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Determination of nicotine, anabasine, and cotinine in urine and saliva samples using single-drop microextraction

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ABSTRACT

A simple, sensitive, and inexpensive singe-drop microextraction (SDME) followed by gas chromatography and flame-ionization detection (GC-FID) was developed for determination of nicotine, anabasine, and cotinine in human urine and saliva samples. The target compounds were extracted from alkaline aqueous sample solution into an organic acceptor drop suspended on the tip of a 25-μL GC microsyringe in the aqueous sample solution. This microsyringe was also used for direct injection after extraction. Under optimized experimental conditions, calibration plots were found to be linear in the range of 0.5–25.0, 0.5–65.0, and 0.5–45.0 mg L^{-1} for nicotine, anabasines and cotinine, respectively. The method detection limit values were in the range of 0.33–0.45 mg L^{-1}. Intra-day and inter-day precisions for peak area ratios were in the range of 1.3–9.2% and 2.0–7.0%, respectively. The proposed procedure was successfully applied to the determination of analytes in spiked urine and saliva samples with satisfactory results. The mean relative recoveries of spiked water samples ranged over 71.2–111.0%, with relative standard deviations varying from 2.3% to 10.0%.

1. Introduction

Nicotine and anabasine are the main pharmacologically active alkaloids found in tobacco [1–3]. These alkaloids are absorbed in human through the skin and the lungs [4,5]. Due to the presence of nicotine in cigarette at relatively high concentrations, its addictiveness properties, and also the primary precursors for the highly carcinogenic tobacco-specific nitrosamines, make these chemicals important from a public health standpoint [1]. Nicotine and its major metabolite cotinine can be found in urine, blood and saliva samples [4,6–8].

Some sample preparation methods have been reported for the pre-treatment of the alkaloids in different samples. Liquid-liquid extraction (LLE) [7,9] and solid phase extraction (SPE) [10] are two of the most useful sample preparation methods prior to gas chromatography [11,12], liquid chromatography [4,13,14], or spectroflouorimetric [15] analysis. However, in addition to time-consuming, tedious, and low sensitivity, LLE often requires large amounts of toxic organic solvents and can be relatively expensive. SPE uses considerably less solvent than LLE, but it can be relatively expensive. Single-drop microextraction (SDME) was developed as a sample preparation method due to its simplicity, efficiency, low cost, negligible volume of solvent used and excellent sample clean-up ability. Basic principle of SDME method has been demonstrated in several studies [16,17].

In this work a simple, sensitive, and inexpensive SDME method has been developed for extraction of nicotine, anabasine, and cotinine in human urine and saliva samples then GC with FID detection was used for quantification of these alkaloids. Experimental parameters influencing the extraction efficiency of alkaloids including extraction organic solvent, pH of sample solution, stirring rate, salting-out, and extraction time were studied.

2. Experimental

2.1. Chemicals and solvents

Nicotine [3-((2 s)-1-methylpyrrolidin-2-yl]pyridine), anabasine [3-(2-piperidyl]pyridine), and cotinine (1-methyl-5-(3-pyridinyl)-2-pyrrolidinone) with purity of >96% were supplied by Fluka (UK). Phenylhydrazine (internal standard, I.S.), chloroform, dichloromethane, trichloroethylene, toluene, benzene, butyl acetate, methanol, and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

2.2. Apparatus

The analysis was performed with a gas chromatograph (model GC-17 Shimadzu, Japan) equipped with a flame-ionization detec-

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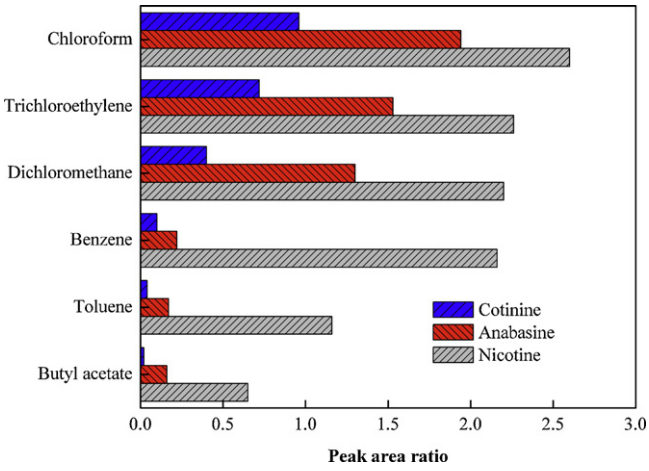

Fig. 1. Effect of extraction organic solvent on the extraction efficiency.

