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Article

Single gas chromatography method with nitrogen phosphorus detector for urinary cotinine determination in passive and active smokers

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Nicotine is a major addictive compound in cigarettes and is rapidly and extensively metabolized to several metabolites in humans, including urinary cotinine, considered a biomarker due to its high concentration compared to other metabolites. The aim of this study was to develop a single method for determination of urinary cotinine, in active and passive smokers, by gas chromatography with a nitrogen phosphorus detector (GC-NPD). Urine (5.0 mL) was extracted with 1.0 mL of sodium hydroxide 5 mol L^{-1}, 5.0 mL of chloroform, and lidocaine used as the internal standard. Injection volume was 1 μL in GC-NPD. Limit of quantification was 10 ng mL^{-1}. Linearity was evaluated in the ranges 10-1000 ng mL^{-1} and 500-6000 ng mL^{-1}, with determination coefficients of 0.9986 and 0.9952, respectively. Intra- and inter-assay standard relative deviations were lower than 14.2 %, while inaccuracy (bias) was less than +11.9%. The efficiency of extraction was greater than 88.5%. Ruggedness was verified, according to Youden's test. Means of cotinine concentrations observed were 2,980 ng mL^{-1} for active smokers and 132 ng mL^{-1}, for passive smokers. The results revealed that satisfactory chromatographic separation between the analyte and interferents was obtained with a ZB-1 column. This method is reliable, precise, linear and presented ruggedness in the range evaluated. The results suggest that it can be applied in routine analysis for passive and active smokers, since it is able to quantify a wide range of cotinine concentrations in urine.

Uniterms: Gas chromatography/with nitrogen phosphorus detector. Urinary cotinine/determination. Urine/toxicological analysis. Smokers/Toxicology.

A nicotina é uma substância presente no cigarro capaz de causar dependência, sendo biotransformada em vários metabólitos nos seres humanos, dentre eles a cotinina urinária, que é considerada um indicador biológico de exposição à nicotina, devido a suas altas concentrações, comparado a outras matrizes. Assim, o objetivo deste estudo foi desenvolver um único método para determinação de cotinina urinária, em amostras de urina de fumantes ativos e passivos, através de cromatografia em fase gasosa com detector de nitrogênio- fósforo (CG-DNF). Para o preparo de amostras foram utilizados 5 mL de urina, 1 mL de hidróxido de sódio 5 mol L^{-1}, 5 mL de clorofórmio, tendo como padrão interno a lidocaína. Na faixa de concentrações de 10-1000 ng mL^{-1} e 500- 6000 ng mL^{-1}, o coeficiente de determinação foi 0,9986 e 0,9952, respectivamente e, o limite de quantificação foi 10 ng mL^{-1}. A precisão intra- e interensaio apresentou desvio padrão relativo (%) menor que 14,2% e a inexatidão foi menor que +11,9%, com uma eficiência de extração de 88,5%. O método apresentou robustez, de acordo com o teste de Youden. As concentrações médias de cotinina observadas foram 2980 ng mL^{-1}, para fumantes ativos e 132 ng mL^{-1}, para fumantes passivos. Os resultados sugerem que o método é confiável, preciso, linear e apresentou robustez, na faixa avaliada, podendo ser aplicado na rotina para análises de amostras de fumantes ativos e passivos, pois é capaz de quantificar uma ampla faixa de concentrações de cotinina urinária.

Unitermos: Cromatografia em fase gasosa/com detector de nitrogênio-fósforo/análise quantitativa. Cotinina urinária/determinação. Urina/análise toxicológica Fumantes/Toxicologia.

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INTRODUCTION

It is well known that tobacco smoke, which contains many toxic and tumorigenic compounds, is recognized as a major cause of mortality and morbidity (Richard, Eian, Graham, 2004). Tobacco is the single most preventable cause of death in the world today. By 2030, the death toll will exceed eight million a year (WHO, 2008).

