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Optimization and validation of a procedure using the dried saliva spots approach for the determination of tobacco markers in oral fluid

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ABSTRACT

Exposure to tobacco smoke is one of the most common causes of premature death worldwide and is the cause of 8 million deaths annually. We have developed, optimized, and validated a procedure for the detection of nicotine, cotinine and trans-3-hydroxycotinine (biomarkers of tobacco exposure) in oral fluid using the dried saliva spots sampling approach and gas chromatography coupled to tandem mass spectrometry, thus allowing the distinction between active and passive smokers. For optimization, four parameters were evaluated, namely extraction solvent, extraction solvent volume, extraction time and spots drying time. During method validation, the parameters selectivity, linearity, precision and accuracy, recovery, stability, and dilution factor were assessed.

Linearity was obtained for all target analytes in the concentration range of 10–200 ng/mL allowing the quantification of compounds up to 1000 ng/mL considering the dilution factor. The method recoveries ranged from 29.2% to 43.30% for nicotine, 66.60–89.10% for cotinine and 80.30–92.80% for trans-3-hydroxycotinine, while achieving intra-day, inter-day and intermediate precision and accuracy values never higher than 10.37% and ±6.62% respectively for all compounds. The herein described analytical method is the first to allow the determination of tobacco biomarkers in oral fluid using dried saliva spots, which is considered a sensitive, simple and low-cost alternative to conventional methods.

1. Introduction

Currently, it is estimated about 1 billion smokers worldwide and this human behaviour is associated with the onset of several respiratory, cardiac and oncological diseases [1,2]. Tobacco consumption is one of the main causes of premature death, being responsible for over 8 million deaths annually due to tobacco smoke exposure. This exposure can either be due to active smoking or to environmental tobacco smoke (ETS). Nicotine (NIC) is the main active compound present in tobacco leaves and in electronic devices for tobacco replacement, being responsible for the establishment of addiction mechanisms [2]. The absorption of NIC is pH-dependent and is enhanced when tobacco smoke reaches a pH higher than 6.5, thus facilitating its absorption through the biological barriers of the oral mucosa and lung alveoli [2,3]. After NIC reaches the bloodstream, it undergoes several biotransformation processes, mainly through the action of enzymes such as cytochrome P450 (CYP2A6), flavin-containing monooxygenase 3 (FMO3), and UDP-glucuronosyltransferase (UGT) present in the liver, leading to the formation of several metabolites [2,3]. From this metabolization, 70–80% of NIC is converted into cotinine (COT), a target biomarker of exposure to tobacco smoke, via CYP2A6 [4]. Subsequently, COT is converted by the same enzyme into trans-3-hydroxycotinine (OH-COT), being considered a complementary marker that allows, together with COT, the distinction between passive and active smokers [3,4]. Although NIC is a specific biomarker of tobacco smoke exposure, its application in monitoring tobacco consumption is limited due to its

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short half-life (1.5–3.5 h) [5]. In this context, several studies point out the metabolites COT and OH-COT as biomarkers of choice since they result specifically from nicotine metabolism, have longer half-lives (6–22 h for COT and 5–8 h for OH-COT) and smaller fluctuation for oral fluid (OF) samples when compared to NIC, since the presence of these biomarkers in OF does not arise from consumption, but instead is solely dependent on the metabolization of NIC within the body [2,5–7].

OF is an alternative biological sample which is becoming increasingly used in the field of toxicology and clinical practice. This sample enables the monitoring of several drugs to conduct exposure level assessment through the quantification of specific biomarkers, as well as to perform physiological and metabolic activity studies, owing to the high correlation with plasma free fraction levels [2,8–10]. Compared to the classic samples, blood and urine, OF is easier to collect due to a non-invasive way, allowing the collection under surveillance, without the need of a specialized health technician [2,8–11]. However, this sample may present some disadvantages, namely the amount available and the influence of the physiological and psychological state on its production [11–13]. Additionally, OF only allows the determination of compounds with short detection windows, thus enabling only exposure assessment of near events prior to the moment of collection (24–36 h before) [2].

Several extraction techniques have been reported for the preconcentration of NIC and metabolites in OF, the most common being solid phase extraction (SPE) [12–14] and liquid-liquid extraction (LLE) [15,16]. Nonetheless, nowadays there is a trend for miniaturized procedures which are considered simpler, faster and minimize wastes. Regarding NIC and metabolites pre-concentration, the use of solid phase microextraction (SPME) [17], liquid-liquid microextraction (LLME) [18] and dried blood spots (DBS) [19] have been reported.

The use of the DSS (dried saliva spots) extraction technique has provided a new approach for the analysis of biomarkers in OF. This type of procedure consists on applying a low volume of OF (50–100 μL) on an appropriate filter paper card, allowing it to dry so it can then be stored and/or to proceed with the extraction of the compounds [20–23]. This type of extraction method has been used in pharmacokinetic studies [24], microbiology [25] and more recently for the determination and monitoring of exposure to different drugs of abuse [21–23] and medication [21]. The first method relying on DSS as an extraction method was developed by Abdel-Rehim, reporting the determination of lidocaine by LC-MS [20]. This technique makes it possible to perform multiple extractions simultaneously through a simple, low-cost and more environmentally friendly procedure, facilitating also its storage and transport [20–23,26].

Several analytical methodologies have been developed to determine NIC, COT and OH-COT in different types of biological matrices, with the most common being liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [27], liquid chromatography coupled with mass spectrometry (LC-MS) [17], gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) [14], gas chromatography coupled with mass spectrometry (GC-MS) [28], high resolution mass spectrometry coupled to a diode array detector (HPLC-DAD) [29] and IONSCAN®-LS ion mass spectrometry (IONSCAN®-LS IMS) [18].