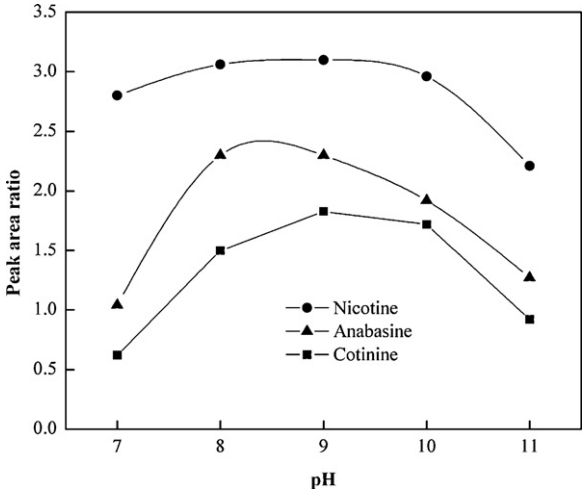

Fig. 2. Effect of pH of sample solution on the extraction efficiency.

tor (GC-FID) and a BP21 capillary column (25 m × 0.32 mm I.D., film thickness 0.5 μm). Helium (99.999%) was used as the carrier gas at a total flow rate of 29 mL min^{-1}. The inlet was operated in split mode with a split ratio of 20:1. The oven temperature was programmed as follows: initial temperature 100 °C (held for 1 min),

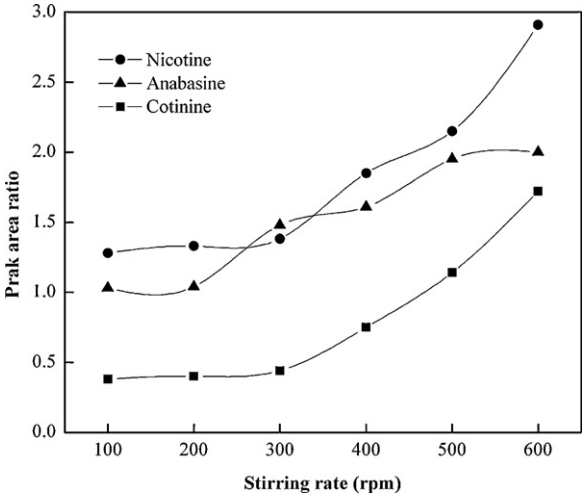

Fig. 3. Effect of stirring rate on the extraction efficiency.

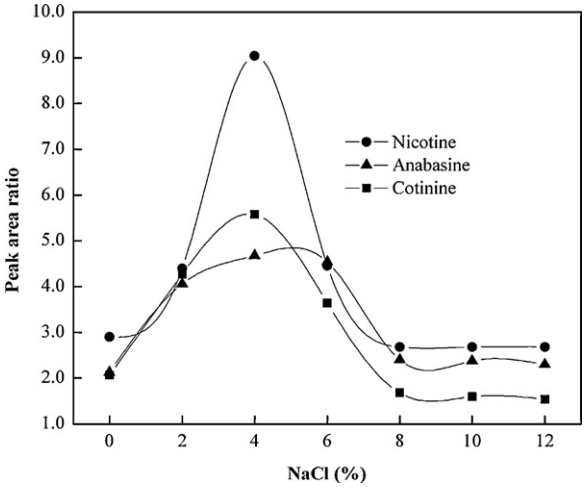

Fig. 4. Effect of NaCl addition on the extraction efficiency.

ramped at 30 °C min^{-1} to 180 °C, ramped at 40 °C min^{-1} to 210 °C (held for 1 min), ramped at 40 °C min^{-1} to 270 °C (held for 5 min). The temperatures of injector and detector were set at 270 and 300 °C, respectively.

