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Determination of biomarkers of tobacco smoke exposure in oral fluid using solid-phase extraction and gas chromatography–tandem mass spectrometry

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ABSTRACT

A new, simple and sensitive method was described for the simultaneous determination of nicotine, cotinine and *trans*-3′-hydroxycotinine in oral fluid samples using solid-phase extraction and gas chromatography/tandem mass spectrometry (GC–MS/MS). This technique was developed using only 0.2 mL of sample, and deuterated analogues were used as internal standards. The method was found to be linear between 0.5 and 1000 ng/mL, with determination coefficients higher than 0.996 for all analytes. Intra- and interday precision and accuracy were in conformity with the criteria normally accepted in bioanalytical method validation. All analytes were stable in the samples for at least 24 h at room temperature, for at least 72 h at 25 °C in processed samples and for at least three freeze/thaw cycles. Absolute recoveries ranged from 89 to 92% for all analytes. GC–MS/MS has demonstrated to be a powerful tool for the simultaneous quantitation of the analytes, providing adequate selectivity and sensitivity. In addition, its performance characteristics allow its routine use in the analysis of biomarkers of tobacco smoke exposure, extending the window of analyte detection in nicotine cessation programs, using a sample amount as low as 0.2 mL of human oral fluid.

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1. Introduction

Nicotine (NIC), a major component in tobacco, is a major addictive substance in cigarette smoke [1]. It is absorbed through the skin and mucosal lining of the mouth and nose or by inhalation in the lungs by both active and passive smokers [1–7]. Nicotine can cross the biological membranes including the blood–brain barrier, and once absorbed is readily and extensively metabolized [3] to a number of metabolites by liver enzymes [1,5,8,9] and to a lesser extent in the lung and kidney [10]. In humans, about 70–80% of nicotine is converted to cotinine (COT) [5] by hepatic cytochrome P450 (CYP2A6) [1,12], which is further metabolized to *trans*-3′-hydroxycotinine (OH-COT) and to other minor metabolites [1,2,12–14].

Previous studies reported that NIC has a short half-life ($t_{1/2}$ = 1–2 h). However, since COT and OH-COT have longer half-lives (18–20 h and 4–8 h, respectively) these are considered potentially useful biomarkers for evaluating tobacco smoke exposure [15–17].

Several methods have been reported for the determination of NIC, COT and related alkaloids in various biological fluids, and those include immunoassays [18–21], gas chromatography (GC) coupled to either flame ionisation (FID) [22] or mass spectrometric (MS) detection [1,12,23–26], high-performance liquid chromatography (LC) coupled to UV detector [27,28] or MS [7,16,29–36]. Hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS) methods have been also reported [33]. A direct-injection thermospray ionisation (TSI) LC–MS method is described for the rapid determination of NIC and 17 of its metabolites in smokers' urine [37].

Oral fluid (OF) is an important alternative matrix to blood and urine [36] for monitoring drug and tobacco exposure, since collection is simple, noninvasive and can be performed by nonmedical personnel under direct observation [6,36]. In addition, drug concentrations in this specimen are highly correlated with those present in plasma, and the window for drug detection is shorter, reflecting recent consumption [36]. Therefore, OF is the preferred biological specimen for many studies [9]. A few papers have dealt with the determination of NIC and metabolites in OF samples, using GC–MS [1] or LC–MS/MS [19,36]; these highlight the fact that, while adequate results can be obtained using less sensitive and less selective detectors such as UV or FID, MS-based methods represent the state

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of the art in this respect. In fact, those MS methods allow both the unequivocal identification of the analytes and the detection of the compounds even at very low concentrations, as often occurs in OF.

Tandem mass spectrometers provide further advantages when analyzing biological specimens, and in general selectivity is improved, because specific product ion fragments are obtained. This also leads to an increase in sensitivity, and lower amounts of the analytes can be detected [38]. This assumes even more relevance when OF is tested, since the analytes' concentrations are usually low in this specimen [39]. This powerful tool has already been used in bioanalysis, namely in the detection of biomarkers of alcohol consumption [40], *in vivo* lipid peroxidation [41] or the detection of the metabolite of Δ^9-tetrahydrocannabinol (carboxy-THC) in hair samples at concentrations in the range of pg/mg [42]. This paper describes a new and sensitive method for the quantitative analysis of NIC and two of its major metabolites (COT and OH-COT) in oral fluid samples by means of solid-phase extraction and GC–MS/MS.