More than 4000 compounds have been identified in tobacco smoke in which nicotine is the principal alkaloid (Hansen *et al.*, 2001). Nicotine, a major chemical found in all tobacco products, is present in both mainstream and sidestream tobacco smoke. In both smokers and non-smokers, nicotine enters the bloodstream when tobacco smoke is inhaled. It is then circulated to various body organs (Benowitz, 1996).

Cotinine is the major proximate metabolite of nicotine and has been widely used as a biomarker of tobacco smoke exposure (Kuo, Yang, Chiu, 2002) and offers several advantages over biochemical markers as an objective indicator of nicotine intake or confirmation of non-smoker status. Its concentrations are not influenced by other substances since it is a specific nicotine biomarker and concentrations within a given individual varies by only 15 to 20% over 24 h (Oddoze, Pauli, Pastor, 1998). Cotinine in urine accounted for less than 15% of total systemic dose of nicotine (Cope *et al*., 1996; Benowitz, 1996).

The half-life of cotinine and nicotine are approximately 19 and 2 h, respectively. Consequently, cotinine, because of its longer half-life, is currently the marker of choice for demonstrating cigarette smoke exposure (Haufroid, Lison, 1998). Its determination in biological fluids has aroused particular interest. These biochemical markers have been used to estimate active smoking behavior, to validate abstinence from smoking and to evaluate the levels and significance of environmental tobacco smoke (ETS) exposure (Benowitz, 1996). Urinary samples are more convenient to collect and urinary cotinine is a well-known biomarker of ETS (Jarvis *et al.*, 1987; Wall *et al.*, 1988; Kuo, Yang, Chiu, 2002).

Many methods have been proposed for the determination of cotinine in human urine. These methods use radioimmunoassay (Kuo, Yang, Chiu, 2002); high performance liquid chromatography (Zuccaro *et al.*, 1995; Oddoze, Pauli, Pastor, 1998; Tyrpien *et al.*, 2000; Abou-Qare, Abou-Donia, 2001; Doctor *et al.*, 2004; Kowalski *et al.*, 2007) and gas chromatography with nitrogen phosphorus detection (Kuo, Yang, Chiu, 2002; Moriya, Hashimoto, 2004). Liquid or gas chromatography coupled mass spectrometry is commonly employed to determine cotinine in passive smokers (Ji Jr. *et al.*, 1999; Meger *et al*., 2002; Man *et al*., 2006; Chadwick, Keevil, 2007). Other less sensitive detectors, such as flame ionization detector (FID), have also been used to quantify cotinine in urine of active and passive smokers. However, the sample preparation needed larger urine volume (25 mL) (Vacchino *et al.*, 2006).

Among these methods, the chromatographic techniques are more preferred than others because they are potentially more sensitive and more specific. This fact is due to sample enrichment by extraction prior to analysis and sample cleanup through chromatographic separation during analysis (Song *et al.*, 2005). In this context, many methods have been proposed for extraction of samples containing cotinine, among then, liquid-liquid extraction (LLE) (Shin *et al.*, 2002; Man *et al.*, 2006; Kowalski *et al.*, 2007) and solid phase extraction (SPE) (Oddoze, Pauli, Pastor, 1998; Moyer *et al.*, 2002; Xu, Iba, Weisel, 2004).

LLE presents the advantages of being a simple method of sample preparation that can use a great number of solvents, pure and available commercially, which supply a wide range of solubility and selectivity (Queiroz, Collins, Jardim, 2001).

The aim of this study was to develop a single method able to determine urinary cotinine, in active and passive smokers, by GC-NPD with a previous LLE.

MATERIAL AND METHODS

Reagents and standard solutions

Cotinine (COT) with approximately 98% purity (Lot no. 055k4053) and lidocaine (LID) with approximately 98% purity (Lot no. 162008), were purchased from Sigma-Aldrich Inc. (St. Louis, USA). Analytical grade isopropyl alcohol and chloroform were purchased from Vetec Ltda. (Rio de Janeiro, Brazil), methyl alcohol from Isofar Ltda. (Rio de Janeiro, Brazil) and sodium hydroxide from Labsynth Ltda. (São Paulo, Brazil).

