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Determination of Cotinine in Urine and Wastewaters by High Performance Liquid Chromatography Coupled with Tandem Mass-Spectrometric Detection

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Abstract—A technique of extracting cotinine in urine and wastewaters, followed by its quantitative determination, using high performance liquid chromatography combined with tandem mass-spectrometric detection is presented. The method is characterized by low detection limits and high levels of efficiency and sensitivity. The optimal conditions for the solid-phase extraction of cotinine from urine and wastewaters are found. This technique makes it possible to reliably estimate the content of cotinine in the urine of active and passive smokers and in wastewaters.

Keywords: cotinine, high performance liquid chromatography, mass-spectrometry, wastewaters, urine, solid-phase extraction

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Tobacco smoking is a serious medical and social problem of our time, causing many diseases and premature mortality. Smoking directly or indirectly causes the death of three million people every year around the world. About 40% of men and 15% of women smoke in Russia, which indicates the presence of a smoking epidemic. The adoption of the antismoking law and the ban on smoking in public places in Russia has made it particularly relevant to determine the real-time number of smokers in populated areas [1].

Nicotine is half-metabolized (the half-decay period is about 2 h) in different ways, mainly based on biotransformation into cotinine (70–80%), for which the half-decay period is 15–20 h; consequently, nicotine itself can be found in the body several days after tobacco consumption [2].

Cotinine can be used as a good indicator of the effect of tobacco smoke on the human body, since its level in urine is proportional to the total effect of tobacco smoke. Although the content of cotinine varies depending on the form of tobacco consumption (electronic cigarettes or nicotine replacement therapy), this compound is widely used as a biological marker for determining the tobacco smoking factor and assessing the effect of tobacco smoke on the environment [3].

A parameter, such as the cotinine content in wastewaters, can be used to estimate the total consumption of tobacco and level of its consumption per capita. Studies in this area will make it possible to determine the number of residents who are serviced by the relevant treatment facilities in real time, which, in turn, will provide significant opportunities for epidemiological studies [4].

The level of cotinine in urine is 300 μg/L for an active smoker and 10 to 200 μg/L for a passive or light smoker. The content of cotinine in domestic wastewaters varies from 1 to 4 μg/L [5]. This low content of cotinine in the study objects requires the development of a sensitive technique of determining cotinine.

The objective of this study is to develop a highly-sensitive method of determining cotinine in urine and wastewaters (without preconcentration) using high performance liquid chromatography (HPLC) combined with mass-spectrometric detection.

EXPERIMENTAL PROCEDURE

Devices and Materials. The study used the following reagents: cotinine and deuterated cotinine (d_3) (Sigma-Aldrich, United States, ≥98%), methanol, acetonitrile, formic acid, sodium hydroxide, disodium phosphate dihydrate, dihydrogen phosphate dihydrate, ammonia (25%), ammonium acetate, ammonium dihydrophosphate, and chloroform (Panreac, Spain, analytical reagent grade), dichloromethane, ethylacetate (Komponent-reaktiv, Russia, analytical reagent grade), acetic acid (Khimmed, Russia, chemically pure reagent), deionized water, and urine and wastewater samples.

Cotinine was determined in urine and wastewaters using an Agilent 1290 liquid chromatograph (Agilent Technologies, United States), which was fitted with an automatic injector and a tandem triple-quadrupole Agilent 6460 mass-spectrometer (Agilent Technologies, United States) equipped with an electrospray ionization source (ESI). The data were collected and chromatograms were processed using the MassHunter software (Agilent Technologies, United States); a Synergi Hydro column (250 × 4.6 mm) with a sorbent grain diameter of 4 μm (Phenomenex, United States) was used for the HPLC analysis. Autosamplers (LABMATE, Poland) with the setting range of the sampling volume from 10 to 100, 20 to 200, 100 to 1000, and 1000 to 5000 μL were used for the sampling aliquots (the allowable relative error limit for measurements was not less than ±5%). Precise portions were weighed using an ExplorerPro scale (OhausCorporation, United States) with an accuracy of 0.0001 g. Samples were centrifuged using an SM-50 centrifuge (Elmi, Latvia). Ultrasound liquid extraction and mobile phase degasation were carried out using a UZV-4.0 ultrasound bath (Sapfir, Russia). The solvent was evaporated using a rotary evaporator with an automated vacuum station (Buchi, Switzerland). pH solutions were measured using a PB-11 pH-meter (Sartorius, Germany). Solid-phase extraction was performed using a vacuum unit (manifold), a Buchi V-100 vacuum pump (Switzerland), and Chromabond SA solid-phase extraction cartridges (sorbent based on silicagel with grafted benzene sulfonic acid groups) with a pore size of 60 μm, a particle size of 45 μm, and a specific surface of 500 m^2/g (Macherey-Nagel, Germany).