2. Experimental

2.1. Reagents and standards

The analytical standards of NIC, NIC-d_4, COT and COT-d_3 were purchased from LGC Promochem (Barcelona, Spain) as a 1 mg/mL solution, except NIC-d_4 (0.1 mg/mL). OH-COT and OH-COT-d_3 were obtained from Toronto Research Chemicals (North York, Canada).

Methanol (Merck Co, Darmstadt, Germany), 2-propanol (Panreac, Barcelona, Spain), hydrochloric acid (Carlo Erba, Milan, Italy), methylene chloride (Analar Normapur, Darmstadt, Germany), and ammonium hydroxide (J.T. Baker, Deventer, Holland) were of HPLC grade. Sodium hydroxide was obtained from Vaz Pereira (Benavente, Portugal). N-methyl-N-(trimethylsilyl) trifluoroacetamide and trimethyl chlorosilane (TMCS) were acquired from Macherey-Nagel (Düren, Germany). Deionized water was obtained from a Millipore purification system. Oasis® MCX extraction cartridges (3 cm^3/60 mg) were purchased from Waters (Milford, MA, USA).

Stock solutions of each standard were prepared at 15 μg/mL by proper dilution with methanol. Standard mixtures of the studied compounds were prepared at 0.002, 0.02 and 1 μg/mL also in methanol. The internal standards' (IS) concentration was 1 μg/mL. All these solutions were stored light protected at 4 °C.

2.2. Biological specimens

Blank oral fluid samples used in all experiments were obtained from laboratory staff.

Authentic samples were collected by the spitting method, and the process was performed without stimulation in about 10 min. These samples were stored refrigerated at −10 °C until analysis.

2.3. Gas chromatographic and mass spectrometric conditions

Chromatographic analysis was performed using an HP 7890A gas chromatography system (Agilent Technologies, Waldbronn, Germany), equipped with a model 7000B triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany), a MPS2 autosampler and a PTV-injector from Gerstel (Mülheim an der Ruhr, Germany). A capillary column (30 m × 0.25-mm I.D., 0.25-μm film thickness) with 5% phenylmethylsiloxane (HP-5 MS), supplied by J & W Scientific (Folsom, CA, USA), was used.

The oven temperature started at 90 °C for 2 min, followed by an increase of 30 °C/min to 190 °C, and finally a new increment of 25 °C/min was used to achieve the final temperature of 250 °C. The temperatures of the injection port and the ion source were set at 250 °C and 230 °C, respectively. Helium was used as carrier gas at a constant flow rate of 0.8 mL/min.

The mass spectrometer was operated with a filament current of 35 μA and electron energy 70 eV in the positive electron ionisation mode. Nitrogen was used as collision gas at a flow rate of 2.5 mL/min. Data was acquired in the multiple reaction monitoring (MRM) mode, using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies).

The transitions were chosen for selectivity and abundance to maximize signal-to-noise ratio in matrix extracts. Table 1 resumes the precursor and product ions and collision energies selected for each analyte. The total separation time was 11.7 min, and the retention times (min) were 6.0 for NIC, 8.7 for COT and 9.8 for OH-COT.

2.4. Sample preparation

Oral fluid samples (0.2 mL) were diluted with 2 mL of 0.5 M sodium hydroxide, 1.8 mL of deionized water and spiked with 10 μL of the internal standards working solution. The mixture was agitated by rotation/inversion movements for 15 min and was added to Oasis® MCX extraction cartridges, previously conditioned with 2 mL of methanol and 2 mL of water. The columns were washed sequentially with 2 of each of the following: deionized water, 1.5 M hydrochloric acid and methanol. After drying under full vacuum for 10 min, the analytes were eluted with 2 mL methylene chloride:2-propanol:ammonium hydroxide (78:20:2, v/v/v); to prevent analyte loss during evaporation, 50 μL of 1% hydrochloric acid in methanol (v/v) was added. The extracts were evaporated to dryness under a gentle stream of nitrogen at room temperature. The residues were dissolved in 65 μL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) with 5% trimethylchlorosilane (TMCS) and derivatisation took place in a dry bath at 85 °C for 45 min. The extracts were transferred to autosampler vials and an aliquot of 2 μL was injected into the GC–MS/MS instrument in the splitless mode.

