 2024 goda), utv. Resheniem Komissii Tamozhennogo soyuza ot 9 dekabrya 2011 goda № 880 [CU TR 021/2011. On safety of food products: technical Regulation of the Customs Union (last amended as of April 22, 2024), approved by the Decision of the Customs Union Commission on December 9, 2011 No. 880]. *KODEKS: electronic fund for legal and reference documentation*. Available at: https://docs.cntd.ru/document/902320560 (October 01, 2024) (in Russian).

[3] Agents Classified by the IARC Monographs, Volumes 1–137. *IARC*. Available at: https://monographs.iarc.who.int/agents-classified-by-the-iarc/ (October 01, 2024) (in Russian); MUK 4.1.3588-19. Izmerenie soderzhaniya N-nitrozoaminov (N-dimetilnitrozoamin, N-metiletilnitrozoamin, N-dietilnitrozoamin, N-dipropilnitrozoamin, N-dibutilnitrozoamin, N-piperidinnitrozoamin) v pishchevoi produktsii (konservy iz myasa, myasorastitel'nye) metodom khromato-mass-spektrometrii: Metodicheskie ukazaniya po metodam kontrolya [Quantification of N-nitrosamines (N-nitrosodimethylamine, N-methylethylnitrosamine, N-diethylnitrosamine, N-nitrosodipropylamine, N-nitrosodibutylamine, N-nitrosopiperidine) in food products (canned meat, meat-and vegetable-based products) by using chromato-mass-spectrometry: methodical guidelines on control techniques]. Moscow, Rospotrebnadzor, 2020 (in Russian); MUK 4.1.3646-20. Izmerenie soderzhaniya N-nitrozoaminov (N-dimetilnitrozoamin, N-metiletilnitrozoamin, N-dietilnitrozoamin, N-dibutilnitrozoamin, N-dipropilnitrozoamin, Npiperidinnitrozoamin, N-pirrolidinnitrozoamin, N-morfolinnitrozoamin, N-difenilnitrozoamin) v pishchevoi produktsii (kopchenye myasnye, myaso- i ptitseprodukty) metodom khromato-mass-spektrometrii: Metodicheskie ukazaniya po metodam kontrolya [Quantification of N-nitrosamines (N-nitrosodimethylamine, N-methylethylnitrosamine, N-diethylnitrosamine, N-nitrosodibutylamine, N-nitrosodipropylamine, N-nitrosopiperidine, N-nitrosopyrrolidine, N-nitrosomorpholine, N-nitrosodiphenylamine) in food products (smoked meat, meat- and poultry-based products) by using chromato-mass-spectrometry: methodical guidelines on control techniques]. Moscow, Rospotrebnadzor, 2021 (in Russian).

[4] Sheweita S.A., Mostafa M.H. N-Nitrosamines and their effects on the level of glutathione, glutathione reductase and glutathione S-transferase activities in the liver of male mice. *Cancer Lett.*, 1996, vol. 99, no. 1, pp. 29–34. DOI: 10.1016/0304-3835(95)04034-X

N-nitrosamines. In its turn, many studies have shown that a lot of them can have negative (both carcinogenic and unidirectional non-carcinogenic) effects on health [14–18]. It is noteworthy that previous studies, which focused on establishing points of departure such as a benchmark level (BMD, BMDL) for further non-carcinogenic risk assessment, do not cover all NAs occurring in food products[5] [19–21] and do not estimate their combined impacts. It should be noted that previous studies with their focus on establishing $BMD_{10}$ for genotoxic effects produced by specific N-nitrosamines [22, 23] revealed less strict levels in comparison with the combined hepatotoxic effects produced by four NA (NDMA, NMEA, NDPA, NDBA) per BMDL [17, 24].

**The aim of this study** was to establish tolerable daily intake for NAs in canned meat-based food for infants and toddlers.