Urine and Wastewater Sampling Preparation. Working solutions were prepared directly on the day of analysis by diluting the aliquots of the initial solution with deionized water. During urine sample preparation, a sample was centrifuged (15000 rounds per 5 min), followed by the 3-fold dilution of the supernatant with deionized water. The resulting solution was subject to centrifugation again (15000 rounds per 5 min). The required amount of the internal standard was then added, followed by extraction. During the preparation of wastewater samples, a sample was centrifuged (15000 rounds per 5 min) and the internal standard solution was added, followed by extraction.

Solid-Phase Extraction. The precise volume of the internal standard solution was added to and mixed with the precise sample volume. While observing the conditions that are recommended by the manufacturer for each sorbent, we compared the cotinine recovery rate using the following cartridges:

Chromabond SA (sorbent based on silicagel with grafted groups of benzene sulfonic acid) and Chromabond C18 (sorbent based on endcapped silicagel with grafted octadecylsilyl groups).

Procedure of solid-phase extraction (SPE) using Chromabond SA:

(1) 3 mL of methanol and 3 mL of water were sequentially drawn through the cartridge for conditioning and balancing the cartridge sorbent;

(2) a 2 mL sample was drawn through the sorbent at a rate of 5 mL/min;

(3) the cartridge was cleaned with successive 3 mL washes of 5% acetic acid, methanol : water (50 : 50), and methanol : acetonitrile : acetic acid (40 : 40 : 20) to remove neutral and acid admixtures;

(4) elution was performed in methanol : acetonitrile : ammonia (2 mL) at a ratio of 47.5 : 47.5 : 5, respectively.

SPE procedure using Chromabond C18:

(1) 4 mL of methanol and 4 mL of water were sequentially drawn through the cartridge for conditioning and balancing the cartridge sorbent;

(2) a 2 mL sample was drawn through the sorbent at a rate of 5 mL/min;

(3) the cartridge was washed with 3 mL of water and the sorbent was then dried by passing air for 3 min;

(4) elution was performed with 1.5 mL of dichloromethane, followed by 1 mL of dichloromethane : isopropanol : ammonia at a ratio of 78 : 20 : 2;

(5) the resulted extract was evaporated on a rotary evaporator at a temperature of 50°C and the solution residue was dissolved in 2 mL of acetonitrile : water (50 : 50).

Conditions for HPLC–MS/MS Analysis. Sample components were separated in the isocratic regime using a Synergi Hydro column (250 × 4.6 mm) with a sorbent grain diameter of 4 μm (Phenomenex, United States) and a Thermo Scientific guard column with a C18 phase (10 × 4 mm, 3 μm). The temperature of the column thermostat was 35°C. The injection volume was 4 μL. The isocratic elution regime was used. The mobile phase was water : acetonitrile (50 : 50) and the flow rate was 0.7 mL/min. The ESI source was used as a source of ionization in the positive ion monitoring regime. The capillary voltage was 3.5 kV. The flow rate of the peripheral air was 11 L/min. The temperature of the peripheral gas was 300°C, the sprayer temperature was 325°C, and the gas (nitrogen) flow rate was

Table 1. Cotinine recovery from model solutions with different concentrations using liquid extraction method. Organic solvent: chloroform ($n = 3$, $P = 0.95$)

Cotinine concentration, mg/L	Recovery, %
0.5	68 ± 8
1	75 ± 7
5	92 ± 7
10	66 ± 8
50	74 ± 6

Table 2. Cotinine recovery from model solutions with different concentrations by SPE method using Chromabond SA cartridges ($n = 3$, $P = 0.95$)

Cotinine concentration, μg/L	Recovery, %	s_r, %
0.1	93 ± 9	4
1.0	95 ± 15	7
10	98 ± 16	8
100	93 ± 12	6
500	92 ± 11	5

8 L/min. The voltage was 5 V in the collision cell and 110 V on the fragmentor. To detect cotinine in the multiple reaction monitoring (MRM) regime, we used MRM transitions:

$$m/z = 177.10 \rightarrow m/z = 80.05;$$

$$m/z = 177.10 \rightarrow m/z = 98.06.$$

The internal standard was detected using MRM transitions:

$$m/z = 180.10 \rightarrow m/z = 80.05;$$

$$m/z = 180.10 \rightarrow m/z = 101.06.$$

The collision energy was 20 eV for the first MRM transition and 30 eV for the second. The scanning time was 200 ms per MRM transition.