2.5. Validation procedure

The procedure was validated in terms of selectivity, linearity, intra- and interday precision and accuracy, absolute recovery and stability. Selectivity was evaluated by analyzing blank oral fluid samples of ten different origins (laboratory staff), and it was checked for interferences at the retention times and selected transitions of the studied compounds. Calibration data was generated by spiking blank oral fluid samples, and the calibration curve was established between 0.5 and 1000 ng/mL (ten calibrators evenly distributed) for all analytes. Five calibration curves were prepared, and the criteria for acceptance included a R^2 value of at least 0.99, and the calibrators' accuracy within a ±15% interval, except at the lower limit of quantitation (LLOQ), for which ±20% was accepted.

The limit of quantitation was defined as the lowest amount of analyte that presented a signal-to-noise ratio of at least 5 and could be measured with adequate precision and accuracy (coefficient of variation of less than 20% and an inaccuracy of ±20%).

Intra-day precision was characterized in terms of relative standard deviation (RSD, %) by analyzing sets of 6 spiked oral fluid samples at 4 different concentrations (0.5, 5, 100 and 500 ng/mL) in the same day. Interday precision was assessed at ten concentrations (0.5, 1, 5, 8 10, 100, 250, 500, 750 and 1000 ng/mL) over a 5-day period. Accuracy was evaluated in terms of mean relative error between the measured and the spiked concentrations for the calibrators and also in the intra- and interday precision assays; the limits of acceptable variability were set at 15% for all concentrations, except at the LLOQ, for which 20% was accepted. Absolute recovery was determined by replicate analysis (n = 6) of samples spiked at three concentrations (10, 100 and 1000 ng/mL),

Table 1
GC–MS/MS parameters (quantitation ions underlined).

Time segment (min)	Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (eV)
4.00	NIC	132.2	105.1	15
			117.1	
	NIC-d_4	164.4	123.4	
7.00	COT	174.6	104.1	15
			118	
	COT-d_3	177.6	150.2	
9.00	OH-COT	248.4	175.1	15
			218.1	
	OH-COT-d_3	251	146	

in which the internal standards were only added after extraction. The obtained peak area ratios were compared to those obtained by spiking blank extracts with the same amounts of all compounds; the latter were used as neat standards.

Processed sample-stability, short-term stability and freeze/thaw stability were studied ($n = 3$) at four concentration levels (2.5, 7, 50 and 400 ng/mL). Spiked oral fluid samples were subjected to different storage conditions, and the obtained results were compared to those obtained after analysis of freshly prepared samples. The analytes were considered to be stable under the tested conditions if the coefficients of variation between the two sets of samples were less than 15%.

3. Results and discussion

3.1. Method validation

The method was validated in a 5-day validation protocol. The validation parameters included selectivity, linearity and limits, intra-and interday precision and accuracy, recovery and stability, and were performed according to the guiding principles of the Food and Drug Administration (FDA) [43] and International Conference on Harmonization (ICH) [44].

3.1.1. Selectivity

Selectivity was studied by analyzing blank oral fluid specimens from 10 different origins. Each blank sample was extracted and analysed for assessment of potential interferences from endogenous components. Quality control samples were prepared and analysed contemporaneously with the samples. Identification criteria included an absolute retention time within 2% or ±0.1 min of the retention time of the same analyte in the control sample and the presence of two transitions per compound. The maximum allowed tolerances for the relative ion intensities between the two transitions (as a percentage of the base peak) were as follows. If the relative ion intensity in the control sample was higher than 50%, then an absolute tolerance of ±10% was accepted; if this value was between 25 and 50%, a relative tolerance of ±20% was allowed; if it was between 5 and 25%, an absolute tolerance of ±5% was accepted and finally, for relative ion intensities of 5% or less, a relative tolerance of ±50% was used [45].