The pH measurements were made with a 780 pH meter (Metrohm, Switzerland) equipped with a combine Ag/AgCl glass electrode. The centurion scientific centrifuge (model K280R, UK) was used for centrifuging.

2.3. SDME procedure

A microsyringe with an angled-cut tip (10 μL, F-LC, SGE, Australia) was used for the extraction procedure. The sample vial, containing 5 mL of aqueous sample solution, analytes, and I.S., was sealed with a screw cap with PTFE silicon septum. Before extraction, a known volume of extraction organic solvent (1 μL) was withdrawn into the microsyringe. The microsyringe was fixed above vial with a clamp. The needle of the microsyringe was inserted through the septum of the sample vial and directly immersed into the sample solution. The plunger of the microsyringe was depressed to expose the extraction organic solvent drop to the sample solution. Then the solution was stirred for 30 min using a magnetic stirrer bar (8.5 mm × 3.0 mm). After the extraction, the drop was retracted back into the microsyringe and immediately injected into the GC injection port for further analysis.

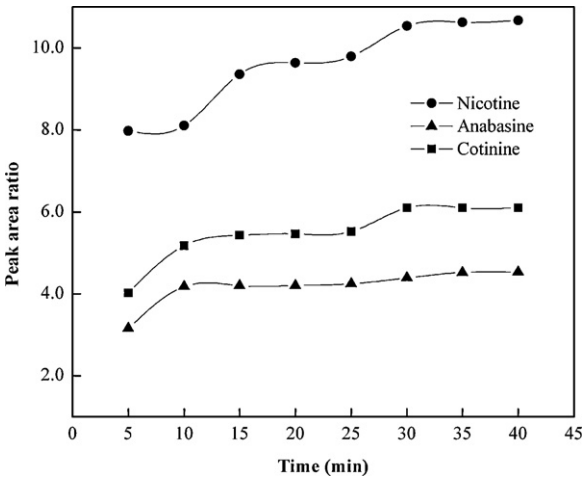

Fig. 5. Effect of extraction time on the extraction efficiency.

Table 1
Linear range, coefficient of determination (R^2), method detection limit (MDL), and Lower limit of quantification (LLOQ) for SDME method for the determination of nicotine, anabasine, and cotinine in urine and saliva samples.

Sample	Compound	Linear range (mg L^{-1})	R^2	MDL (mg L^{-1})[a]	LLOQ (mg L^{-1})
Urine	Nicotine	0.5, 2.0, 7.0, 10.0, 15.0, 20.0, 25.0	0.994	0.37	1.23
	Anabasine	0.5, 2.0, 7.0, 15.0, 20.0, 25.0, 40.0, 50.0, 55.0, 60.0, 65.0	0.993	0.45	1.50
	Cotinine	0.5, 2.0, 7.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0	0.993	0.43	1.43
Saliva	Nicotine	0.5, 2.0, 7.0, 10.0, 15.0, 20.0, 25.0	0.994	0.33	1.10
	Anabasine	0.5, 2.0, 7.0, 15.0, 20.0, 25.0, 40.0, 50.0, 55.0, 60.0, 65.0	0.993	0.40	1.33
	Cotinine	0.5, 2.0, 7.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0	0.993	0.34	1.13

[a] MDL are calculated as three times the standard deviation of ten replicated runs of urine and saliva samples spiked with 0.5 mg L^{-1} of all analytes.

Table 2
Results from determination of intra-day and inter-day precisions of nicotine, anabasine, and cotinine in urine and saliva samples determined by standard addition method.