RESULTS AND DISCUSSION

Urine is a complex biological liquid, which, in addition to water (97%), includes different organic and inorganic compounds, such as mineral salts, protein degradation products (urea, amino acids, and creatinin), as well as microelements, inactivated hormones, and enzymes. Wastewaters contain a significant proportion of chemical substances (contaminations) as part of waterborne fine particles, which form the wastewater's disperse phase. The waterborne substances also include many mineral pollutants: sand, clay, slags, and vegetable and animal organic substances (plant, fruit, grass, and vegetable residues, paper, etc.). Therefore, the choice of conditions for extracting cotinine is an important stage of the analysis.

The Choice of Liquid–Liquid Extraction Conditions

This study carried out a comparative analysis of extractants, such as tetrachloromethane, chloroform, dichloromethane, and ethylacetate. Cotinine solution (5 mg/L) was used as study samples. The maximum recovery (87 ± 5%) was obtained using chloroform. The recovery rate was estimated at equal cotinine concentrations. Based on the results given in Table 1, it was concluded that the liquid extraction method is not suitable for determining cotinine in a wide range of concentrations, since the recovery rate depends on its concentration.

The Choice of a Sorbent for Solid-Phase Extraction

It was established that the use of the Chromabond C18 cartridge with octadecylsilyl groups makes it possible to recover only 57% of cotinine, while the recovery rate using the Chromabond SA cartridge is 98%. It should be noted here that the recovery rate does not depend on the concentration of cotinine in the range of concentrations under study. The further research used the Chromabond SA cartridge, where cotinine is retained according to the ion-exchangeable mechanism.

To estimate the reproducibility of cotinine recovery by the SPE method, we prepared a series of model cotinine solutions with a concentration of 0.1; 1; 10; and 100 and 500 μg/L. The results are given in Table 2.

The Choice of the Stationary Phase

This research compared the following chromatographic columns:

Agilent Zorbax Eclipse C18 (150 × 4.6 mm, the size of particles is 5 μm);

Phenomenex Synergi Fusion-RP (250 × 4.6 mm, the size of particles is 4 μm);

Agilent Poroshell HILIC (250 × 4.6 mm, the size of particles is 4 μm); and

Phenomenex Synergi Hydro (250 × 4.6 mm, the size of particles is 4 μm).

Since cotinine is a hydrophilic compound, it is reasonable to use a stationary phase with polar endcapping, which provides a good retention of nonpolar and highly polar compounds. Figure 1 shows that the Phenomenex Synergi Hydro column has the highest peak intensity. The efficiency of the columns was compared by calculating the number of perfect plates per m of column. The maximum number of perfect plates (34200 ± 700) was obtained using the Phenomenex Synergi Hydro column.

To reduce the analysis time, we changed the temperature of the HPLC column thermostat from 25 to 45°C. The selected temperature of the column thermostat was 35°C.