Using these criteria, all the analytes were successfully and unequivocally identified in all the quality control samples, whereas in the blank samples no analyte could be identified. A representative ion chromatogram of a spiked oral fluid sample is shown in Fig. 1, and Fig. 2 represents a chromatogram obtained by analysis of a blank sample.

3.1.2. Calibration curves and limits

Linearity of the method was established between 0.5 and 1000 ng/mL (ten evenly distributed calibrators) ($n = 5$), and determination coefficients higher than 0.996 were obtained for all analytes. The calculated concentration of each calibrator had to be within a ±15% interval of target except for the LLOQ, where ±20% was accepted.

Along with each calibration curve, quality control (QC) samples at low (LQCs: 2.5 and 7 ng/mL), medium (MQC: 50 ng/mL) and high (HQC: 400 ng/mL) levels were also extracted and analysed ($n = 3$). Due to the high calibration range adopted, the calibration curves had to be divided into two linear ranges, from 0.5 to 10 ng/mL and from 10 to 1000 ng/mL (Table 2), in order to fulfill the above-mentioned criteria.

The LLOQ was defined as the smallest concentration of analyte that could be measured reproducibly and accurately (coefficient of variation of less than 20% and calculated concentration within a ±20% interval from the target level).

Despite of being capable of detecting discrete peaks at concentrations lower than 0.5 ng/mL, the analytes could not be successfully identified at those concentrations (by application of the above-mentioned identification criteria). This was due to the fact that deuterated analogues were used as IS, and as such have slightly contributed to the signal of the qualifiers, impairing identification. For this reason, our method's limits of detection (LODs) were considered to be 0.5 ng/mL, the same as the LLOQs.

These results are adequate, given the small sample volume 0.2 mL used. For instance, Concheiro et al. [36] have obtained slightly higher limits, using a somewhat higher sample volume (0.25 mL) and liquid chromatography–tandem mass spectrometry. Shakleya and Huestis [6] were able to detect 0.1 and 0.3 ng/mL of

Table 2
Linearity data.

Compound	Linearity (ng/mL)	Slope	Intercept	R^2
NIC	0.5–10	0.0222 ± 0.020	0.0643 ± 0.054	0.9982 ± 0.001
	10–1000	0.0011 ± 0.001	0.2365 ± 0.261	0.9964 ± 0.001
COT	0.5–10	0.0387 ± 0.024	0.0408 ± 0.029	0.9990 ± 0.001
	10–1000	0.0204 ± 0.012	0.3682 ± 0.333	0.9982 ± 0.001
OH-COT	0.5–10	0.2345 ± 0.021	0.1378 ± 0.148	0.9981 ± 0.001
	10–1000	0.0634 ± 0.035	4.0310 ± 1.400	0.9964 ± 0.002

Mean values ± standard deviation.