This work describes the entire process of optimization of extraction conditions for both NIC and its major metabolites COT and OH-COT in OF using DSS and their subsequent quantification by GC-MS/MS.

2. Experimental

2.1. Reagents and standards

Standard solutions of NIC, COT and respective deuterated analogues (NIC-d4 and COT-d3) were acquired from LGC Promochem (Barcelona, Spain) at a concentration of 1 mg/mL, except for NIC-d4 (100 μg/mL). OH-COT and its respective internal standard (OH-COT-d3) were purchased from Toronto Research Chemicals (York North, Canada) at a concentration of 1 mg/mL. Trimethylchlorosilane (TMCS) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were supplied by Macherey-Nagel (Düren, Germany), from which a derivatisation solution of MSTFA with 5% TMCS was prepared. Whatman™ 903 protein saver cards were obtained from Sigma-Aldrich (Sintra, Portugal). Working solutions were prepared by diluting the respective analytical standards in methanol (MeOH), thus preparing a mix of NIC, COT and OH-COT at concentrations of 10 and 100 μg/mL. A mix of deuterated standards was prepared at 1 μg/mL in MeOH, and an individual solution of NIC-d4 at 0.5 μg/mL was also prepared in the same solvent. All solutions were stored protected from light at 4 °C.

2.2. Biological samples

The blank OF samples used throughout the experimental procedures were obtained from laboratory staff members who are neither smokers nor exposed to tobacco in their routine.

The authentic samples were obtained from students of Universidade da Beira Interior (UBI) after reading and accepting informed consent (Ethical Committee project: CE-UBI-Pj-2021–046:ID1005). The specimens were collected by spitting without the use of stimulating devices or specific collection devices for this purpose. All samples after collection were frozen at −20 ºC.

2.3. GC-MS/MS conditions

Chromatographic analysis was accomplished using an HP 7890A gas chromatographic system, coupled with a triple quadrupole mass spectrometer model 7000B (both from Agilent Technologies, Waldbronn, Germany), an 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-5MS), purchased from J & W Scientific (Folsom, CA, USA) was employed.

The oven temperature started at 90 ºC, maintained for 2 min, followed by a steady increase of 30 ºC/min until 190 ºC, and a second temperature ramp is performed with an increase of 25 ºC/min until a temperature of 250 ºC is reached. Three μL of the derivatized extract was injected in the splitless mode using helium as carrier gas at a constant flow rate of 0.9 mL/min. The mass spectrometer was set with a filament current of 35 μA and electron energy 70 eV in electron positive ionization (EI+) mode. Data were acquired in the multiple reaction monitoring (MRM) mode using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies). Table 1 shows the detection conditions used in MRM for each compound, indicating the quantifier and qualifier transitions, retention time, collision energy and dwell time.

2.4. Sample preparation

The extraction process designed for the studied compounds was carried out in two steps. Once the samples were thawed, a clean-up

Table 1
Retention time and GC–MS/MS parameters.

Analyte	Retention time (minutes)	Transitions (*m/z*)	Collision energy (eV)	Dwell time (μs)
NIC	5.91	160.4–130.0	5	100
		160.4–119.0	5	100
NIC-d4[a]	5.90	164.8–123.1	10	100
COT	8.59	177.2–69.1	20	100
		177.2–99.1	5	100
COT-d3[a]	8.60	180.1–101.3	10	100
OH-COT	9.68	246.4–116.9	5	100
		246.4–173.1	5	100
OH-COT-d3[a]	9.67	233.2–201.2	20	100

[a] Internal standard; quantitative transition were underlined.

process by protein precipitation (PP) by addition of 5 μL of frozen acetonitrile (ACN) to 100 μL of sample is performed, and afterwards the samples are homogenized for 10 s in vortex and centrifuged for 10 min at 3500 rpm. Subsequently, extraction by DSS is performed following these steps: application of 50 μL of OF into Whatman™ 903 protein saver cards, followed by a drying step for 1 h at a temperature of 25 ºC; then, an additional application of 50 μL of sample is performed, after which it will dry for 1 h at the same temperature. The whole spots are then cut with scissors around the defined circle and placed individually in a falcon tube where 1 mL of acidified methanol (MeOH pH 5.0) and 50 μL of each of the internal standard solutions (internal standard mix NIC-d4, COT-d3 and OH-COT-d3 at 1 μg/mL; NIC-d4 at 0.5 μg/mL) is added. The compounds were then extracted for 5 min at 70 rpm in a roller mixer and then transferred to assay tubes to which 50 μL of a 1% HCl in MeOH is subsequently added. The extracts were evaporated to dryness under a gentle nitrogen stream, after which 20 μL of the derivatization solution (MSTFA+5%TMCS) was added followed by a microwave derivatization process for 2 min at 800 W. Finally, a 3 μL aliquot of the derivatized sample is manually injected into the GC-MS/MS.