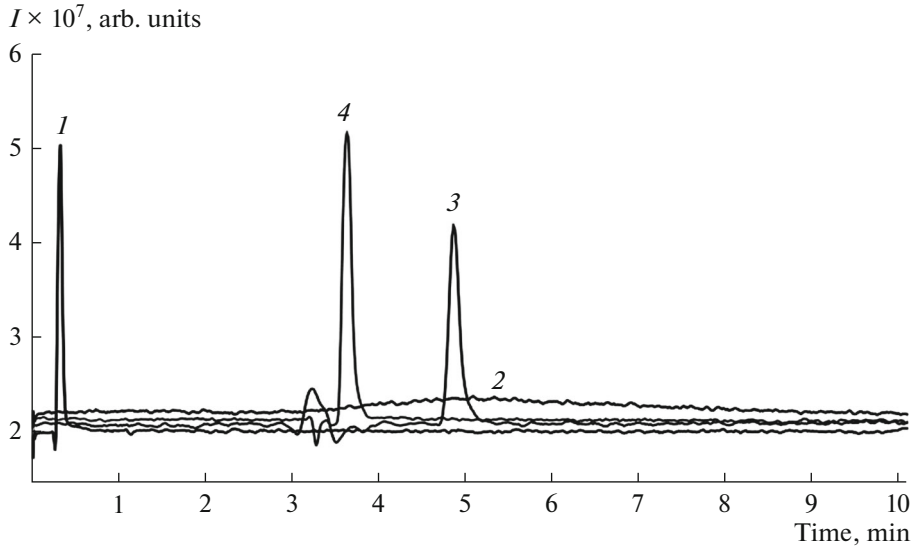

Fig. 1. Overlap of chromatograms obtained using following columns: (*1*) Zorbax Eclipse C18, (*2*) Synergi Fusion-RP, (*3*) Poroshell HILIC, (*4*) Synergi Hydro; injection volume: 4 μL, $c_{cot.}$ = 1 μg/L, mobile phase: 0.1% formic acid : acetonitrile (50 : 50), mobile phase rate: 0.7 mL/min. Total ion current detection regime, ESI.

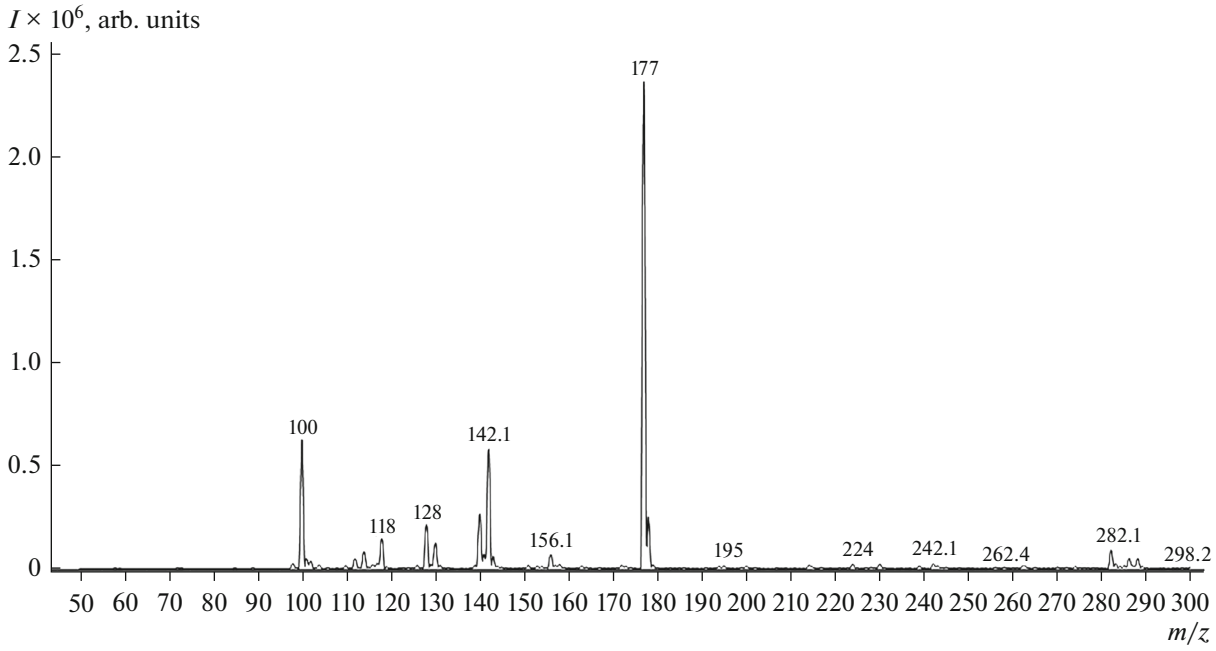

Fig. 2. Cotinine solution mass-spectrum ($c_{cot.}$ = 100 μg/L). Synergi Hydro RP 80A column (250 × 4.6 mm × 4 μm). Injection volume 4 μL. $c_{cot.}$ = 1 μg/L. Mobile phase: 0.1% formic acid : acetonitrile (50 : 50); mobile phase rate: 0.7 mL/min. Total ion current detection in m/z value range from 50 to 300.

The Choice of Conditions for Mass-Spectrometric Detection

The temperature of the peripheral and drying gas, gas flow rate, sprayer pressure, and capillary voltages were selected according to the manufacturer's recommendations.

The Choice of MRM-transitions, Collision Energy, and Voltage on the Fragmentor

The optimal conditions for mass-spectrometric detection in the direct injection regime were selected using cotinine solution (100 μg/L) in the ion scanning regime (from m/z = 50 to m/z = 300).

