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Quantitative Estimation of Volatile N-Nitrosamines in Tobacco Smoke Using Validated GC-MS Method and its Uncertainty Evaluation, Illustrated by Determination of N-Nitrosomethylethylamine

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ABSTRACT: The present work describes an efficient, sensitive and rapid GC-MS method for quantitative estimation of nine volatile N-nitrosamines diluted in methanol as a sample solution, which can be used to determine the above-mentioned compounds in tobacco smoke or in sample solutions obtained from solid/liquid material using extraction. The concentration of sample solution should not be less than 0.5 μg mL^{-1} (Limit of quantitation of this method) for each N-nitrosamine. The uncertainty of this method is estimated based on validation data, which is illustrated by determination of N-nitrosomethylethylamine in tobacco smoke of the commercial best-selling local cigarette brand. The uncertainty value was used as the acceptance criteria for evaluation of the method precision. The determined quantity of N-nitrosomethylethylamine varying from 108 to 124ng per cigarette is very high, which can be caused by high nitrate and tar content in local tobacco. © *2015 Bull. Georg. Natl. Acad. Sci.*

Key words: *volatile N-nitrosamine, GC-MS, uncertainty evaluation.*

Development of modern industry causes increasingly serious pollution in the environment, where human lives in, constituting a catastrophic health risk including cancer. Anti-cancer is thus one of the challenges for the scientists in the 21st century in the realm of life science. Removal of carcinogen from the environment is an important step. Nitrosamines are probably the most widespread carcinogens existing in the workplace, processed meats, cigarette smoke, cosmetics, pesticides, rubber products, beer and are even produced in the stomach by reaction of secondary amines and nitrite (NO_2^-) both taken from foods. The first analytical studies on N-nitrosamines in tobacco smoke originated from the laboratory of Georg Neurath. N-nitrosamines in tobacco smoke originate from tobacco transfer into smoke from thermal degradation of nitrosamino acids and from pyrosynthesis during smoking. There are more than one hundred publications describing the presence of volatile, non-volatile and tobacco-specific N-

nitrosamines and N-nitrosamino acids in tobacco, tobacco smoke and environmental tobacco smoke [1-4].

The "classical" nitrosamine analysis was performed for many years by gas chromatography using a thermal energy analyzer (TEA) as a detector. Today, with increased sensitivity requirements, the detection limits of the TEA and its complex operation no longer comply with the required needs for low detection limits and sample throughput. Also, several analytical methods were employed in the past for quantitative determination including colorimetry, spectrophotometry, polarography, capillary electrochromatography, micellar electro-kinetic capillary chromatography and high performance liquid chromatography [5-9]. Chromato-Mass spectrometric methods increasingly replaced the above-mentioned TEA [10-14]. Consequently, there is a need to develop and validate reliable chromato-mass spectrometric methods for determination of N-nitrosamines in environment and food products.

For consistent interpretation of the measurement results, it is necessary to evaluate the confidence that can be placed in the presentation of an analytical result, which must be accompanied by indication of the data quality. This information is essential for interpretation of the analytical result. Method validation is an essential component of the measures a laboratory should implement in order to produce reliable analytical data. Besides common method performance characteristics obtained in the validation process, testing laboratories shall have and apply procedures for estimating the uncertainty of measurements (International Organization for Standardization 2005). This clearly means that the analytical result cannot be viewed only as a separate value. There are several possibilities to estimate the uncertainty, as reported in the literature. The measurement uncertainty is estimated mainly by the top-down or bottom-up approaches. In the top-down approach, the major sources of uncertainty are identified and evaluated, while in the bottom-up approach, all the uncertainty sources are systematically evaluated and only those with significant contributions are used to derive the measurement uncertainty. The top-down approach is time-consuming and requires extensive knowledge of the analytical procedure, but it enables identification of major uncertainty sources and consequently reduction of total measurement uncertainty. Another relatively quick and easy way of uncertainty estimation is the in-house validation that includes the determination of the method performance parameters.