2.5. Validation procedure

The developed method was fully validated according to the SWGTOX guidelines [30]. The validation process was carried out over 5 days, and the studied parameters were linearity, limit of detection (LOD), lower limit of quantification (LLOQ), selectivity, inter-day, intra-day and intermediate precision and accuracy, recovery, stability and dilution factor. The method's selectivity indicates the ability to detect the compounds under study even in the presence of other interferences present in the biological sample used. Several interferences may be found in OF sample, such as bacteria, food residues, mineral salts, enzymes, among others [8–10]. Extraction eliminates most of these, but some may remain in the final extract. In order to verify if the method is selective, it is necessary to check, considering the retention time and transitions defined, if it is not affected by matrix components [31]. In order to establish a calibration curve for each of the compounds under study, the relationship between the peak area of each compound and the respective internal standard (IS) was evaluated, and subsequently related to the concentration of each of the calibrators. The acceptance criteria for the calibration line were as follows: calibrator's accuracy within ±15% (except at the LLOQ, where ±20% was accepted) and a coefficient of determination (R^2) of at least 0.99 [30,31]. The method's LOD was defined as the lowest analyte concentration with acceptable chromatography, in which all transitions were present with signal-to-noise ratios of at least 3 and retention time within ±0.2 min of the average retention time of the calibrator (n = 10). Precision and accuracy are two essential requirements to ensure that the values obtained from the analytical method of quantification are as close as possible to the real values and that its performance remains stable. The parameters of precision and accuracy were evaluated using the coefficient of variation (CV) and relative error (RE) respectively. The acceptance criterion for RE is ±15% for each concentration, and for CV the defined criterion is that it cannot exceed 15% for each concentration [30,31]. Stability studies are an important parameter when developing analytical procedures, since they allow to deal with several problems that arise in the routine of a laboratory, which may affect the reliability of the results [30,31]. Stability was studied under four different parameters: processed sample stability, room temperature stability, freeze-thaw stability and long-term stability. Two acceptance criteria were used to evaluate stability: CV of less than 15% and RE ±15%. To evaluate the different stability parameters, 3 QCs at different concentrations (low, medium and high concentration levels) must be used [30,31].

3. Results and discussion

3.1. Optimization of the extraction procedure

Compound extraction using DSS follows a broadly similar procedure to comparable extraction approaches applied to other types of matrices, generally referred to as DMS (dried matrix spots) [26]. This type of extraction implies the clipping of each spot into an individual tube, to which extraction solvents and internal standards is added [21–23,26]. In the developed work, four parameters were optimized, which have been defined as necessary and critical for the efficient performance of the developed methodology. The extraction conditions were adjusted to the optimal ones based on the results obtained along each of the optimization steps.

The first optimization step was the study of the extraction solvents, in which 9 different solvents were tested (n = 3). Hexane, methanol: acetonitrile (MeOH:ACN) (50:50; v/v), acidified acetonitrile (ACN pH 5.0), MeOH, MeOH pH 5.0, acetonitrile (ACN), ethyl acetate (AET), isopropanol (ISOP), and dichloromethane (DCM) were tested. To carry out this study the following preliminary conditions were defined: 2 mL volume of extraction solvent, overnight drying at room temperature and 15-minute extraction in the roller mixer. Using the SPSS software (version 27) the values obtained for NIC, COT and OH-COT using different extraction solvents were analysed, using a non-parametric test (Friedman's test) with a significance value < 0.05 to this end. Although this study did not show significant variations between the analytical values obtained after extraction with different solvents, MeOH pH 5.0 was chosen as the most suitable extraction solvent exhibiting higher relative areas for the combined extraction of all compounds, in particular cotinine (the most important biomarker), presenting in general cleaner chromatograms and fewer interferences.

Keeping the initial optimization conditions for the extraction process, establishing MeOH pH 5.0 as extraction solvent (Fig. 1A), the next step of optimization was carried out, in which the necessary volume for an adequate extraction was determined. Three different volumes of solvent were tested (n = 3): 1, 2 and 3 mL. After performing the statistical study, no significant differences were observed (p-values in the Friedman's test of 0.457, 0.097 and 0.896 for NIC, COT and OH-COT respectively) between the different volumes, and therefore the smallest volume of solvent (1 mL) was chosen (Fig. 1B).

The next optimized parameter was the contact time between the solvent and the spot (extraction time). For that, 3 different extraction times were analysed (n = 3): 5, 15 and 30 min. Statistical results of these extraction assays indicate that there was no significant difference between the different extraction times for COT and OH-COT, with p-values of 0.097 and 0.717 respectively; however, significant difference between 5 min and 30 min (p-value = 0.043, value adjusted by Bonferroni correction) was observed for NIC, and as such the shortest extraction time (5 min) was selected (Fig. 1C). This allowed reducing extraction time to a minimum.

Lastly, DSS drying time after sample application was evaluated. To this end, three drying times were evaluated (n = 3): 1 and 3 h, and overnight. No significant differences were observed in the drying times (p-value of 0.097, 0.717, 0.368 for NIC, COT and OH-COT respectively). As such, the shortest drying time was chosen (1 h) (Fig. 1D), thus allowing faster analysis.

The internal standard was added after extraction in all optimization experiments.

3.2. Method validation

3.2.1. Selectivity

The selectivity study was performed through the analysis of 10 OF samples of different origin, belonging to individuals not exposed to tobacco smoke, which were compared to blank samples spiked at the lowest limit of quantification (LLOQ) and analysed by the developed