Sample	Compound	Added (mg L^{-1})	Intra-day (n = 5)		Inter-day (n = 25)	
			Found (mg L^{-1})	RSD (%)	Found (mg L^{-1})	RSD (%)
Urine	Nicotine	0.5	0.60	3.4	0.56	4.2
		7.0	6.62	9.2	6.80	7.0
		20.0	20.14	4.7	20.17	3.6
	Anabasine	0.5	0.48	6.9	0.48	6.3
		7.0	7.17	1.3	7.17	2.1
		20.0	21.30	6.1	21.18	4.2
	Cotinine	0.5	0.52	3.1	0.54	2.3
		7.0	6.63	4.3	6.74	2.0
		20.0	21.30	1.6	21.00	2.6
Saliva	Nicotine	0.5	0.58	4.7	0.55	4.1
		7.0	7.14	7.6	7.04	5.5
		20.0	21.00	6.3	20.10	4.3
	Anabasine	0.5	0.42	6.3	0.45	2.6
		7.0	6.88	3.2	7.10	3.5
		20.0	20.3	2.1	20.10	5.1
	Cotinine	0.5	0.58	4.8	0.51	3.0
		7.0	7.10	2.7	6.93	4.6
		20.0	20.30	3.3	20.10	2.5

2.4. Preparation of standard solution and samples

Stock standard solutions of three analytes (1000 mg L^{-1}) and I.S. (500 mg L^{-1}) were prepared in methanol. Working solutions were obtained by appropriate dilution of the stock standard solution. Calibration standards were made at different concentration ranges. Each one was prepared in three replicates. All solutions were stored in a refrigerator in the dark.

Urine and saliva samples were kindly donated by volunteers. The samples were filtered using Whatman No. 42 filter paper, centrifuged before analysis and then processed immediately or stored in a refrigerator in the dark. For analysis aliquots of 100 μL of urine and 0.5 mL of salvia samples were transferred to 5 mL vials, fortified with analytes and I.S., diluted up to volume with double distilled water (pH 9) and submitted to SDME-GC-FID analysis.

3. Results and discussion

In this study the effects of several important parameters influencing the extraction efficiency such as extraction organic solvents, pH of sample solution, stirring rate, salting-out, and extraction time were studied. Chromatographic peak area of analyte and that of I.S. (peak area ratio) was used to assess the extraction efficiency under experimental conditions tested. Solutions of 10, 30, 40, and 500 mg L^{-1} of nicotine, cotinine, anabasine, and I.S., respectively, were used for optimization of the proposed SDME procedure.

3.1. Effect of extraction organic solvent

The selection of an appropriate extraction organic solvent is essential for the SDME method. The extraction organic solvent has to meet certain requirements such as (a) immiscibility with water, (b) low volatility, (c) extraction capability of target compounds, and (d) good chromatographic behavior. Based on these considerations, several water-immiscible organic solvents including, chloroform, trichloroethylene, dichloromethane, butyl acetate, benzene, and toluene were tested. The experiments were performed using 1 μL microdrop extraction from 5 mL working solutions (pH 10) at a stirring rate of 600 rpm for 20 min. Average peak area ratios as a function of extraction organic solvent were shown in Fig. 1. The

Table 3
Recovery of SDME method in urine and saliva samples spiked with nicotine, anabasine, and cotinine determined by standard addition method (n = 5).

Sample	Analyte	Recovery (RSD) (%)			
		4.0 (mg L^{-1})	7.0 (mg L^{-1})	15.0 (mg L^{-1})	35.0 (mg L^{-1})
Urine	Nicotine	73.5 (6.0)	100.9 (8.0)	98.6 (2.7)	–
	Anabasine	85.2 (2.3)	82.3 (8.6)	74.4 (8.0)	74.5 (4.5)
	Cotinine	94.5 (2.7)	91.6 (4.6)	93.8 (9.9)	94.5 (6.3)
Saliva	Nicotine	76.0 (3.3)	99.5 (10.0)	97.8 (9.5)	–
	Anabasine	111.0 (6.8)	79.3 (3.6)	62.4 (8.5)	71.2 (8.8)
	Cotinine	100.8 (4.8)	100.2 (9.6)	81.4 (6.4)	78.4 (5.8)