Stock solutions of cotinine and lidocaine (internal standard) were prepared in isopropyl alcohol at 1 mg mL^{-1} and stored at -20°C, protected from light. These solutions were used for at least one month. Working solutions were freshly prepared in isopropyl alcohol immediately before analysis. Throughout the study, water was obtained from a Milli-Q system by Millipore (São Paulo, Brasil).

Instrumentation and chromatographic conditions

The GC system consisted of a GC model Clarus 400, equipped with NPD from Perkin Elmer® with Totalchrom Workstation® Software. Chromatographic analysis was performed in a ZB-1 column Phenomenex® (100%

polydimethylsiloxane, 30 m x 0.53 mm i.d.; 5 μm film thickness). Nitrogen was used as a gas carrier at a pressure of 4.3 psi. One microliter injection volume using splitless mode was injected manually, at an injector temperature of 260 °C. The oven temperature was programmed from 180 °C increasing by 30 °C/minute to 250 °C for 0.5 min, increasing by 15 °C/min to 259 °C for 0.1 min and then increasing by 0.1 °C/min to 260 °C. The detector temperature was 280 °C. The total run time was 13.4 min.

Urine samples

Cotinine-free urine, sourced from non-smokers not exposed to environmental nicotine, was used as a blank matrix to perform validation parameters. Urine samples from forty-one active smokers, ten passive smokers and eighteen unexposed non-smokers were used for application of this method. This study was approved by the Ethics Committee of Federal University of Alfenas (23087.002423/2008-12). An informed consent term was obtained from each volunteer.

All samples were collected in polyethylene urine containers and density was measured at each sampling time with a refractometer (Atago®) for standardization of cotinine levels. Samples were then frozen and stored at -20 °C until analysis.

Sample preparation

Before extraction, samples were thawed and equilibrated to room temperature. Urine (5.0 mL) was placed in a 15 mL centrifuge glass tube. Sodium hydroxide (5 mol L^{-1}, 1 mL), lidocaine as the internal standard (500 or 1000 μg mL^{-1}, 0.05 mL) and chloroform (5.0 mL) were added and the mixture was mixed for 15 minutes in a bench top shaker. After centrifugation for 15 minutes at 840 g, the aqueous layer was discarded and 4.5 mL of organic phase was transferred into a conical glass tube. The extract was evaporated in a stream of nitrogen, within a bath at 40 °C. The residue was reconstituted in 0.05 mL of isopropyl alcohol.

Method validation

Appropriate validation is necessary to ensure the suitability of analytical methods for the purpose (Kowalski *et al*., 2007), and the confidence parameters of the present method were assayed according to the Guidance for Industry of the FDA for Bioanalytical Method Validation (2001). The parameters evaluated in the present study were: linearity, limit of quantification, recovery, intra- and inter-assay precision, accuracy and ruggedness.

The linearity was studied in two linear ranges (10-1000 ng mL^{-1} and 500-6000 ng mL^{-1}) to minimize errors from a wide range of urinary cotinine concentrations. Urine samples were spiked with COT at concentrations 10; 100; 250; 500; 750; 1000 ng mL^{-1} and 500; 1000; 2000; 3000; 4000; 6000 ng mL^{-1}, and each treatment was assayed in six replicates. Concentration of internal standard was adjusted according to the range evaluated (500 μg mL^{-1}, for 10-1000 ng mL^{-1} of cotinine or 1000 μg mL^{-1} for 500-6000 ng mL^{-1}, of cotinine).

These samples were analyzed according to the item sample preparation. Calibration curves were constructed by plotting peak area ratios of analyte and its internal standard versus original concentrations, and evaluated by linear least square regression analysis.

The limit of quantification was estimated after successive dilutions of cotinine solutions, until obtention of a concentration in which the peak area was ten times the signal-to-noise ratio (S/N= 10), provided by the blank extract (N= background noise).

Recovery was determined by three replicate analyses of samples after the additional spiking of a known mass of the analyte, in the non-contaminated samples, at three levels. The results were compared with those obtained when the analyte was spiked after the clean-up procedure of the sample, at the same levels.