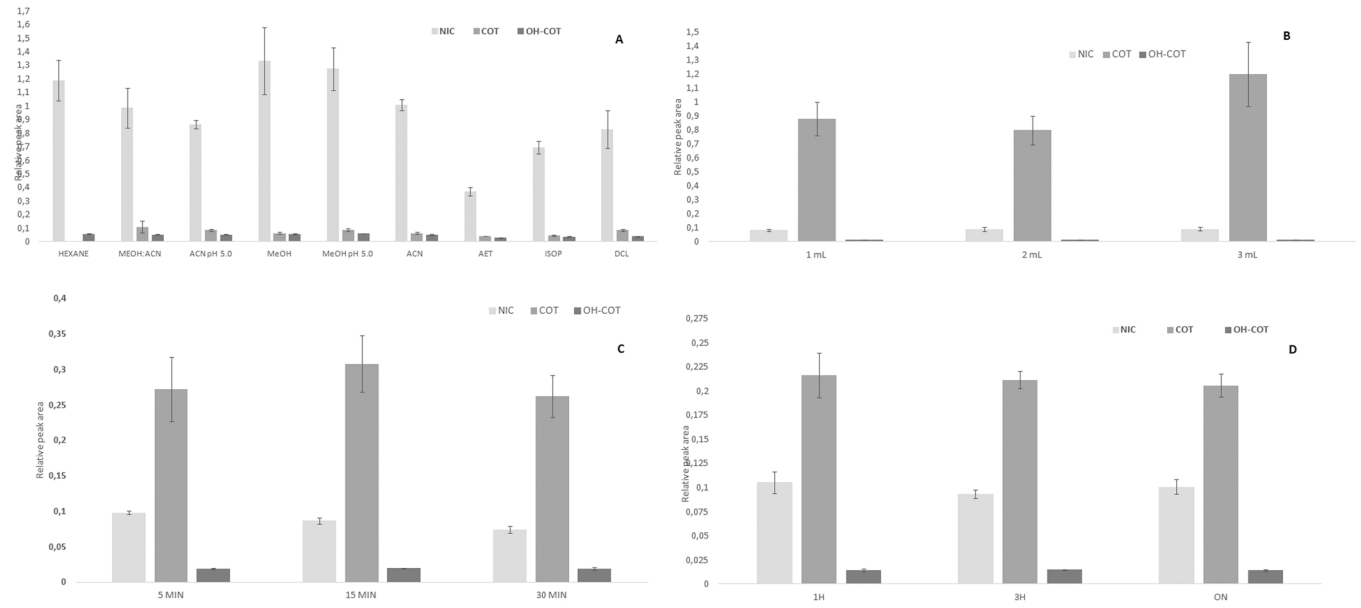

Fig. 1. Effects of extraction solvent (A); effects of extraction solvent volume (B); effects of extraction time (C) and effect of drying time of NIC, COT and OH-COT on the DSS (D). Legend: ACN (acetonitrile); AET (ethyl acetate); DCL (dichloromethane); ISOP (isopropanol); MeOH (methanol).

methodology. This comparison may be seen in Fig. 2. The World Anti-Doping Agency (WADA) technical document for separation by GC-MS/MS [32] was used.

3.2.2. Calibration curve and limits

Blank samples were spiked in a concentration range from 10 to 200 ng/mL for all compounds. In order to evaluate the linearity of the method, 7 calibrators were evenly distributed within this range (n = 5). To fulfil validation criteria, weighted least squares regression (1/x) was adopted. Calibration data is presented in Table 2. The LOD was 5 ng/mL for all compounds.

OF is an uncommon sample for the detection of tobacco biomarkers in general; however, when compared to other samples, it presents some advantages. According to a study with multiple biological matrices, Pérez-Ortuño et al. [15] obtained lower LLOQ values for NIC and COT, although using a 5 times larger volume (0.5 mL) of biological sample after salivary stimulation with a sweet (Smint®), LLE as extraction technique and a more sensitive analytical equipment (LC-MS/MS). Another study performed by Feng et al. [13] for NIC and COT analysed distinct levels of exposure to tobacco smoke, using a Quantisal™ device for sampling, saving it in a buffer, and using 0.5 mL of OF, obtaining lower LLOQ using SPE (Phenomenex Trace B) as extraction technique. Also using SPE (Clean Screen® ZSDAU020) and 0.5 mL of OF mixed with 2 mL of sodium acetate buffer, Kim et al. [12] obtained LLOQ of 5 ng/mL for NIC, COT and OH-COT by GC-MS.

Nevertheless, the obtained limits allow, considering an established consensus among several authors, to differentiate between active and passive consumption, using lower volumes of OF, which is the main advantage of the herein presented study [2].

3.2.3. Intra-day, inter-day and intermediate precision and accuracy

For the assess of intra-day precision and accuracy, at the LLOQ and 3 different quality control samples (QCs) (low, intermediate and high) (n = 6) were analysed on the same day, obtaining CVs ranging from 2.18% to 6.15%, and REs from ±0.21 to ±6.62% for all compounds (Table 3).

In a set of 5 different runs (n = 5), inter-day precision and accuracy were assessed for each of the calibrators used to define the calibration curve, obtaining CVs from 0.57% to 10.37%, and RE from 0 to ± 6.50% for all compounds (Table 3). To assess the precision and accuracy parameters for intermediate quantification values of the calibration curve, 3 QCs with concentrations of 20, 100 and 180 ng/mL were evaluated during a period of 5 days in triplicate for each one of the concentrations (n = 15), obtaining CV values below 8.32%, and RE between ±0.62 and ±4.80% for all compounds (Table 3).

3.2.4. Recoveries

The evaluation of the recovery of the developed extraction methodology for all compounds was performed at the concentrations of 25, 75 and 150 ng/mL, and the results are presented in Table 4. The obtained recoveries ranged between 29.2% and 43.3% for NIC, 66.6% and 89.1% for COT and between 80.3% and 92.8% for OH-COT.