**Materials and methods.** The present study continues and enlarges previous research works with their focus on experimental substantiation of N-nitrosamines toxicity in food for infants and toddlers per health risk criteria[6] [18, 24]. A reference level (BMDL) was established for NAs identified in canned meat-based food for infants and toddlers within the research work (2018)[6] using the methodology for NAs quantification [2] in analyzed food products. Quantification of nitrosamine levels in canned meat-based food for infants and toddlers covered NDMA, MENA, DENA, DBNA, NPNA, NPIP, NPYR, NMOR, and NDPHA. An exposure scenario involving combined intake of several N-nitrosamines was used in the present study,

In the experiment[6], Wistar rats were used as an experiment model. Their age (between 4 and 8 weeks) was equivalent to the analyzed human age (between 6 months and 3 years) during the whole imitation experiment [25]. The animals were divided into three groups, one control and two test (experimental) ones. The test groups were given canned meat with various N-nitrosamine levels, minimal (Test Group 1) and maximum (Test Group 2) ones [24]. Thus, Test Group 1 ($n$ = 30) was given canned meat with the total N-nitrosamine level equal to 8.6 μg/kg, which consisted of 1.3 μg/kg N-nitrosodimethylamine (NDMA), 1.3 μg/kg N-methylethylnitrosamine (NMEA), 2.0 μg/kg N- nitrosodibutylamine (NDBA), 3.1 μg/kg nitrosodipropylamine (NDPA), 0.3 μg/kg N- nitrosopyrrolidine (NPYR) and 0.6 μg/kg N- nitrosopiperazine (NPIP). Test group 2 ($n$ = 30) was given canned meat with the total N-nitrosamine level equal to 16.1 μg/kg accordingly (2.0 μg/kg NDMA, 2.6 μg/kg NMEA, 1.0 μg/kg NDBA, 5.2 μg/kg NDPA, 2.2 μg/kg NPYR and 3.1 μg/kg NPIP). The control group ($n$ = 30) was given only pelleted feed. The remaining chemicals (DENA, NMOR, NDPHA) were not detected in the analyzed canned meat.

In addition, according to the experiment design[6], all experimental animals were given pelleted feed, which had been preliminary analyzed to identify N-nitrosamine levels in it. The established concentrations (μg/kg) were as follows: NDMA, 0.83; NMEA, 0.83; NDPA, 4.6; NDBA, 3.8; NPIP, 0.24; NPYR, 7.0. These data were applied to estimate exposure levels in the experiment. Such serum liver markers as AST, ALT, GGT activity, levels of total bilirubin and alkaline phosphatase were selected as biomarkers of hepatotoxic effects since they reflect disruption in liver function [26].

[5] Toxicological Profile for N-Nitrosodimethylamine (NDMA). USA, Atlanta, Agency for Toxic Substances and Disease Registry, 2023, 231 p.

[6] Gigienicheskaya otsenka vozdeistviya nezayavlennykh proizvoditelem kontaminantov myasnykh konservov dlya detskogo pitaniya dlya detei rannego vozrasta, sozdayushchikh potentsial'nyi risk dlya zdorov'ya, v tom chisle, na baze rezul'tatov eksperimenta na laboratornykh zhivotnykh: otchet o nauchno-issledovatel'skoi rabote (zaklyuchitel'nyi, etap 2018 goda) [Hygienic assessment of impacts exerted by contaminants in food meat-based products for infants and toddlers, which are undeclared by manufacturer and create potential health risks, based, among other things, on the results obtained by experiments involving laboratory animals: report on accomplished research work (the concluding stage, 2018)]; NIOTRK Registration Number AAAA-A18-118052890007-1, Perm, 2018, 58 p. (in Russian).

Individual N-nitrosamine doses in canned meat-based food for infants and toddlers were estimated in conformity with the Guide R 2.1.10.3968-23[7].

We used the Kolmogorov – Smirnov test and Lilliefors test to estimate whether the values of the analyzed biochemical blood indicators conformed to normal distribution.