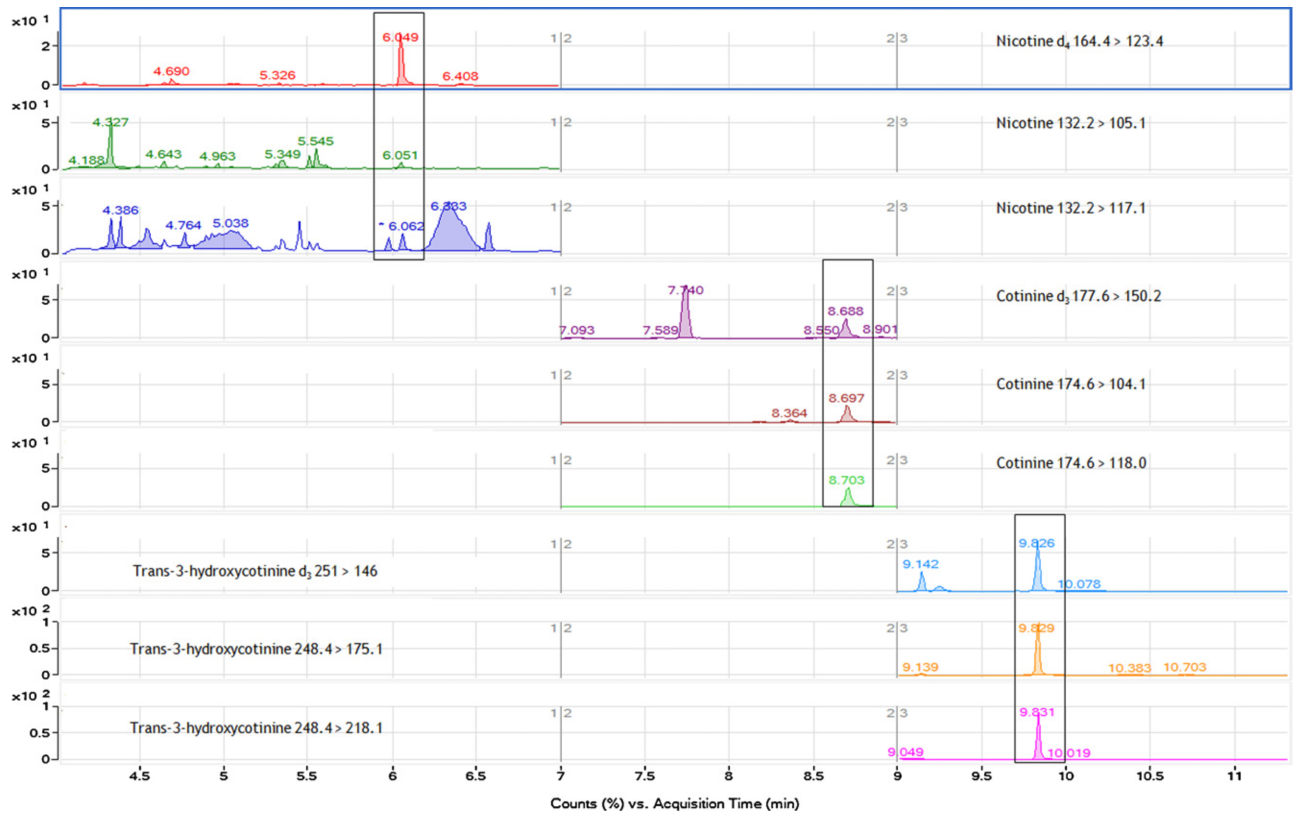

Fig. 1. Ion chromatogram of a spiked sample (100 ng/mL).