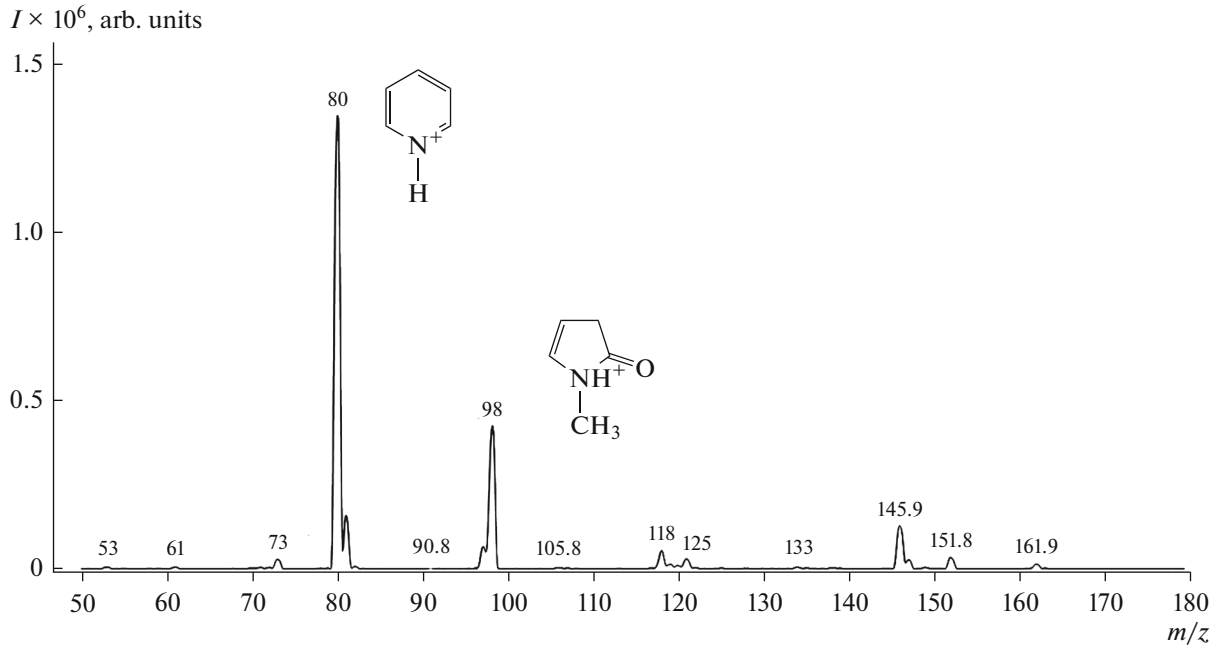

Fig. 3. Mass-spectrum of electronic cotinine ionization (CE = 50 eV). Synergi Hydro RP 80A column (250 × 4.6 mm × 4 μm). Injection volume is 4 μL. C = 1 μg/L. Mobile phase: 0.1% formic acid : acetonitrile (50 : 50); mobile phase rate: 0.7 mL/min.

The parent ion weight determined by the most intensive peak was 177.10 (Fig. 2). The highest sensitivity can be achieved using the MRM regime in tandem mass-spectrometric detection. Figure 3 shows the mass-spectrum of daughter ions at a collision energy of 50 eV.

MRM-transition with maximum intensity is usually selected for quantitative analysis. We also selected additional transition with the maximum m/z value for confirmation. Therefore, the following MRM-transitions were used for the quantitative cotinine analysis:

$$m/z = 177.10 \rightarrow m/z = 80.05;$$

$$m/z = 177.10 \rightarrow m/z = 98.06.$$

The intensity of the signal of a certain MRM-transition is greatly influenced by the value of collision energy (CE). Its choice generally depends on the stability of the precursor ion and the required level of its fragmentation. The value of CEs that provide the maximum intensity of selected MRM-transitions was determined by constructing correlations between the intensity of their signals and the CE value (Fig. 4). Collision energies of 30 and 20 eV were selected for transitions m/z = 177.10 → m/z = 80.05 and m/z = 177.10 → m/z = 98.06, respectively.