The non-parametric Mann – Whitney U-test was employed to compare the groups and identify significant differences (the significance level was set at $p < 0.05$, median (*Me*) and interquartile range ($Q_{25}$–$Q_{75}$)). Relationships between serum liver markers and a dose (a sum of N-nitrosamines) were estimated by regression analysis, which was conducted using Statistica 10.0. Statistical significance of the model and coefficients was estimated using the F-test. Quality of the model was estimated per the determination coefficient ($R^2$). The lower bound of the benchmark N-nitrosamine doses (BMDL) in canned meat-based food for infants and toddlers (data windowing) was established in conformity with the procedure for determining and substantiating safe standards for chemical levels in food products per health risk criteria approved by the Eurasian Economic Commission (EEC)[8]. Tolerable daily intake (TDI) was also calculated per the procedures approved by the EEC.

**Results and discussion.** Individual doses for the sum of all identified N-nitrosamines, which were consumed with canned meat-based food for infants and toddlers, were established depending on the experimental groups within the ranges between $1.39 \cdot 10^{-4}$ and $1.92 \cdot 10^{-4}$ (Test Group 1) and between $1.5 \cdot 10^{-4}$ and $2.03 \cdot 10^{-4}$ (Test Group 2) mg/kg of body weight.

Hepatotoxic effects produced by N-nitrosamines were estimated considering individual variability in liver markers. To exclude any influence of previous intergroup differences on the experimental results, background values of the analyzed serum liver markers (AST, ALT, GGT activity, levels of total bilirubin and alkaline phosphatase) were determined in all three animal groups (two test groups and the control). The test results are provided in Table 1.

Since some indicators were revealed to not conform to normal distribution (per the Kolmogorov – Smirnov test and Lilliefors test), the group indicators were compared using the non-parametric Mann – Whitney U-test.

Statistical analysis ($p > 0.05$) of background values of the liver markers did not establish any significant intergroup differences per all analyzed indicators. Despite some growth in median ALT and alkaline phosphatase levels in Test groups 1 and 2 against the control, the differences were not significant.

In the experiment, total individual doses of six N-nitrosamines (6 NAs) consumed with canned meat-based food for infants and toddlers varied between 24.8 and 333 ng/kg of body weight depending on the animal group. Biochemical blood indicators were estimated upon completion of the experimental period (28 days), during which the animals were given canned meat with N-nitrosamines in it (Table 2).

Thus, the non-parametric Mann – Whitney U-test established significant differences ($p < 0.05$) in levels of ALT, AST, GGT, alkaline phosphatase and total bilirubin between the control and both test groups (1 and 2) as well as between two test groups. These findings indicate that the analyzed canned meat, which contained various levels of N-nitrosamines, produced hepatotoxic effects. This

[7] R.2.1.10.3968-23. Rukovodstvo po otsenke riska zdorov'yu naseleniya pri vozdeistvii khimicheskikh veshchestv, zagryaznyayushchikh sredu obitaniya [Health Risk Assessment upon Exposure to Chemical Pollutants in the Environment]. Moscow, the Federal Service for Surveillance over Consumer Rights Protection and Human Wellbeing, 2023, 221 p. (in Russian).

[8] Metodicheskie ukazaniya po ustanovleniyu i obosnovaniyu gigienicheskikh normativov soderzhaniya khimicheskikh primesei, biologicheskikh agentov v pishchevoi produktsii po kriteriyam riska dlya zdorov'ya cheloveka [Methodical Guidelines on determining and substantiating safe standards for chemical levels and biological agents in food products per health risk criteria]. *EEC*. Available at: https://eec.eaeunion.org/upload/medialibrary/3ae/MU-po-ustanovleniyu-i-obosnovaniyu-gigienicheskikh-normativov.pdf (October 07, 2024) (in Russian).