An efficient, sensitive and rapid method for routine detection and quantitation of volatile N-nitrosamines (nine volatile N-nitrosamines - N-nitrosodimethylamine - NDMA, N-nitrosomethylethylamine - NMEA, N-nitrosodiethylamine - NDEA, N-nitrosodipropylamine - DPNA, N-nitrosodibutylamine - NDBA, N-nitrosopiperidine - NPIP, N-nitrosopyrrolidine - NPYR, N-nitrosomorpholine - NMPA, N-nitrosodiphenylamine – NDPA) diluted in methanol as a sample solution was developed and validated which can be used to determine above-mentioned compounds in tobacco smoke or in sample solutions obtained from solid/liquid material using extraction. The concentration of sample solution should not be less than 0.5 μg mL^{-1} (limit of quantitation of this method) for each N-nitrosamine. This method was used to determine volatile N-nitrosamines in tobacco smoke. For this study the tobacco of the commercial best-selling local cigarette brand "Pirveli" was selected.

The purpose of the present work was to estimate detailed measurement uncertainty for this method illustrated by determination of one of the volatile N-nitrosamines namely N-nitrosomethylethylamine. The obtained uncertainty value was used as the acceptance criteria for evaluation of the method precision, more concretely, the percentage difference between two inter-day determinations of N-nitrosamines, which should not be more than expanded uncertainty value. The method was validated according to the international - ICH and Eurachem guidelines [15,16].

Experimental Procedures

The chromatography analysis was performed using Agilent 6890 - Inert MSD 5975 Quadrupole GC-MS System (Agilent Technologies, USA). System control, data collection and data processing were accomplished using HP Chemstation software. The chromatographic condition was optimized using the Carbowax/20M (30 m x 0.25 mm x 0.25μm) column; Gas carrier – He; injection mode – splitless; Injection temperature – 220^{0}C; volume - 1μL; oven program - 45^{0}C for 3 min (isocratic), then 20^{0}C/min to 220^{0}C (gradient) and 220^{0}C for 8.25 min for standard solution (total run time - 20 min) and 18.25 min (total run time – 30 min) for sample solution (isocratic); average velocity – 36 cm sec^{-1}; flow rate – 1.0 mL min^{-1}, constant flow; ionization mode – El; mass resolution setting – normal; source temperature - 230^{0}C. The statistical analysis and the evaluation of uncertainty of analytical procedure were performed using Microsoft Excel 2010 according to NATA, ISO, EUROLAB. Analysis was carried out in recommended environmental conditions: temperature t = 20 ± 2^{0}C and relative humidity RH = 40 - 60%, which is an important factor influenced uncertainty estimation.

In order to obtain an estimate of the uncertainty associated with the measurement result the following tasks were to be performed: to specify the measurand; to identify the sources of uncertainty; to calculate the uncertainty components associated with each potential source of uncertainty identified; to calculate the standard uncertainty, applying the appropriate coverage factor, to give an expanded uncertainty. The following sources of uncertainty were identified: analytical balance, repeatability, equipment, measuring glassware, measuring pipette. Expanded uncertainties of solution preparation - U_{SP} (A type of uncertainty) and analytical procedure (repeatability measurement) - U_{AP} (B type of uncertainty) were estimated separately.

The expanded uncertainty for determination of N-nitrosamine was calculated by the formula:

$$U = \sqrt{U_{AP}^2 + U_{SP}^2}, \quad (1)$$

The expanded uncertainty of each uncertainty component was calculated by the formula: $U = k \times u$, where k is the probability distribution factor (coverage factor), which equals 1.73 for equal distribution and 2 for normal distribution; u – the combined standard uncertainty of each component.

The combined standard uncertainty was calculated by the formula:

$$u = \sqrt{\sum_i u_i^2}, \quad (2)$$

where u_i is the standard uncertainty of each source.

The standard uncertainty of analytical procedure was calculated by the formula:

$$u_{(AP)i} = \frac{RSD \times t(f;95\%)}{\sqrt{n}}, \quad (3)$$

where RSD is the relative standard deviation % of the peak area of N-nitrosamine obtained from the standard/sample solution, t – student t-distribution value (probability one sided - $P_{1,\%} = 95\%$) for the calculated degree of freedom (f) which was calculated by the formula: $f = m \times (n - 1)$; where, m – the number of injected solutions and n – injection number of each solution.