In the methodology developed by Da Fonseca et al. [14] for the determination of exposure biomarkers, the authors have obtained recoveries higher than 89.2%, 84.6% and 86.7% for NIC, COT and OH-COT respectively, using 0.2 mL of sample and SPE. In another study by Pérez-Ortuño et al. [15], 0.5 mL of OF was used, and the samples were collected by stimulation using Smint® followed by spitting and LLE with dichloromethane, obtaining recoveries for NIC and COT greater than 90%.

Although the recoveries obtained for NIC are relatively low compared to those obtained for COT and OH-COT and to those obtained by other authors, our recoveries were sufficient to reach the desired LLOQ, and therefore we can say that the recoveries proved to be acceptable for the developed method, bearing in mind that this is the first method developed for multiple compounds using this method.

3.2.5. Stability

To evaluate the different stability parameters, 3 QCs at different concentrations (20, 100 and 180 ng/mL) were used [30]. The assessment of stability in processed samples (Table 5) was performed after the analysis of the 3 QCs under normal conditions, making a re-injection of the same vials (n = 3) after 24 h at room temperature, obtaining values below 3.76% for CV and ±12.56% for RE. Room temperature stability (Table 5) was determined by analysing samples of OF that had been previously spiked (n = 3) and left at room temperature for 24 h, followed by the standard DSS extraction process. When comparing the values obtained with fresh QCs at the same concentration, it was observed that the CV did not exceed 8.63%, obtaining a RE always below ±10.87%. The freeze-thaw stability (Table 5) was performed after 4

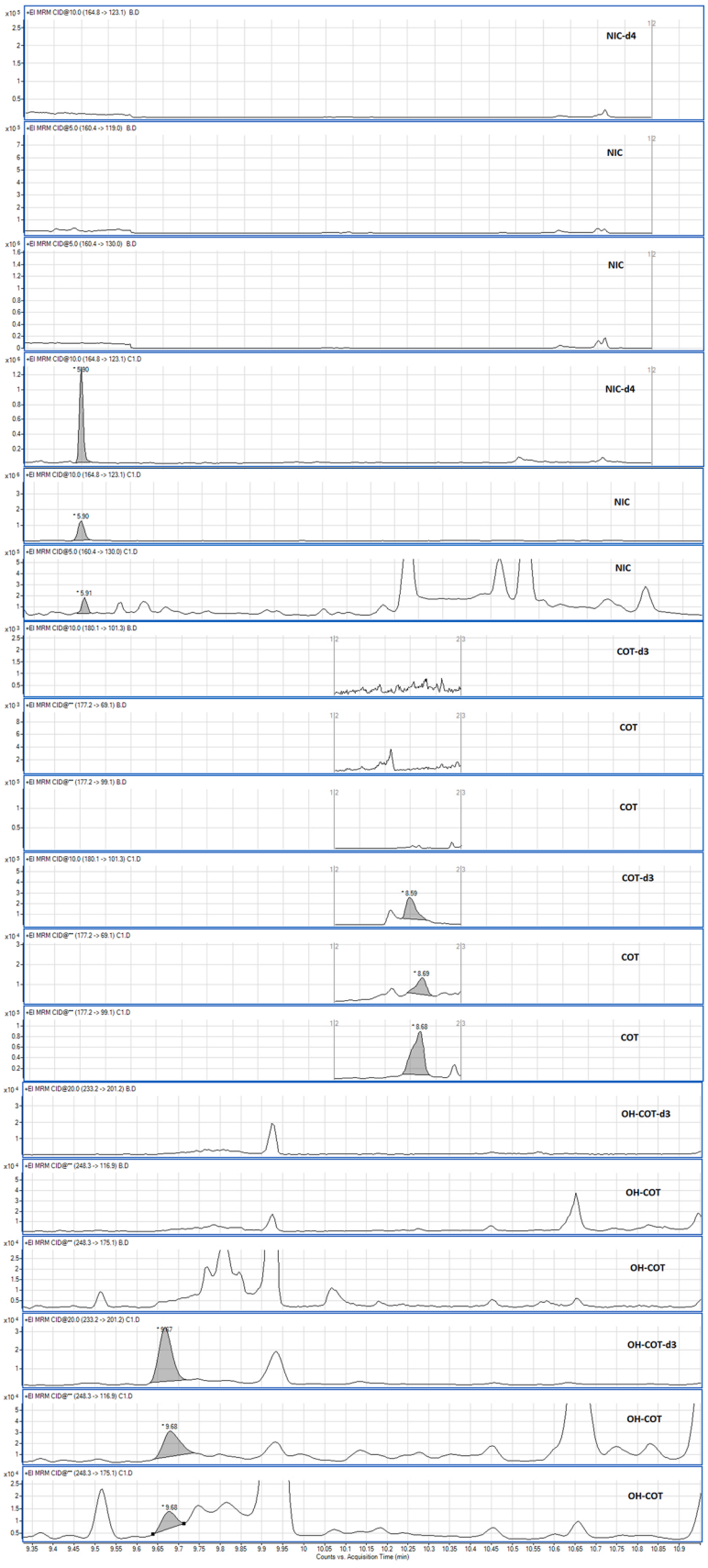

Fig. 2. Chromatogram of a blank sample and a fortified sample at the LLOQ for all compounds.

Table 2
Linearity data (n = 5).

Analyte	Linear range (ng/mL)	Linearity[a]		R^{2a}	LOD (ng/mL)	LLOQ (ng/mL)
		Slope	Intercept			
NIC	10–200	0.0011 ± 0.0001	0.0103 ± 0.0042	0.9973 ± 0.0012	5	10
COT	10–200	0.0012 ± 0.0002	0.0137 ± 0.0112	0.9964 ± 0.0015	5	10
OH-COT	10–200	0.0553 ± 0.0076	0.7745 ± 0.3621	0.9942 ± 0.0021	5	10

[a] Mean values ± standard deviation. The weighting factor was 1/x for all analytes.