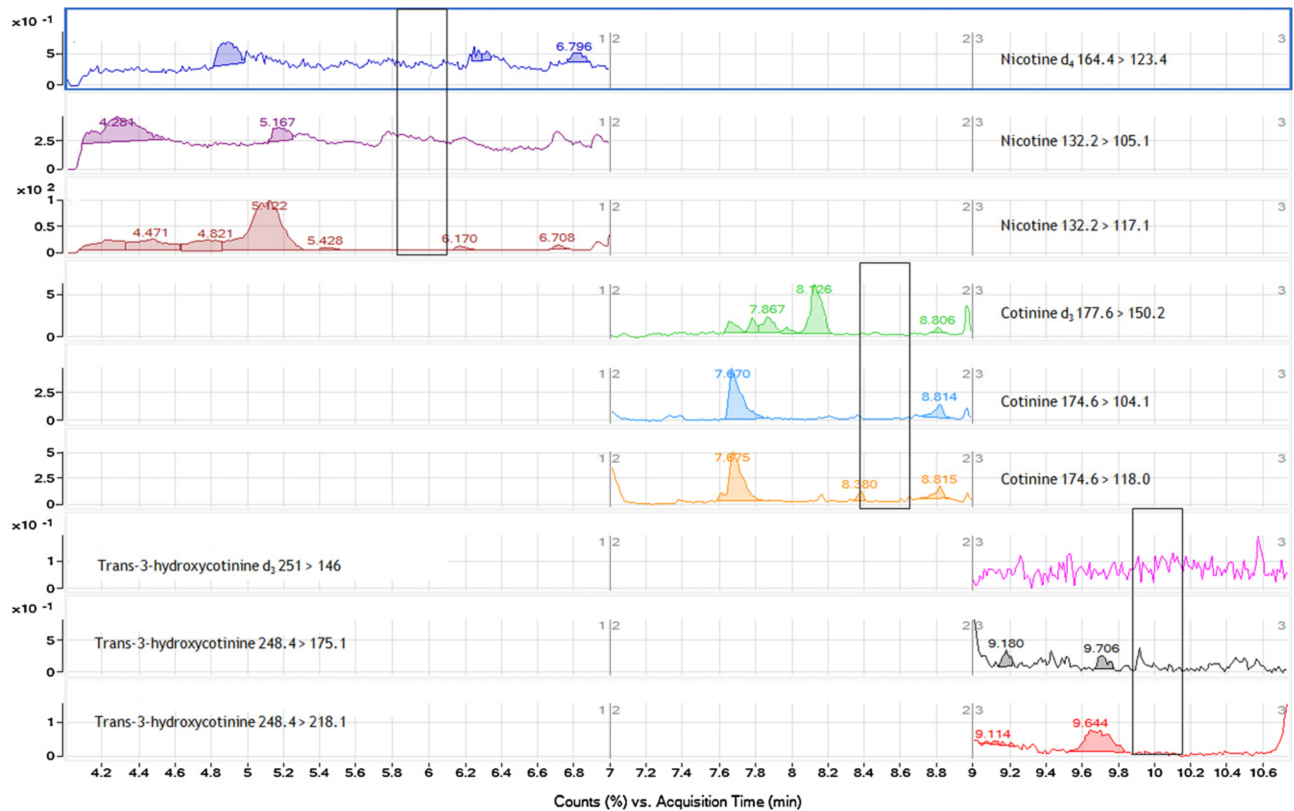

Fig. 2. Ion chromatogram of blank sample.

Table 3
Intra-day precision and accuracy ($n = 6$).

Compound	Spiked	Measured	CV%	RE%
NIC	0.5	0.52 ± 0.02	4.73	3.45
	5	4.83 ± 0.32	6.82	−3.36
	100	97.40 ± 8.28	8.51	−2.60
	500	495.63 ± 2.25	4.49	−0.87
COT	0.5	0.5 ± 0.02	5.16	−0.42
	5	4.93 ± 0.29	6.07	−1.45
	100	93.95 ± 1.46	1.56	−6.05
	500	500.04 ± 4.85	4.97	0.01
OH-COT	0.5	0.53 ± 0.02	3.79	6.58
	5	5.04 ± 0.25	5.10	0.83
	100	99.15 ± 9.53	9.62	−0.85
	500	499.31 ± 7.69	7.55	−0.14

All concentrations in ng/mL; CV, coefficient of variation; RE, relative error [(measured concentration – spiked concentration/spiked concentration) × 100].

OH-COT and COT, respectively, with LOQs of 0.5 and 0.2 ng/mL for those compounds, yet using twice the sample volume. Using 0.5 mL of oral fluid, Miller et al. [19] report LOQs of 1 ng/mL for all analytes.

Therefore, our LLOQs are lower, particularly if one takes into account the lower sample volume used. This fact highlights the selectivity of tandem mass spectrometry, allowing reducing the background noise usually observed in bioanalysis.

3.1.3. Intra- and interday precision and accuracy

Intraday precision and accuracy (relative error, %) for NIC and metabolites were determined by analysis of six independent replicates at four concentrations across the dynamic range of the assay: 0.5, 5, 100 and 500 ng/mL. The obtained CVs were typically below 10% for all compounds at all concentrations, while relative errors were within a ±7% interval (Table 3).

Interday precision and accuracy were evaluated at ten concentrations within a 5-day period. The calculated CVs were lower than 12% for all compounds at all concentration levels, while accuracy was within a ±10% interval. These data are presented in Table 4.

Intermediate precision (combined intra- and interday) was evaluated using the QC samples (LQC, MQC and HQC) prepared and analysed simultaneously with the calibration curves on 5 different days (15 measurements for each concentration). The CVs were typically below 7% for all compounds at all concentrations, while accuracy was within ±6% of the nominal concentration (Table 5).

3.1.4. Extraction recovery

Recovery (%) was calculated at 10, 100 and 1000 ng/mL ($n = 6$). Oral fluid samples were spiked at the intended concentrations and the IS were added after the SPE procedure. The peak area ratios were compared to blank extracts in which both the analytes and IS were added after SPE (neat standards). Values higher than 89% were obtained for all analytes, and are presented in Table 6.