The standard uncertainty of solution preparation was calculated by the formula:

$$u_{(SP)i} = \frac{a}{x \times \sqrt{3}} \cdot 100\%, \quad (4)$$

where a is the resolution/deviation of measuring advice/glassware/pipette; x – the measured value.

Standard solution preparation: 0.25 mL of 2000 μg mL^{-1} N-nitrosamines mix standard (Supelco USA) was accurately measured and transferred to a 10 mL volumetric flask and was diluted up to the mark with the diluent (Methanol). Then it was mixed well and filtered through 0.45 μm syringe filter (50 μg mL^{-1}).

Sample solution preparation: Sample solutions were prepared using a specially constructed laboratory instrument composed of the following parts: 1. Specially made quartz tube for burning tobacco; 2. Specially made glassware with bubbler on glacial bath for N-nitrosamine absorption (methanol was used as

a solvent); 3. Vacuum pump. The smoke from tobacco burning in quartz tube was conducted through the solvent absorbing all N-nitrosamine compounds without any loss. The obtained sample solution was filtered through 0.45 μm syringe filter.

The standard and sample solutions were prepared in dark glassware, protected from light and were analysed immediately. The standard solutions were stored in refrigerator during analysis.

The concentration (C_u), μg mL^{-1} of N-nitrosamine in sample solution was calculated by the formula:

$$C_u = \frac{A_u \times C_s \times V \times P}{A_s \times 10 \times 100}, \quad (5)$$

where A_u is the peak area of N-nitrosamine obtained from the chromatogram of sample solution; A_s – peak area of N-nitrosamine obtained from the chromatogram of standard solution; C_s – concentration of N-nitrosamine in standard, μg mL^{-1}; V – volume of standard, mL; P – purity of standard, %.

The quantity (X), μg/cigarette of each N-nitrosamine in tobacco smoke was calculated by the formula:

$$X = \frac{C_u \times W_c \times V}{W_T}, \quad (6)$$

where C_u is the determined concentration, μg mL^{-1} of N-nitrosamine in sample solution; W_c – average mass of weighed cigarette (calculated on 20 units); V – volume of solvent (methanol); W_T – mass of weighed tobacco.

Chromatographic system suitability was checked by five replicate injections (n = 5) of standard solution. Main parameters including RSD, % of peak areas (acceptance criteria: < 2.0%), RSD, % of retention times (acceptance criteria: < 1.0%), the resolution between all the nearest peaks (acceptance criteria: > 2.0), the tailing factor (acceptance criteria: < 2.0) and the number of theoretical plates (acceptance criteria: > 2000) were measured. The precision was estimated by measuring repeatability (I day) and time-dependent intermediate precision (II day) on five replicate injections of standard solution and on three individual determinations of N-nitrosamines in sample solution. The precision was checked by RSD, % of determined concentrations (μg mL^{-1}) and RSD, % of retention times for three individual determinations of N-nitrosamines which should not be more than 10.0% and 1.0%, respectively, also by the percentage difference between two inter-day determinations of N-