Table 3
Inter-day, intraday, intermediate precision and accuracy.

Analyte	Concentration (ng/mL)	Inter-day (n = 5)			Intra-day (n = 6)			Intermediate (n = 15)		
		Measured[a] (ng/mL)	CV (%)	RE (%)	Measured[a] (ng/mL)	CV (%)	RE (%)	Measured[a] (ng/mL)	CV (%)	RE (%)
NIC	10	10.00 ± 0.54	5.40	0.00	9.87 ± 1.15	3.20	-1.30			
	20							19.88 ± 1.47	7.40	-0.62
	40	40.20 ± 2.85	7.08	0.50	40.08 ± 1.92	4.80	0.21			
	70	69.03 ± 6.20	8.99	-1.38						
	100							95.49 ± 4.71	4.93	-4.51
	110	105.35 ± 6.47	6.14	-4.23	106.48 ± 3.53	3.31	-3.20			
	140	134.18 ± 7.13	5.32	-4.16						
	170	175.93 ± 4.25	2.42	3.49						
	180							178.44 ± 11.63	6.52	-0.87
	200	206.38 ± 3.87	1.87	3.19	194.47 ± 9.92	5.10	-2.76			
COT	10	10.65 ± 0.50	4.65	6.50	10.03 ± 0.89	3.32	0.30			
	20							19.80 ± 1.52	7.69	-1.02
	40	38.08 ± 3.95	10.37	-4.79	41.65 ± 2.27	5.44	4.13			
	70	67.01 ± 4.42	6.60	-4.28						
	100							95.20 ± 5.73	6.01	-4.80
	110	106.86 ± 6.18	5.78	-2.85	106.94 ± 5.01	4.69	-2.78			
	140	137.97 ± 4.76	3.45	-1.45						
	170	172.57 ± 6.50	3.77	1.48						
	180							177.90 ± 11.76	6.61	-1.17
	200	205.91 ± 1.18	0.57	2.96	194.15 ± 7.93	4.09	-2.93			
OH-COT	10	10.00 ± 0.60	5.96	0.01	10.27 ± 0.57	2.18	2.70			
	20							20.31 ± 1.40	6.92	1.54
	40	39.53 ± 2.42	6.13	-1.18	41.74 ± 2.12	5.07	4.35			
	70	70.16 ± 6.74	9.60	0.23						
	100							98.91 ± 8.23	8.32	-1.09
	110	108.31 ± 9.62	8.89	-1.54	117.28 ± 7.21	6.15	6.62			
	140	138.17 ± 4.60	3.33	-1.31						
	170	166.98 ± 2.07	1.24	-1.78						
	180							181.88 ± 14.45	7.94	1.04
	200	205.86 ± 12.78	6.21	2.93	201.56 ± 10.68	5.30	0.78			

[a] Mean values ± standard deviation; CV – coefficient of variation; RE – relative error.

Table 4
Absolute recoveries (n = 3).

Analyte	Concentration (ng/mL)	Recovery[a] (%)
NIC	25	29.20 ± 13.60
	75	43.30 ± 3.73
	150	39.20 ± 2.22
COT	25	73.40 ± 8.00
	75	89.10 ± 10.47
	150	66.60 ± 4.16
OH-COT	25	81.70 ± 1.40
	75	80.30 ± 8.22
	150	92.80 ± 2.01

[a] Mean values ± standard deviation.

cycles of freezing and thawing of QC samples, after which the compounds under study were extracted using DSS (n = 3), obtaining CV of less than 6.76% and RE below ±9.98% when compared to fresh QCs analysed on the same day. Long-term stability (Table 5) was evaluated based on the application of spiked QCs (n = 3) in the spots, which were left at room temperature during the period of 1, 2, 3 and 4 weeks; after each of these periods, samples were extracted and analysed. The CV and RE obtained for the data resulting from the analysis after a period of 1–4 weeks were under 10.16% and ±12.0% for NIC, 16.06% and ±56.43% for COT and 13.26% and ±12.24% for OH-COT respectively; this shows that extracting simultaneously the three compounds after the same period of sample application time is not feasible without affecting the results. However, evaluating individually the CV and RE values for each of the weeks after sample application, it is possible to verify that only the values obtained in the 3rd and 4th week for COT exceeded the values indicated in the used guidelines. Thus, it was concluded that NIC and OH-COT remain stable after application in DSS for 4 weeks, while COT only remains stable for a period of 2 weeks after application in DSS. Leite et al. [33] have studied cotinine stability under different storage conditions for 90 days, and observed a good agreement between the levels for all studied conditions; average differences ranged from −11–24 ng/mL, and no buffer or preservatives were used. There are neither studies in the literature on the stability of nicotine in DSS for times longer than 4 weeks, nor studies where stabilizers or preservatives were used to increase the analytes' stability, as they exist in what concerns abused drugs. One of the most recent paper on this matter was published by Marchei et al. in 2020 [34], in which the stability of a number of psychoactive drugs in both neat and in buffered OF samples was evaluated. The authors concluded that several factors contributed to the stability in OF, namely type of analyte, presence of a stabilizer, duration of storage and temperature.

Table 5
Stability evaluation.