Concheiro et al. [36] have obtained similar values (90–94%), while Miller et al. [19] report recovery values of 93–96% for COT. The higher recovery values for this sample are reported by Shakleya and Huestis [6] (98–113%), while Jacob et al. [11] have obtained 53–65%.

3.1.5. Stability

Stability was assessed using blank oral fluid QC samples, spiked at the above-mentioned LQC, MQC and HQC concentrations, which were extracted using the above mentioned procedure ($n = 3$). To study stability in processed samples, the extracts were left standing at room temperature in the autosampler for 24 h. Those samples were compared to freshly prepared samples, and the obtained coefficients of variation were less than 11% for all compounds, meaning that the analytes are stable in the autosampler for at least 24 h at room temperature.

Short-term stability was evaluated at the same concentration levels ($n = 3$). Oral fluid samples were spiked and were left at room temperature for 24 h, time after which they were extracted and compared with freshly spiked samples, and the obtained coefficients of variation were less than 7% for all compounds, meaning

Table 4
Inter-day precision and accuracy ($n = 5$).

Compound	Spiked	Measured	CV%	RE%
NIC	0.5	0.51 ± 0.05	10.92	1.62
	1	0.92 ± 0.02	2.72	−8.11
	5	5.17 ± 0.14	2.73	3.45
	8	7.92 ± 0.24	3.06	−0.96
	10	10.00 ± 0.16	1.60	0.14
	100	101.58 ± 9.17	9.03	1.58
	250	238.70 ± 12.93	5.42	−4.52
	500	480.06 ± 9.33	6.11	−3.99
	750	723.07 ± 2.17	2.79	−3.59
	1000	1007.34 ± 2.14	2.00	0.73
COT	0.5	0.48 ± 0.05	11.50	−3.24
	1	1.07 ± 0.05	5.55	6.78
	5	4.92 ± 0.16	3.30	−1.70
	8	7.96 ± 0.11	1.46	−0.49
	10	10.05 ± 0.14	1.48	0.53
	100	95.38 ± 6.76	7.09	−4.62
	250	241.30 ± 9.37	8.03	−3.48
	500	499.34 ± 6.54	1.31	−0.13
	750	738.41 ± 14.25	1.93	−1.55
	1000	1012.96 ± 8.20	0.81	1.30
OH-COT	0.5	0.47 ± 0.04	9.62	−5.81
	1	1.02 ± 0.08	8.02	1.87
	5	4.96 ± 0.16	3.27	−0.73
	8	8.14 ± 0.20	2.52	1.79
	10	9.90 ± 0.17	1.78	−0.98
	100	109.99 ± 3.16	2.88	9.99
	250	262.70 ± 9.25	7.33	5.08
	500	518.09 ± 9.37	1.81	3.62
	750	757.97 ± 14.17	1.87	1.06
	1000	981.20 ± 7.75	0.79	−1.88

All concentrations in ng/mL; CV, coefficient of variation; RE, relative error [(measured concentration – spiked concentration/spiked concentration) × 100].

Table 5
Intermediate precision and accuracy ($n = 15$).

Compound	Spiked	Measured	CV%	RE%
NIC	2.5	2.54 ± 0.13	5.40	1.62
	7	6.87 ± 0.38	5.66	−1.82
	50	52.80 ± 2.41	4.57	5.61
	400	399.93 ± 14.63	3.66	−0.02
COT	2.5	2.43 ± 0.16	6.8	−2.74
	7	7.22 ± 0.42	5.88	3.13
	50	48.69 ± 2.34	4.81	−2.63
	400	393.41 ± 14.75	3.75	−1.65
OH-COT	2.5	2.52 ± 0.16	6.51	0.79
	7	6.82 ± 0.39	5.86	−2.62
	50	50.17 ± 3.05	6.08	0.33
	400	420.82 ± 13.42	3.19	5.20

All concentrations in ng/mL; CV, coefficient of variation; RE, relative error [(measured concentration – spiked concentration/spiked concentration) × 100].

Table 6
Absolute recovery (%).

Concentration (ng/mL)	NIC	COT	OH-COT
10	89.4 ± 6.9	98.4 ± 2.5	86.7 ± 3.6
100	89.2 ± 1.5	84.6 ± 4.3	89.2 ± 0.5
1000	98.1 ± 4.5	84.7 ± 13.8	99.8 ± 1.8

Mean values ± standard deviation.