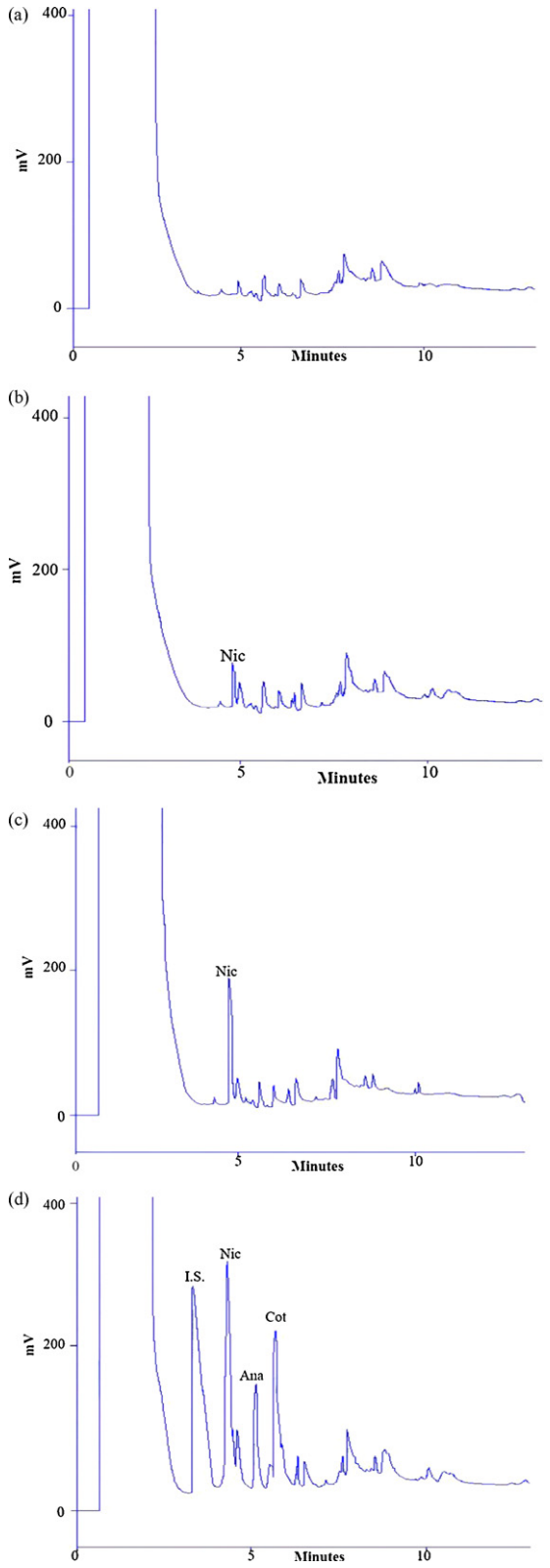

Fig. 6. GC-FID chromatograms of alkaloids obtained by SDME under optimized conditions: (a) blank saliva (non-smoker); (b) blank saliva (passive-smoker); (c) blank saliva (active-smoker); (d) spiked blank saliva with 0.5 mg L^{-1} of each analyte (active-smoker). Peaks: (I.S.) internal standard; (Nic) nicotine; (Ana) anabasine; (Cot) cotinine.

Table 4
Nicotine, anabasine, and cotinine concentration levels by smoking status ($n = 3$).

Sample	Smoking status	Concentration (mg L^{-1})		
		Nicotine	Anabasine	Cotinine
Saliva	Non-smoker	–	–	–
	Passive-smoker	0.96[a]	–	–
	Active-smoker	1.82[b]	–	–
Urine	Non-smoker	–	–	–
	Passive-smoker	0.38	–	0.26
	Active-smoker	1.62[c]	–	3.20

[a] Sampling time after 1 h environmental tobacco exposure.
[b] Sampling after 1 h smoking three cigarettes.
[c] Sampling after 4 h smoking three cigarettes.

results demonstrated that chloroform provided the higher extraction efficiencies, whereas butyl acetate had the lowest extraction efficiency for all analytes. Therefore, chloroform was selected as the most appropriate extraction organic solvent for subsequent SDME experiments.

3.2. Effect of pH of sample solution

The extraction efficiency of a weak organic base or acid depends on pH value of sample solution. The pH value of sample solution was investigated at 7, 8, 9, 10, and 11 using 1 μL microdrop extraction from 5 mL working solutions at a stirring rate of 600 rpm for 20 min. Fig. 2 shows the extraction efficiencies as a function of pH, which demonstrates that, the analytical signals improved by increasing the pH from 7 to 9 followed by decreasing from 9 to 11. Based on the above results, a pH of 9 was used for subsequent experiments.