Analytes	Concentration (ng/mL)	Processed samples stability (n = 3)		Room temperature stability (n = 3)		Freeze/thaw stability (n = 3)		Long-term stability (n = 3)							
								Week 1		Week 2		Week 3		Week 4	
		CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)
NIC	20	2.84	-5.10	5.57	-8.72	0.46	-2.08	4.96	-3.14	6.40	0.52	6.87	4.23	10.16	-4.99
	100	0.45	-12.06	5.06	-6.79	4.78	-6.25	3.16	-10.75	2.48	-12.00	6.25	-8.28	7.19	-7.93
	180	2.41	-12.56	8.63	-5.39	1.08	-6.88	6.83	-6.60	4.64	-10.27	4.75	-11.18	3.76	-9.86
COT	20	3.43	-11.80	5.62	-6.24	3.12	-8.10	2.28	-0.32	7.58	-9.09	16.06	-24.82	10.70	-56.43
	100	2.39	-6.51	4.04	-7.34	2.55	-4.93	2.30	-10.97	3.91	-11.19	4.31	-5.86	4.82	-10.60
	180	3.27	-10.26	1.30	-10.87	5.15	-6.33	7.04	-5.53	1.38	-13.00	4.09	-12.61	5.07	-10.48
OH-COT	20	2.21	-8.54	4.43	-8.55	5.09	-5.02	4.73	2.64	9.80	1.18	10.19	-5.59	13.26	-2.11
	100	3.76	-5.54	5.97	-7.95	6.76	-7.06	8.58	-0.93	9.69	-1.97	1.72	-10.46	7.81	-6.72
	180	1.34	-4.84	3.89	-10.72	3.52	-9.98	6.67	-8.19	0.42	-2.24	5.65	-9.26	3.18	-12.24

CV – coefficient of variation; RE – relative error.

3.2.6. *Dilution integrity*

Dilution integrity is an important parameter for the analysis of real samples whose concentration value exceeds the upper limit of quantification (ULOQ) of the calibration curve of the developed method. Therefore, a dilution factor of 1:5 was tested at a concentration of 1000 ng/mL for the three compounds. A blank sample of OF spiked at that concentration was used in order to study this parameter and was then applied to DSS after dilution. The obtained CV and RE values were below 6.84% and ±8.46% respectively for the three compounds. The values are summarized in Table 6.

3.2.7. *Authentic samples*

After the complete optimization and validation of the extraction method, it was subsequently applied to samples of OF from volunteer students of the Universidade da Beira Interior (Table 7). After analysing the samples, it was found that the samples of eight of the volunteers must have come from active smokers, because the detected COT levels were above the cut-off of 10 ng/mL for OF indicated by Florescu et al. [6], which was further corroborated by the presence of high concentrations of NIC and OH-COT. The method was further validated by comparing the obtained concentrations to those obtained using neat sample and extraction by SPE, a procedure developed by Da Fonseca et al. [14] (Table 7). The use of OF samples as biological matrix for the determination of the degree of exposure has been growing over the last years mainly due to the easy availability of this type of matrix; however, the development of methods using low sample volumes presents a significant advantage. The study in real samples developed was limited in the number of samples given the current pandemic situation, requiring a larger set of samples of active or passive smokers to determine the accuracy of the method and draw further conclusions. Although other methods reach lower LLOQs than those herein obtained, all methods allow distinguishing between active and passive smokers. Kim et al. [35] also described in another article the usefulness of the ratio between COT and OH-COT for the identification of active consumers in real samples. Fig. 3 represents the chromatogram obtained from the analysis of one of those samples.

Table 6
Dilution integrity (n = 3).

Analytes	Concentration (ng/mL)	Dilution factor (1:5)		
		Measured[a] (ng/mL)	CV (%)	RE (%)
NIC	1000	953.81 ± 65.20	6.84	-4.62
COT	1000	915.36 ± 19.98	2.18	-8.46
OH-COT	1000	947.3 ± 42.78	4.52	-5.26

[a] Mean values ± standard deviation; CV – coefficient of variation; RE – relative error.

4. Conclusion

The method developed for the identification of tobacco biomarkers nicotine, cotinine and trans-3-hydroxycotinine in OF samples using DSS as extraction method by GC-MS/MS analysis was fully optimized and validated. The recovery and clean-up procedure accomplished allowed to achieve satisfactory extraction values ranging from 29.2% to 92.8%, given the sample volume used. The developed method represents a new extraction approach, allowing the use sample volumes as low as 100 μL, which can be useful when sample amount is limited. In addition, the herein described procedure is fast when compared to other methods using the same type of sample, allowing multiple extractions in shorter periods of time, thus constituting an advantage towards its applicability in the routine of an analytical laboratory. Furthermore, since it employs less solvent volumes, this methodology is more environmentally friendly, also contributing to a reduction of the costs per analysis. Considering that through the spitting collection methodology the volume obtained is often greater than necessary, this type of methodology can be considered as a first approach in the detection of such compounds, allowing a subsequent analysis with the remaining volume or to perform other complementary tests. Although the sample volume used was small, quantification limits of 10 ng/mL were obtained for all compounds, and it is possible to analyse the studied compounds for a period of 2 weeks after sample application to the spot without affecting the performance and reliability of the methodology. This is the first method developed that combines the extraction of these 3 compounds using DSS and GC-MS/MS. This method allows the distinction between passive and active consumers in real samples, being a very sensitive method useful in cases where there is a limited volume of OF sample. Furthermore, it enables various extraction parameters to be changed to adapt easily to a laboratory's routine.

Compliance with ethical standards

The present study was approved by the ethics committee Universidade da Beira Interior (CE-UBI-Pj-2021-046) and has been conducted according to ethical standards. The analysed authentic samples belonged to individuals who provided an informed consent for their use, and all analyses were carried out according to the ethical standards of the institution.