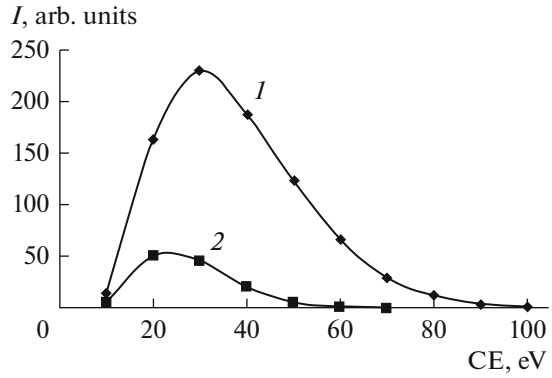

Fig. 4. Dependence of intensity of transition signals: (*1*) m/z = 177.10 → m/z = 80.05; (*2*) m/z = 177.10 → m/z = 98.06, on collision energy values.

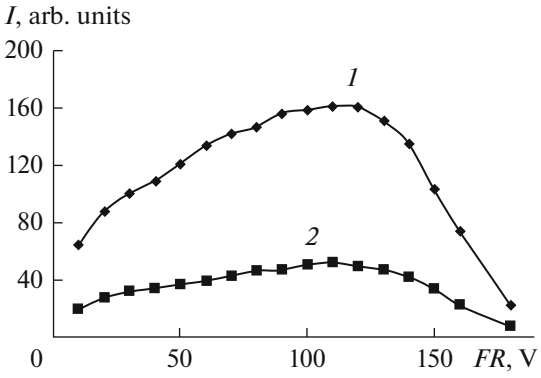

Fig. 5. Dependence of intensity of cotinine signal on fragmentor voltage for selected MRM-transitions: (*1*) m/z = 177.10 → m/z = 80.05; (*2*) m/z = 177.10 → m/z = 98.06.

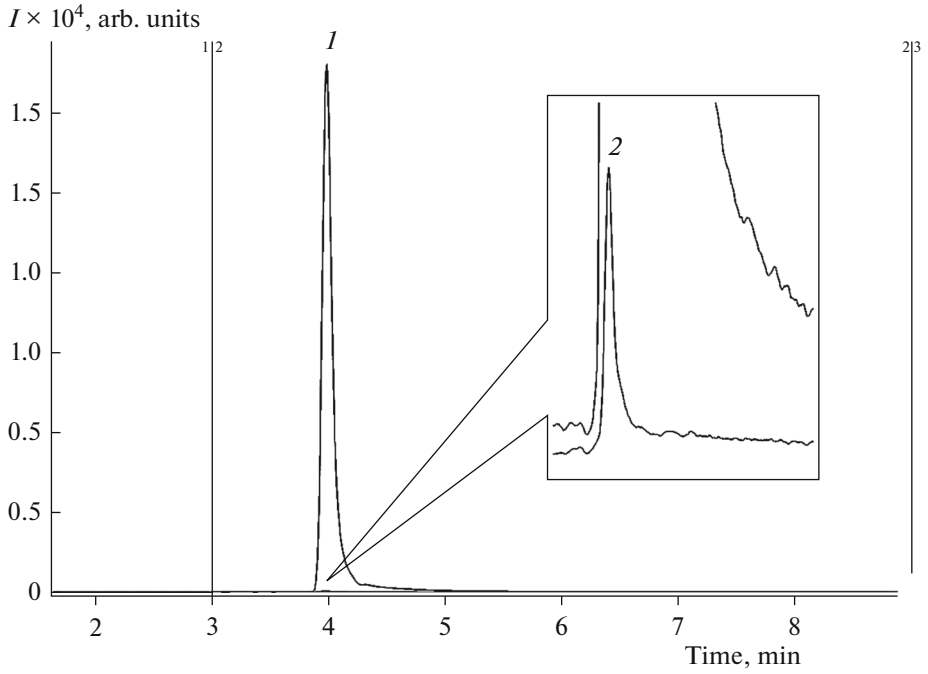

Fig. 6. Overlap of chromatograms of urine of active smoker (*1*) and passive smoker (*2*). Column: Synergi Hydro RP 80A (4.6 × 250 mm; 4 μm). Mobile phase: acetonitrile : water in 50 : 50 ratio. Flow rate is 0.7 mL/min. Column temperature is 35°C. Detection in multiple reaction monitoring regime at positive ionization. Transition m/z 177.10 → 80.05 (30 eV); m/z 177.10 → 98.06 (20 eV). Injection volume is 4 μL.