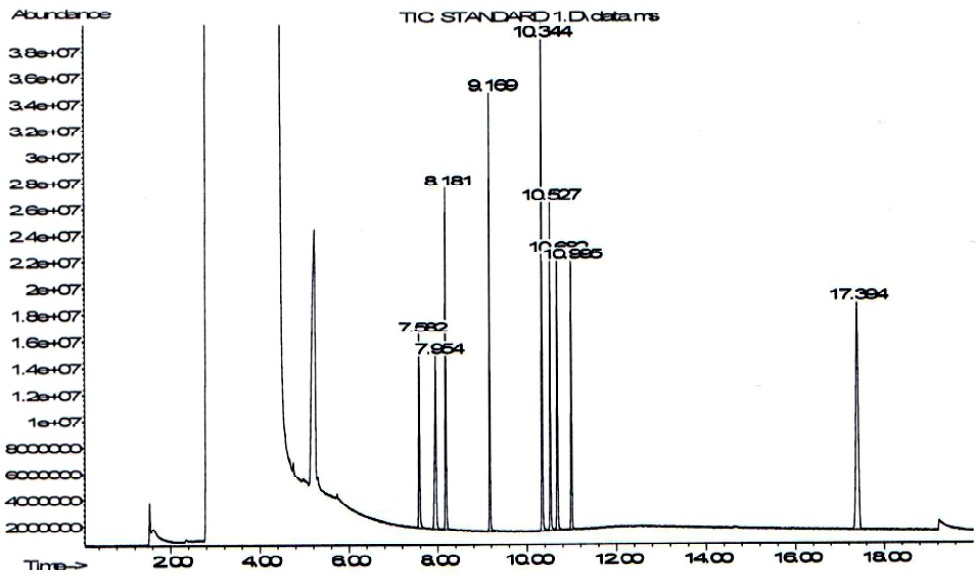

Fig. 1. The chromatogram of 50 μg mL^{-1} standard solution: Retention Time (RT), in minutes:
7.582 - N-nitrosodimethylamine - NDMA, 7.954 - N-nitrosomethylethylamine - NMEA, 8.181 - N-nitrosodiethylamine - NDEA, 9.169 - N-nitrosodipropylamine - DPNA, 10.344 - N-nitrosodibutylamine - NDBA, 10.527 - N-nitrosopiperidine - NPIP, 10.682 - N-nitrosopyrrolidine - NPYR, 10.995 - N-nitrosomorpholine - NMPA, 17.394 - N-nitrosodiphenylamine – NDPA.

Table 1. Precision results for NMEA

Standard solution				
The number of injections(n)	Peak area repeatability - I day		Intermediate precision - II day	
	Peak area	Retention time	Peak area	Retention time
1	100343457	7.950	98643654	7.964
2	100300025	7.951	99111165	7.961
3	97465435	7.951	98653465	7.962
4	97745364	7.952	99128963	7.963
5	97140244	7.950	97333942	7.963
Average	98598905	7.951	98574238	7.963
RSD %	1.610	0.011	0.743	0.014

Sample solution						
The number of sample solution	Peak area repeatability - I day			Intermediate precision - II day		
	Peak area	Retention time	Concentration, μg mL^{-1}	Peak area	Retention time	Concentration, μg mL^{-1}
1	1285585	7.583	0.651	1385585	7.965	0.701
2	1299213	7.582	0.658	1399213	7.963	0.708
3	1226052	7.581	0.620	1265921	7.960	0.641
Average	1270283	7.582	0.643	1350240	7.963	0.684
RSD %	3.063	0.013	3.063	5.432	0.032	5.432
Percentage difference						6.18

nitrosamines which should not be more than expanded uncertainty value (acceptance criteria). The precision (repeatability) as one of the validation parameters was used to estimate standard uncertainty of analytical procedure (u_{AP}).

Results and discussion

RSD % of peak areas for all N-nitrosamine was below 2.0%; RSD % of retention times – below 1.0%; the resolution between the two nearest peaks was more than 2.0; the tailing factor was less than 2.0; the number of theoretical plates was more than 2000. This indicates that the chromatographic system is suitable for determination of all nine N-nitrosamine compounds. Fig. 1 shows the chromatogram of 50 μg mL^{-1} standard solution.

The precision results (Table 1) show that the calculated RSD % of determined concentrations (three individual determinations) of NMEA in sample solutions, the RSD % of peak areas and the RSD % of retention times obtained from standard solutions chromatograms, the RSD % of retention times obtained from three sample solutions chromatograms comply with the acceptance criteria.

The calculated percentage difference between two inter-day determinations for N-nitrosomethylethylamine (6.18% for the determined concentrations, μg mL^{-1} and 6.90% for the calculated quantities, ng/cigarette) complies with the acceptance criteria, more precisely: it is not more than expanded uncertainty value (7.81%).

The uncertainty results are given in Table 2. The budget shows that the expanded uncertainty value of analytical procedure is a major contributor for the expanded uncertainty value of this method.