Intra- assay precision was assessed using three replicates of each concentration of linear range, on the same day. Inter-assay precision was evaluated for three replicates analyzed on separate days (n=6). The results were expressed as a percentage of relative standard deviation (% RSD).

Accuracy was established by spiking urine samples with 30, 500, 1000, 3000 and 6000 ng mL^{-1} of COT (n= 3/ concentration). After extraction and chromatographic analysis, results were compared to the theoretical added values.

Ruggedness was evaluated through the Youden approach, which is based on a fractional factorial design according to the Official Journal of the European Communities (2002). For this purpose, eight determinations were carried out, combining the nominal and with-variation parameters. The variables evaluated are described in Table I. The experiments are described in Table II, and Table III contains the formula used to evaluate the variation effect.

RESULTS

Figure 1 shows the urinary chromatograms of passive, active and non-smoker volunteers obtained with the

TABLE I - Robustness parameters

Parameters	Nominal (+)	Variation (-)
Sample volume (mL)	5.0	4.0
NaOH concentration (mol L^{-1})	5.0	4.5
Agitation in vortex	Yes	no
Time in bench top shaker (min)	15	10
Time in centrifuge (min)	15	10
Detector temperature (°C)	280	275
Gas carrier pressure (psi)	4.3	4.2

TABLE II - Combinations assayed for nominal or variable parameters

Parameters	Combination assayed							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample volume (mL)	+	+	+	+	-	-	-	-
NaOH concentration (mol L^{-1})	+	+	-	-	+	+	-	-
Agitation in vortex	+	-	+	-	+	-	+	-
Time in bench top shaker (min)	+	+	-	-	-	-	+	+
Time in centrifuge (min)	+	-	+	-	-	+	-	+
Detector temperature (°C)	+	-	-	+	+	-	-	+
Gas carrier pressure (psi)	+	-	-	+	-	+	+	-
Results	a	b	c	D	e	f	g	h

TABLE III - Variation effects evaluation

Factor	Formula for variation effect
Sample volume	(a+b+c+d)/4 – (e+f+g+h)/4
NaOH concentration	(a+b+e+f)/4 – (c+d+g+h)/4
Agitation in vortex	(a+c+e+g)/4 – (b+d+f+h)/4
Time in bench top shaker	(a+b+g+h)/4 – (c+d+e+f)/4
Time in centrifuge	(a+c+f+h)/4 – (b+d+e+g)/4
Detector temperature	(a+d+e+h)/4 – (b+c+f+g)/4
Gas carrier pressure	(a+d+f+g)/4 – (b+c+e+h)/4

validated method. Lidocaine was used as the internal standard at concentrations of 500 µg mL^{-1} in a) non smokers; and at 1000 µg mL^{-1} in b) and c), passive and active smokers, respectively. The method revealed satisfactory chromatographic separation between the analyte and interferents, I requiring only a short time for chromatography (less than 14 min) and proved suitable for determining the cotinine in urine in routine analysis.

LOQ is the lowest concentration of an analyte on a calibration curve, and for this method the concentration 10 ng mL^{-1} resulted in a signal-to-noise ratio of 10, and