The fragmentor voltage can significantly influence the sample ionization yield and, accordingly, the sensitivity of determination. Correlations between the intensity of the cotinine signal and fragmentor voltage were constructed (Fig. 5). The optimal potential of fragmentation was selected by comparing the intensity of signals of MRM-transitions from 10 to 180 at a potential gradient of 10 V. The optimal value of fragmentor voltage was 110 V.

Linearity

The metrological characteristics of the proposed approach were assessed by constructing a calibration curve to determine cotinine using the HPLC–MS/MS method under selected conditions for a series of 12 test solutions with the content of cotinine at a concentration range from 0.1 to 1000 μg/L.

The equation of linear regression of the dependence of the peak area on cotinine concentration, as well as the metrological parameters of the equation of linear regression, was calculated by the least square method (Table 3).

The peaks corresponding to cotinine were revealed in a "blank" solution. The blank solution was obtained by the SPE of a water sample with the addition of deuterated cotinine. Under normal conditions, nicotine from the atmosphere can be transformed into environmental cotinine, which can explain its presence in solution solvents and reagents. The content of cotinine in the blank solution is characterized by good reproducibility ($s_r = 11$); the further calculations were made taking into account the presence of cotinine in the background concentrations.

At low concentrations (50 ng/mL or lower), the influence of the background content of cotinine is significant. During the analysis of real objects, it is reasonable to consider two linear sections: from 0.1 to 10 μg/L and from 5 to 1000 μg/L. The detection limit is 0.03 μg/L without preconcentration.

Table 3. Linear ranges of calibration curve during determination of cotinine by HPLC–MS/MS method ($n = 3$, $P = 0.95$)

Linear range number	Linear range	Calibration curve equation
1	0.1–10 μg/L	$S_i/S_{IS} = (65.1 \pm 5.8) \times 10^{-2} \times c_i + (0.18 \pm 0.23)$ ($P = 0.95$; $n = 3$. $r = 0.999$)*
2	5–1000 μg/L	$S_i/S_{IS} = (62.3 \pm 1.0) \times 10^{-2} \times c_i + (1.46 \pm 5.20)$ ($P = 0.95$; $n = 3$. $r = 0.998$)*

* S_{IS}, area of chromatographic peak of internal standard (deuterated cotinine, 0.91 μg/L).

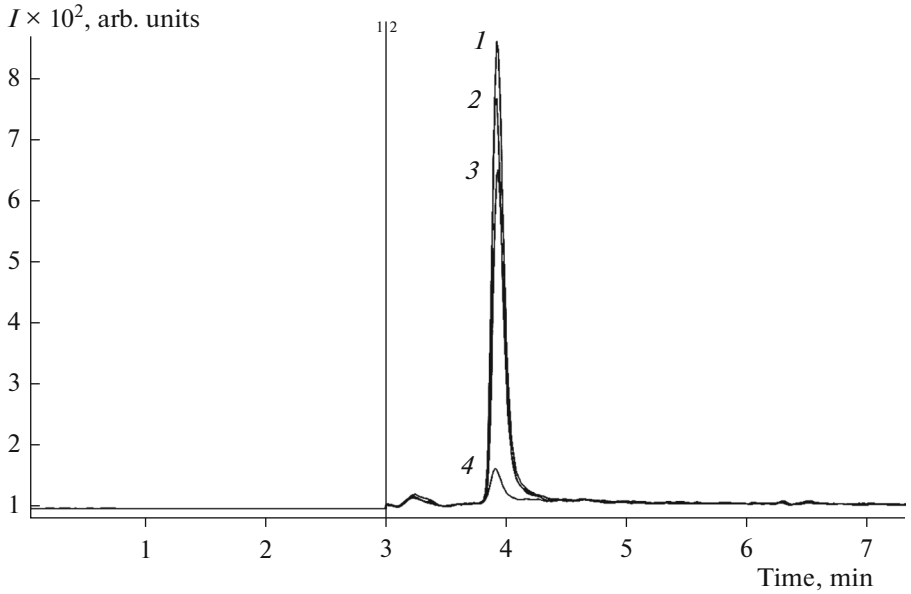

Fig. 7. Overlap of chromatograms of wastewater samples collected in Moscow oblast: (*1*) channel no. 1, (*2*) channel no. 2, (*3*) channel no. 3, (*4*) blank solution. Column: Synergi Hydro RP 80A (4.6 × 250 mm; 4 μm). Mobile phase: acetonitrile : water in 50 : 50 ratio. Flow rate is 0.7 mL/min. Column temperature is 35°C. Detection in multiple reaction monitoring regime at positive ionization. Transition m/z 177.10 → 80.05 (30 eV); m/z 177.10 → 98.06 (20 eV). Injection volume is 4 μL.