Table 7
Method applicability.

Sample	Age	Gender	Concentration (ng/mL)		
			NIC	COT	OH-COT
#1	13	M	136.4	2.9	1.4
#2	13	M	162.3	5.0	1.6
#3	13	F	56.7	4.5	1.1
#4	12	F	n.d.	4.8	1.8
#5	11	F	2.7	3.7	1.9
#6	16	M	n.d.	0.7	n.d.
#7	13	F	2.5	n.d.	n.d.
#8	18	F	269.1	54.9	3.9
#9	13	F	4.4	7.7	0.8
#10	13	F	n.d.	4.0	0.9
#11	12	M	66.8	0.9	n.d.
#12	12	M	52.6	n.d.	n.d.
#13	12	M	n.d.	1.2	0.6
#14	16	F	n.d.	n.d.	n.d.
#15	12	M	n.d.	n.d.	n.d.
#16	13	M	n.d.	n.d.	n.d.
#17	15	F	3.0	9.4	4.5
#18	12	F	3.3	2.2	0.7
#19	13	F	192.0	8.3	4.7
#20	12	M	164.5	2.3	0.9
#21	12	F	n.d.	n.d.	n.d.
#22	12	F	n.d.	n.d.	n.d.
#23	15	F	n.d.	n.d.	n.d.
#24	13	M	n.d.	n.d.	n.d.
#25	13	M	n.d.	n.d.	n.d.
#26	13	M	357.3	n.d.	n.d.
#27	16	F	186.6	n.d.	n.d.
#28	11	F	2.8	n.d.	n.d.
#29	11	M	n.d.	0.7	0.5
#30	15	F	n.d.	n.d.	n.d.
#31	12	M	n.d.	n.d.	n.d.
#32	12	F	n.d.	2.1	0.8
#33	13	M	n.d.	n.d.	n.d.
#34	12	M	n.d.	n.d.	n.d.
#35	12	F	77.6	n.d.	n.d.
#36	12	M	117.1	n.d.	n.d.
#37	12	M	280.8	1.7	0.5
#38	12	F	153.4	0.6	n.d.
#39	12	M	43.5	1.4	n.d.
#40	12	F	184.4	0.9	n.d.

n.d., not detected.

that the analytes are stable in the samples for at least 24 h at room temperature.

Freeze/thaw stability was evaluated as follows. Oral fluid samples were spiked at the intended concentrations, and were stored at −20 °C for 24 h, period after which they were thawed unassisted at room temperature. When completely thawed, the samples were re-frozen for 12–24 h under the same conditions. This freeze/thaw cycle was repeated twice more, and the samples were analysed after the third cycle. The obtained peak areas were compared to those obtained by analysis of freshly prepared samples, and the analytes were stable for at least 3 freeze/thaw cycles (the obtained CVs were less than 8% for all compounds).

3.2. Method applicability

After validation, the herein described procedure was applied to 40 authentic samples obtained at the local school (Frei Heitor Pinto, Covilhã, Portugal), belonging to both males and females aged 11–18. Oral fluid concentrations of the studied compounds ranged from 2.5 to 357.3 ng/mL for NIC, 0.6 to 54.9 ng/mL for COT and 0.5 to 4.7 ng/mL for OH-COT. In 12 of the samples no analytes were detected. These values are presented in Table 7. It is desired to apply the method to a higher number of authentic samples, for a better interpretation of the levels obtained for each of those biomarkers of tobacco smoke exposure.

4. Conclusions

A simple and sensitive procedure employing GC–MS/MS was developed and fully validated for the qualitative and quantitative determination of nicotine and two metabolites in oral fluid samples. The used sample volume was as low as 0.2 mL, which is important, since most of the times sample availability is of concern.

Method selectivity, linearity, intra- and interday precision and accuracy, limits and recovery were adequate, allowing analyte detection even at very low concentrations, which are expected in non-smoking populations.

For those reasons, the method may be used for the monitoring of environmental tobacco smoke exposure, and also to extend the window of drug detection in nicotine cessation programs. This will help in identifying individuals at high risk of developing smoking-related diseases, and those amenable to smoking cessation programs as well.

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