The determined quantities of N-nitrosomethylethylamine in studied tobacco smoke are shown in Table 3. The quantity of NMEA varying from 108 to 124 ng per cigarette is very high compared to the quantities in the tobacco smoke of light and blended cigarettes from North America and Western Europe. The high level of NMEA is probably caused by high nitrate and tar content in local tobacco. Hence, our hypothesis is that the cigarette smokers in Georgia have an especially high risk for lung and liver cancer. Georgia has no legislation that regulates cigarette package

labeling in respect to smoke yields. Therefore the carcinogenic potential of cigarette smoke should be determined by the tar and nitrate content. However, as the mechanism of cancer induction for nitrosamines including NMEA is different from that of other carcinogens, we have suggested that the abundant carcinogenic nitrosoamines in the smoke of commercial cigarettes ought to be declared as an additional risk factor for cancer.

Conclusion

The quantity of N-nitrosomethylethylamine (which varies from 108 to 124 ng/cigarette) was determined in tobacco smoke of local cigarette brand using validated GC-MS method with the estimation of uncertainty. The analytical data support our hypothesis that the exceptionally high values of N-nitrosomethylethylamine as one of the potential carcinogen is associated especially with the increased risk for lung

Table 2. The budget of uncertainty

Expanded uncertainty of solution preparation (B type of uncertainty)									
Source	#	Component	Value	Deviation	Unit	Degree of freedom - f	Probability - $P_{1,\%}$	Probability distribution factor- k	Standard uncertainty - u_i, %
Standard solution	1	0.5 mL glass pipette	0.25	0.005	mL	∞	100	1.73	1.1557
	2	10 mL measuring flask	10	0.025	mL	∞	100	1.73	0.1443
Sample solution	3	5 mL pipette	5	0.030	mL	∞	100	1.73	0.3464
	4	Balance - Sartorius LE 323S-OCE	16650	0.100	mg	∞	95	1.73	0.0003

Expanded uncertainty of analytical procedure (A type of uncertainty)										
Source	#	Component – measuring equipment	RSD of peak areas, %	Injection number - n	Number of solution - m	Degree of freedom - f	Student coefficient - t (f; P_1 %)	Probability - $P_{1,\%}$	Probability distribution factor- k	Standard uncertainty - u_i, %
Standard solution	1	Agilent GC-MS System	1.610	5	1	4	2.132	95	2.00	1.5349
Sample solution	2		3.063	3	3	6	1.943	95	2.00	3.4364

Combined standard uncertainty of solution preparation, u_{SP} %	1.21
Expanded uncertainty of solution preparation, U_{SP} %	2.10
Combined standard uncertainty of analytical procedure, u_{AP} %	3.76
Expanded uncertainty of analytical procedure, U_{AP} %	7.53
Expanded uncertainty, U %	7.81

Table 3. Calculated quantities of NMEA, ng per cigarette with expanded uncertainty

The number of sample	The quantity of NMEA, ng/cigarette	
	I day	II day
1	114	123
2	115	124
3	108	113
Average	112	120
Percentage difference	6.90	
Quantity, ng/cigarette with expanded uncertainty value	112 ± 9 (±7.81 %)	120 ± 9 (±7.81 %)

and liver cancer among people in Georgia who smoke cigarettes of local production. This method can be used to apply successfully for routine analysis in environment including tobacco smoke and food safety monitoring laboratories for quantitative determination of nine volatile N-nitrosamines.

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ანალიზური ქიმია

თამბაქოს გამონაბოლქვში აქროლადი N-ნიტროზამინების რაოდენობრივი განსაზღვრა ვალიდირებული ქრომატო-მასსპექტრომეტრული მეთოდის გამოყენებით და მისი განუსაზღვრელობის შეფასება N-ნიტროზომეთილეთილამინის მაგალითზე