3.3. Effect of stirring rate

Stirring rate reduces the time required to reach thermodynamic equilibrium and increases the extraction efficiency. Faster stirring rate could be employed to improve the extraction efficiency, since agitation permits the continuous exposure of the extraction surface to fresh aqueous samples [18–20]. The effect of stirring rate on the extraction efficiencies of alkaloids was studied in the range 100–600 rpm using 1 μL microdrop extraction from 5 mL working solutions (pH 9) for 20 min. As demonstrated in Fig. 3, the extraction efficiencies increased with increasing of stirring rate and the highest peak area ratios were reached at a stirring rate of 600 rpm. Due to instability of microdrop, stirring rate above 600 rpm was not evaluated. Therefore, all further experiments were performed with stirring rate of 600 rpm.

3.4. Salting-out effect

The effects of ionic strength were extensively evaluated in traditional LLE; generally addition of a certain amount of salt can decrease the solubility of hydrophilic compounds in the aqueous phase through a salting-out effect and consequently enhance their partitioning into the organic phase [21,22]. In order to investigate the effect of salinity on the extraction efficiencies, varied amounts of NaCl (0–12%) were added to 5 mL of working solutions (pH 9) at a stirring rate of 600 rpm for 20 min. Fig. 4 shows that for all the three analytes, the analytical signals increased with addition of NaCl concentration up to 4% and then decreased with further increasing NaCl concentration. Hence, a salt concentration of 4% was chosen for further experiments.

Table 5
Comparison of SDME method with reported methods for the determination of nicotine, anabasine, and cotinine in urine and/or saliva.

Parameter	This work	Ref. [7]	Ref. [9]	Ref. [23]	Ref. [4]
Biological fluids	Saliva/urine	Urine	Saliva/urine	Urine	Saliva/urine
Sample volume (mL)	0.5/0.1	0.5	0.5/5.0	1.0	0.1/0.2
Extraction organic solvent (s)	1 μL $CHCl_3$	3 mL CH_2Cl_2–ether	7 mL ethyl ether	0.5 mL $CHCl_3$–methanol	1.5 mL methanol
LOD	($mg\,L^{-1}$)	($\mu g\,L^{-1}$)		($\mu g\,L^{-1}$)	($\mu g\,L^{-1}$)
Nicotine/saliva	0.33	–	–	–	0.3–1.24
Anabasine/saliva	0.40	–	–	–	0.035–0.82
Cotinine/saliva	0.34	–	–	–	0.015–0.34
Nicotine/urine	0.37	0.2	–	0.2	0.03–1.24
Anabasine/urine	0.45	–	–	–	0.035–0.82
Cotinine/urine	0.43	0.2	–	0.5	0.015–0.34
R^2					
Nicotine/saliva	0.994	–	0.998	–	0.9969
Anabasine/saliva	0.993	–	–	–	0.9982
Cotinine/saliva	0.993	–	0.998	–	0.9994
Nicotine/urine	0.994	0.9996	0.998	0.997	0.9969
Anabasine/urine	0.993	–	–	–	0.9982
Cotinine/urine	0.993	0.9986	0.998	0.997	0.9994
Recovery (%)					
Nicotine/saliva	76.0–99.5	–	89.1–98.2	–	88.2–93.1
Anabasine/saliva	62.4–111.0	–	–	–	88.5–97.4
Cotinine/saliva	78.4–100.8	–	97.1–99.8	–	86.3–88.0
Nicotine/urine	73.5–100.9	91.6–105.0	92.1–97.7	82.4–100.9	88.2–93.1
Anabasine/urine	74.4–85.2	–	–	–	88.5–97.4
Cotinine/urine	91.6–94.5	85.5–101.5	96.3–98.2	95.1–104.4	86.3–88.0
Run time (min)	13	16	8	7	10

3.5. Effect of extraction time

Since the proposed method is an equilibrium extraction procedure, the maximum amount of analyte can be extracted by the extraction organic solvent and the better repeatability after equilibrium is obtained. Effect of extraction time was examined over the range from 5 to 40 min under the above optimized experimental conditions. As shown in Fig. 5, the extraction efficiencies increased with increasing extraction time from 5 to 30 min and reached equilibrium at 30 min. After 30 min, the curve reached a plateau and no increase in the extraction efficiency was observed with additional time. Therefore, an extraction time of 30 min was selected for subsequent experiments.