Table 1

Biochemical blood indicators of 4-week old rats (equivalent to the human age of 6 months) calculated using the median (*Me* [$Q_{25}$; $Q_{75}$])

| Biochemical blood indicators | Control | Test Group 1 | Test Group 2 |
|---|---|---|---|
| AST (U/l) | 126 [124; 130] | 129 [121.5; 132] | 129 [124; 133] |
| ALT (U/l) | 49 [42; 53.5] | 55 [38; 63.5] | 55 [44.5; 60.5] |
| GGT (U/l) | 3 [0; 5] | 3 [0; 3] | 3.1 [3; 3.5] |
| Alkaline phosphatase (U/l) | 417 [385; 436] | 439[390; 485.5] | 440 [423; 500.5] |
| Total bilirubin (μmol/l) | 0.8 [0.5; 1.1] | 0.8 [0.7; 1.0] | 0.8 [0.7; 1.0] |

Table 2

Biochemical blood indicators of 8-week old rats (equivalent to the human age of 3 years) after 28 days of being fed with meat-based canned food for infants and toddlers with N-nitrosamines in it (*Me* [$Q_{25}$; $Q_{75}$])

| Biochemical blood indicators | Control | Test Group 1 | Test Group 2 |
|---|---|---|---|
| AST (U/l) | 129 [119.5; 131.5] | 209 [194.5; 219] | 231 [215; 264.5] |
| ALT (U/l) | 70 [64; 72.5] | 80 [73; 88] | 111 [97; 130] |
| GGT (U/l) | 3 [2; 3] | 8 [7.5; 8] | 9 [8; 9.5] |
| Alkaline phosphatase (U/l) | 219 [189.5; 271] | 299 [287.5; 391] | 379 [290; 459] |
| Total bilirubin (μmol/l) | 0.8 [0.6; 1.0] | 0.8 [0.6; 1.2] | 1.4 [1.3; 1.5] |

was manifested though an authentic increase in activity of all analyzed enzymes in both Test Groups against the control.

Linear regression analysis established a significant relationship between a dose per the sum of all identified NA and GGT levels ($R^2$ = 0.725, $F$ (1.43) = 113.31, $p < 0.000001$), AST levels ($R^2$ = 0.703, $F$ (1.43) = 101.55, $p < 0.000001$), ALT levels ($R^2$ = 0.448, $F$ (1.43) = 34.97, $p < 0.000001$), and alkaline phosphatase levels ($R^2$ = 0.310, $F$ (1.43) = 19.32, $p$ = 0.000071), as well as a moderate relationship between total bilirubin levels and a dose per the sum of all identified NA ($R^2$ = 0.249, $F$ (1.43) = 14.29, $p$ = 0.00048).

The regression analysis results clearly indicate there is a relationship between N-nitrosamine doses and disrupted liver function judging per such liver markers as GGT, AST, ALT, alkaline phosphatase and total bilirubin. The determination coefficients ($R^2$) for the analyzed markers show that a considerable part of changes in their levels is explained by exposure to different doses. The observed changes in levels of the analyzed liver markers are associated with exposure to N-nitrosamines after feeding the experimental rats with canned meat-based food for infants and toddlers with N-nitrosamines in it for 28 days. Therefore, our findings make it possible to assume that these liver markers can be used as biomarkers of NA effect.

Mathematical modeling of a relationship between growing likelihood of hepatotoxic effects produced by the total N-nitrosamine dose was performed using the data windowing to establish BMDL for N-nitrosamines in canned meat-based food for infants and toddlers. This approach allowed considering potential non-linearity of the relationship and local variations in levels of biomarkers of effect.

As a result, the BMDL equal to 0.000175 mg/kg of body weight was established for the sum of NDMA, NMEA, NDPA, NDBA, NPIP and NPYR solely for the GGT level. This value indicates that if the total concentration of all N-nitrosamines is below this level, no significant increase in the GGT level can be observed.

Table 3

Parameters of the model that describes the relationship between more apparent hepatotoxic effect (GGT) and the total dose of all N-nitrosamines

| Likelihood of effect | Indicator | Marker of hepatotoxic effect | $b_0$ | Error | $b_1$ | $F$ | $p$ | $R^2$ | NL (mg/kg of b.w.) |
|---|---|---|---|---|---|---|---|---|---|
| Growing level | Nitrosamine dose (per their sum) | GGT | 1.175219 | 0.000152 | 590.24 | 97.01 | 0.000 | 0.626 | 0.000175 |

Parameters of the statistical model that describes the Dose – Response relationship for the sum of all N-nitrosamines and likelihood of a growing γ-glutamyl transferase level are provided in Table 3.