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წარმოდგენილ შრომაში აღწერილია მეთანოლიან საკვლევ ნიმუშებში ცხრა აქროლადი N-ნიტროზამინის რაოდენობრივი განსაზღვრის ეფექტური, მგრძნობიარე და სწრაფი ქრომატო-მასსპექტრომეტრული მეთოდი, რომელიც შესაძლებელია გამოყენებულ იქნეს, როგორც თამბაქოს გამონაბოლქვში, ასევე მყარი ან თხევადი მასალიდან ექსტრაქციის გზით მიღებულ საკვლევ ნიმუშებში აღნიშნული ნივთიერებების განსაზღვრისათვის. თითოეული N-ნიტროზამინისთვის

საკვლევი ხსნარი არ უნდა იყოს 0,5 მკგ/მლ-ზე მცირე კონცენტრაციის (რაოდენობრივი განსაზღვრის ზღვარი). შეფასებულია მოცემული მეთოდის განუსაზღვრელობა, რომელიც განხორციელდა ვალიდაციის შედეგად მიღებულ მონაცემებზე დაყრდნობით და ნაჩვენებია ქართული წარმოების სიგარეტის თამბაქოს გამონაბოლქვში N-ნიტროზომეთილეთილამინის განსაზღვრის მაგალითზე. განუსაზღვრელობის სიდიდე გამოყენებულ იქნა მეთოდის სიზუსტის შესაფასებლად. დადგენილი N-ნიტროზომეთილეთილამინის რაოდენობა, რომელიც მერყეობს 108–124 ნგ დიაპაზონში 1 სიგარეტზე გადაანგარიშებით, არის ძალიან მაღალი და საჭარაუდოდ გამოწვეული უნდა იყოს ადგილობრივ თამბაქოში ნიტრატებისა და კუპრის მაღალი შემცველობით.

REFERENCES

1. *Hiramoto K., Ohkawa T., Kikugawa K.* (2001) Free Rad. Res. **35**: 803–813.
2. *Scanlan A. R.* (2000) Nitrosamines and Cancer, the Linus Pauling Institute. Available at http://lpi.oregonstate.edu/f-w00/nitrosamine.html.
3. *Mangino M. M., Scanlan A. R.* (1981) ACS Symposium Series **174**: 229–245.
4. *Neurath G., Pirmann B., Lüttich W., Wichern H.* (1964) Contributions to Tobacco Research **3**: 251-262.
5. *Qian M., Wei X. H., Chao W., Hua B., Cheng X. G., et al.* (2011) Chinese Journal of Analytical Chemistry **39**: 1201-1207.
6. *Walters C. L., Johnson E. M., Ray N.* (1970) Analyst **95**: 585-589.
7. *Matyska M. T., Pesek J. J., Yang L.* (2000) J. Chromatogr. A **887**: 487-503.
8. *Filho P. J. S., Valcarcel M., Rios A., Zanin K. D., Caramoa E. B.* (2003) J. Chromatogr. A **985:** 503-512.
9. *Bellec G. F., Cauvin M. C., Calve K. L., Dreano Y., Gouerou H., Menez J. F., Berthou F.* (1996) J. Chromatogr. A **727**: 83-92.
10. *Chen A., Huebschmann H. J., Fangyan L., Foong C. Y., Harn C. S.,* (2012) High Sensitivity Analysis of Nitrosamines Using GC-MS/MS Alpha Analytical Pte. Ltd., Thermo Fisher Scientific, Health Sciences Authority, HAS Singapore.
11. *Brunnemann D. K., Hoffmann D.* (1991) Crit. Rev. Toxicol. **21**: 235-240.
12. *Sannino A., Bolzoni L.* (2013) Food Chem. **141** (4): 3925-3930.
13. *Seyler T. H., Kim J. H., Hodgson J. A., Cowan E. A., Blount B. C., Wang L.* (2013) J. Anal. Toxicol. **37**(4): 195-202.
14. *Zhao H., Wang X., Wang P., Zhou Y., Xue C., Jiang L.* (2013) Chinese Journal of Chromatography **31**(3): 223-227.
15. ICH Harmonized tripartite guideline: Validation of analytical procedures: text and methodology, Q2 (R1) (2005).
16. *Ermer J., Miller J. H.* (2005) Method Validation in Pharmaceutical Analysis, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 403 p.

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