3.6. Statistical analysis

Statistical analysis was performed using computer program OriginPro (version 7.5). The results obtained from urine and saliva samples show that there was significant difference between concentrations of nicotine, anabasine, and cotinine in urine and concentrations of these compounds in saliva samples.

3.7. Method validation

Under the above mentioned optimum experimental conditions, the proposed method was validated by linearity, method detection limit (MDL), lower limit of quantification (LLOQ), precision, and accuracy. The linearity of three compounds was established using blank urine and saliva samples fortified at different concentration ranges of 0.5–25.0, 0.5–65.0, and 0.5–45.0 $mg\,L^{-1}$, for nicotine, anabasine, and cotinine, respectively. The method detection limit (MDL) values were calculated as three times the standard deviation of ten replicate runs of urine and saliva samples spiked with low concentration of analytes. The MDL values were in the range of 0.33–0.45 $mg\,L^{-1}$. The lower limit of detection (LLOQ) values were calculated as ten times the standard deviation of ten replicate runs of urine and saliva samples spiked with low concentration of analytes. The LLOQ values were in the range of 1.10–1.50 $mg\,L^{-1}$.

The linear ranges, coefficient of determinations (R^2), MDLs, and LLOQs of analytes are presented in Table 1. As can be seen, the proposed method has low MDLs and can be used for trace analysis of analytes in urine and saliva samples.

The intra-day and inter-day precisions of the assay were evaluated by analyses urine and saliva samples spiked at three concentration levels (0.5, 7.0, and 20.0 $mg\,L^{-1}$) on the same day and the five consecutive days. As it can be seen from Table 2, the intra-day and inter-day precisions in urine were in the range of 1.3–9.2% and 2.0–7.0%, respectively. The intra-day and inter-day precisions in saliva were in the range of 2.1–7.6% and 2.6–5.5%, respectively.

In order to determine the accuracy and the extraction recovery of the proposed method, standard addition test was performed. In which, the mixed standard solutions of target compounds were prepared with different concentration levels. Different standard solutions of different concentration levels were added to known volume of urine and saliva samples, respectively. The resulting samples were extracted with SDME method and analyzed by GC-FID. Five replicate extractions were performed for each concentration level, and the ratio of measured and added amounts was used to calculate the extraction recovery. The results of Table 3 show that the recoveries, measured at three concentration levels, varied from 71.2% to 111.0% with RSDs less than 10.0%.

In order to test the applicability of the proposed SDME method in real sample analysis, the determination of nicotine, anabasine, and cotinine in urine and saliva samples were performed by standard addition method. Urine and saliva samples were spiked with three analytes at concentration of 0.5 $mg\,L^{-1}$. Representative chromatograms of saliva sample extracts are shown in Fig. 6. As it can be seen, no significant interference peaks were found at the retention position of analytes. Average concentrations of nicotine, anabasine, and cotinine in urine and saliva samples of non-smoker, passive-smoker, and active-smoker are summarized in Table 4.

Table 5 indicates biological fluid sample volume, extraction organic solvent (type and volume), the limit of detection (LOD), coefficient of determination (R^2), recovery, and run time using single step extraction method [7], one step liquid-liquid extraction [9], liquid-liquid extraction (LLE) [23], automated in-tube solid phase microextraction [4], and single-drop microextraction (this work) methods for the determination of nicotine, anabasine, and cotinine in urine and/or saliva samples. The proposed method provides similar quantification extraction efficiency, with advantages of being

simple and using smaller volume of extraction organic solvent (at μL level).

4. Conclusions

A single-drop microextraction method followed by GC-FID was developed for the determination of nicotine, anabasine, and cotinine in human urine and saliva samples. The proposed method is simple, inexpensive, sensitive, and accurate. The results from validation indicate the proposed method can be applied for routine the determination of nicotine, anabasine, and cotinine in urine and saliva samples.

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