Previously, a BMDL was established as equal to 0.633 μg/kg of bodyweight [24]. A BMDL established for the same effect in this study is rather different (0.175 μg/kg of bodyweight). This difference in the results is due to a different range of analyzed N-nitrosamines: the previous study estimated combined effects produced by four NAs (NDMA, NMEA, NDBA, and NDPA), whereas exposure to six NAs (NDMA, NMEA, NDPA, NDBA, NPIP, and NPYR) was analyzed in the present study. A lower BMDL established in the present study can be due to both a wider range of identified NAs and potentially synergy between them [17].

The experiment results were extrapolated from the animal model on the human body in order to establish tolerable daily intake (TDI); the extrapolation considered the modifying factor 40 (which reflects deviation from the ideal experimental conditions) as a product of several factors: a minimal difference between experimental exposure and actual consumption (factor 2); using BMD as a point of departure for establishing TDI (factor 2); interspecies extrapolation of the results determined by the experiment accomplished by using only one animal species (factor 10)[9]. The TDI for the sum of six NAs was established as equal to 4.38 ng/kg of body weight following the use of all modifying factors.

Use of the same modifying factors for the sum of four N-nitrosamines made it possible to establish TDI equal to 15.8 ng/kg of body weight. For reference, the European Medical Agency (EMA) fixed TDI equal to 18 ng/kg of body weight per DEN for all identified NAs [27].

The present study showed that combined exposure to a wider range of N-nitrosamines with unidirectional health effects led to greater negative impacts against exposure to a smaller range of NAs described in the previous study. This determines a lower TDI value for the combined NAs exposure.

Therefore, adequate risk assessment and establishment of truly safe levels require estimating the total intake of all identifiable NAs in canned meat-based food for infants and toddlers. Our findings emphasize the necessity to conduct further investigation in order to get a more precise insight into interactions between various NA and their combined toxic effects.

**Conclusion.** Six NA (NDMA, NMEA, NDPA, NDBA, NPIP, and NPYR) were identified by chemical analysis in canned meat-based food for infants and toddlers. The reference level (BMDL) per genotoxicity effect was established based on NA quantification

[9] Metodicheskie ukazaniya po ustanovleniyu i obosnovaniyu gigienicheskikh normativov soderzhaniya khimicheskikh primesei, biologicheskikh agentov v pishchevoi produktsii po kriteriyam riska dlya zdorov'ya cheloveka [Methodical Guidelines on determining and substantiating safe standards for chemical levels and biological agents in food products per health risk criteria]. *EEC*. Available at: https://eec.eaeunion.org/upload/medialibrary/3ae/MU-po-ustanovleniyu-i-obosnovaniyu-gigienicheskikh-normativov.pdf (October 07, 2024) (in Russian).

and levels of liver markers in blood serum as equal to 0.178 μg/kg of body weight a day per their sum. This is considerably lower than the previously published value (0.633 μg/kg of body weight a day), which was established by the similar study where only four NAs were covered (NDMA, NMEA, NDBA, and NDPA) Accordingly, after the same modifying factors were applied for six NA, the tolerable daily intake (TDI) was established as equal to 4.38 ng/kg of body weight a day, which is below the TDI established per the sum of four NA (15.8 ng/kg of body weight a day). The study findings show that negative health impacts tend to grow under combined exposure to a wider range of N-nitrosamines due to their unidirectional effects, which considers exposure to a greater number of NAs in comparison with the previous study. This emphasizes the necessity to consider the whole range of identified NAs in risk assessment.

In addition, the study findings confirm that it is advisable to develop safe standards for the sum of NDMA and DENA; however, it is also necessary to consider developing safe standards for the analyzed range of six NAs in food products

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