Precision

The precision of the technique was assessed based on the repeatability of the analysis that was performed by the same specialist (specialist 1) on six samples on the same date using the same equipment. The intermediate precision was also determined in the analysis of the same sample at the same laboratory with the complete repetition of the procedure of its preparation; however, measurements in this analysis were carried out by another specialist (specialist 2). The repeatability was assessed by calculating the relative standard deviation (RSD), which was obtained from successive determinations performed by specialist 1 on the same date. The intermediate precision was assessed at an established confidence interval according to Fisher's F test and Student's t test. The results are given in Table 4. It is shown that the technique has a good level of repeatability (the RSD was less than 5%) and good intermediate precision, since all the acceptance criteria are met.

Table 4. Intermediate precision of cotinine determination

Sample number	Specialist 1	Specialist 2
	S_{cot}, arb. units (c_{cot} = 500 μg/L)	
1	1460	1580
2	1490	1600
3	1490	1620
4	1520	1520
5	1570	1610
6	1590	1540
Mean	1520	1578
Parameter	Specialist 1	Specialist 2
SD	50.60	40.21
RSD, %	3.33	2.55
Calculated Fisher's F test, F_{calc}	1.58	
Fisher's F test in the table, F_{table} (0.95; 5.5)	5.05	
Calculated Student's t test, t_{calc}	2.21	
Student's t test in the table, t_{table} (0.95; 10)	2.23	

Table 5. Results of chromatographic determination of cotinine in real objects (n = 3, P = 0.95)

Object (sample)	Concentration, μg/L %	s_r, %
Wastewater (no. 1)	1.4 ± 0.1	9
Wastewater (no. 2)	1.0 ± 0.1	11
Wastewater (no. 3)	1.5 ± 0.1	9
Urine (no smoking)	0.8 ± 0.2	14
Urine (active smoking)	996 ± 21	6

Solution Stability

We studied the stability of the model solution at room temperature (25°C). The sample was analyzed immediately after its preparation and 30 and 60 min after preparation and its analysis was then repeated until the difference with the initial measurement was over 5%. It was established that the solution maintained its stability for 48 h.

Robustness

The study of the robustness of the technique established that the variation of chromatography parameters, such as the mobile phase composition (acetonitrile ±5%), column temperature (±5°C), and the mobile phase rate (±0.2 mL/min), does not significantly influence the amount of the detected cotinine in the objects under study. The relative deviation does not exceed 10.0%.

As an example of the analysis of real objects, we analyzed the urine of an active smoker and a subject who was not exposed to tobacco, as well as wastewater samples that were collected at three water treatment stations in Moscow oblast. The results of the research are given in Table 5. The overlap of the chromatograms that were obtained from urine analysis and wastewater analysis is given in Figs. 6 and 7, respectively.

Therefore, we have developed a method of determining cotinine by high performance liquid chromatography combined with tandem mass-spectrometric detection using the internal standard of deuterated cotinine. The metrological characteristics of cotinine determination by the HPLC–MS/MS method with preliminary sample preparation were assessed using the SPE method. This method is characterized by low detection limits (0.1 μg/L) and good reproducibility (s_r = 6–14%). It can be used for determining cotinine in urine and wastewaters. The content of cotinine that was detected in the urine of an active smoker and in wastewaters was 1 mg/L and 1 μg/L, respectively (without preconcentration).

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REFERENCES

1. Rozhanets, V.V., *Vopr. Narkol.*, 2016, nos. 7–8, p. 54.
2. Pianezza, M.L., Sellers, E.M., and Tyndale, R.F., *Nature*, 1998, vol. 393, no. 6687, p. 750.
3. Abdallaha, I.A., Hammell, D.C., Stinchcomb, A.L., and Hassan, H.E., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2016, vol. 1020, p. 67.
4. Medana, C., Aigotti, R., Sala, C., Dal Bello, F., Santoro, V., Gastaldi, D., and Baiocchi, C., *Spectroscopy*, 2016, vol. 14, no. 2, p. 20.
5. Sommerfeld, K., Łukasik-Głębocka, M., Kulza, M., Drużdż, A., Panieński, P., and Zielińska-Psuja, B., *Forensic Sci. Int.*, 2016, vol. 262, p. e15.

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