CRediT authorship contribution statement

Hernâni Marques: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Tiago Rosado:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Mário Barroso:** Conceptualization, Methodology, Writing – review & editing. **Luis Passarinha:** Conceptualization, Methodology, Writing –

Table 7
Authentic samples.

Sample number	DSS			SPE		
	Concentration (ng/mL)					
	NIC	COT	OH-COT	NIC	COT	OH-COT
1	195.29	74.12	68.43	194.60	74.81	67.52
2	Negative	Negative	Negative	Negative	Negative	Negative
3	Negative	Negative	Negative	Negative	Negative	Negative
4	46.03	43.45	22.51	46.94	38.87	23.55
5	Negative	Negative	Negative	Negative	Negative	Negative
6	Negative	Negative	Negative	Negative	Negative	Negative
7	13.71	Negative	Negative	14.71	Negative	Negative
8	179.54	42.14	38.76	174.55	41.84	39.20
9	34.13	17.93	13.81	33.79	18.48	13.20
10	19.48	Negative	Negative	18.64	Negative	Negative
11	44.11	16.28	14.12	42.26	15.16	13.38
12	47.12	95.47	36.07	48.21	92.75	33.58
13	101.11	105.65	55.90	105.54	107.50	53.56
14	22.83	Negative	Negative	23.12	Negative	Negative
15	118.51	66.40	55.28	122.54	63.85	51.58

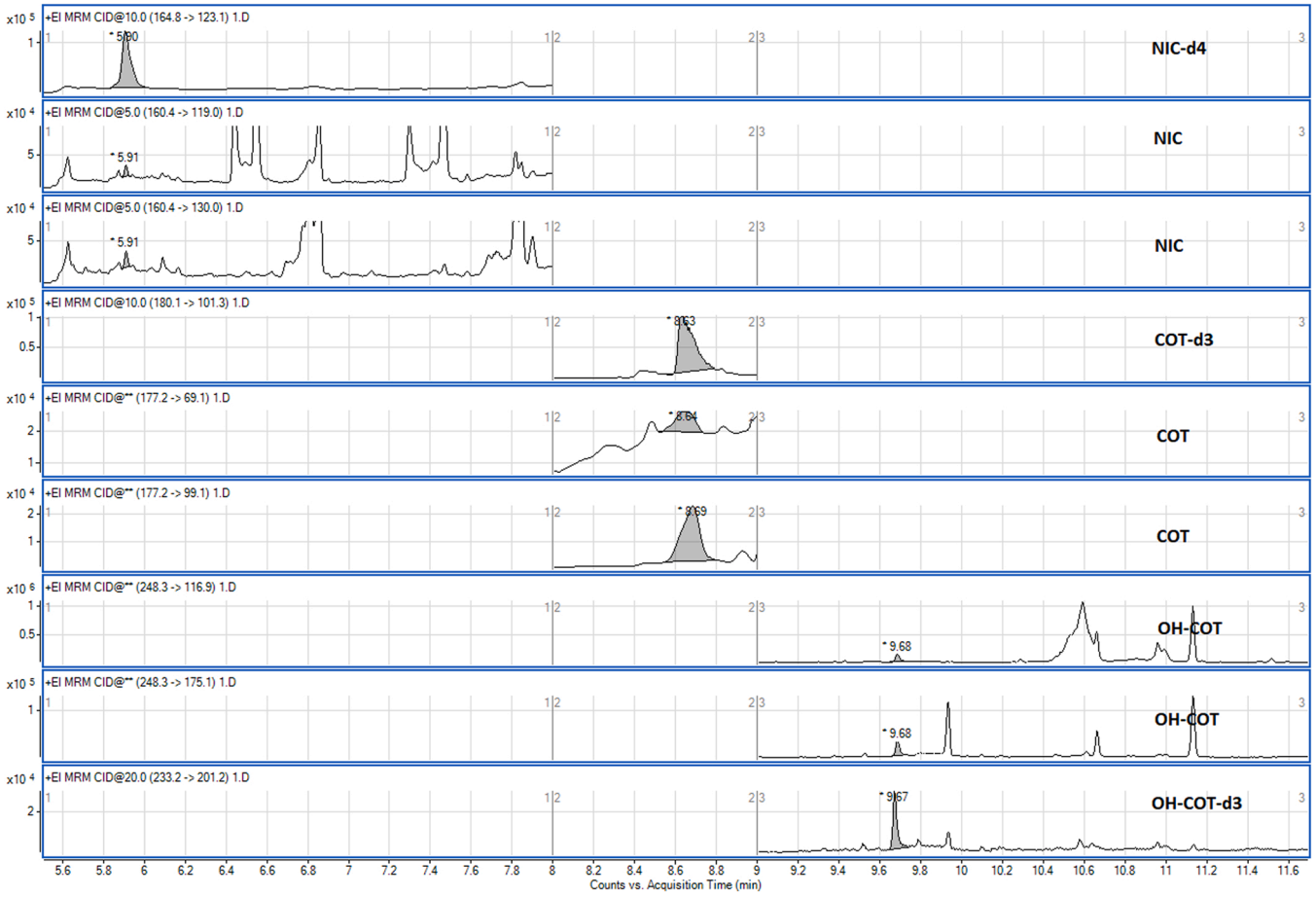

Fig. 3. Chromatogram obtained by analysis of one positive authentic sample (NIC: 195.29 ng/mL; COT: 74.12 ng/mL and OH-COT: 68.43 ng/mL).

review & editing, Supervision. **Eugenia Gallardo:** Conceptualization, Methodology, Writing – review & editing, Supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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