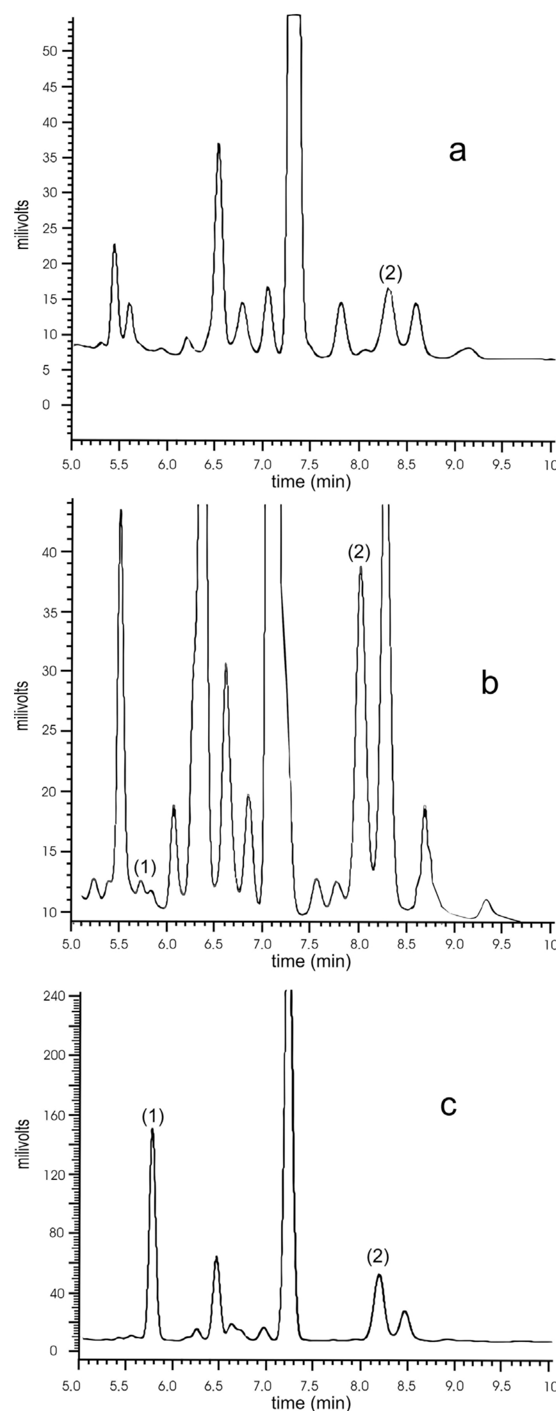

FIGURE 1 - Chromatograms after LLE of urine: a) non smokers; b) passive smokers; c) active smokers. (1) Cotinine: rt 5.7 min; k' 2.0 min; α 1.25 (calculated between cotinine and immediately before peak) (2) Lidocaine (IS): rt 8.2 min; k' 3.3 min; α 1.1 (calculated between cotinine and immediately after peak). Chromatographic conditions: ZB-1 column, injector temperature at 260 °C, the oven temperature programmed with initial temperature of 180 °C, ramp 1: 30 °C/ minute to 250 °C for 0.5 min; ramp 2: 15 °C/ min to 259 °C for 0.1 min and ramp 3: 0.1 °C/min to 260°C, detector temperature at 280 °C. Nitrogen as gas carrier at 4.3 psi.

TABLE IV - Linearity of method

COTININE		
Linear Range (ng mL^{-1})	**10-1000**	**500-6000**
Slope (a)	0.0036 ± 0.0002	0.0012 ± 7.53 x10^{-5}
Intercept (b)	0.0936 ± 0.061	0.2979 ± 0.203
Determination Coefficient (R^2)	0.9986	0.9952

TABLE V - Recovery and accuracy for the analysis of cotinine in urine, by CG-NPD after ELL

COTININE		
Urine concentration (ng mL^{-1})	**Relative Recovery (mean, %)**	**Accuracy (bias, %)**
30	102.2	+11.9
500	99.2	+0.6
1000	88.5	+2.9
3000	104.7	+7.6
6000	108.2	-1.7
Mean	**100.6**	**+4.3**

TABLE VI - Intra- and inter-assay precision for the analysis of cotinine in urine, by CG-NPD after ELL.

Urine concentration (ng mL^{-1})	Intra-assay(n=3) RSD (%)	Inter-assay (n=6, 2 days) RSD (%)
10	3.1	14.2
500	4.5	3.4
1000	6.3	4.6
3000	8.5	2.1
6000	3.9	6.7

the % relative standard deviation (n=5) obtained was 8.99%. Tables IV, V and VI show the results of the method validation.

Ruggedness was assessed using the Youden approach. Eight determinations were made using a combination of the factors with variations (see Table I). Variation influence was evaluated by comparing the values obtained by the formulas in Table III, with those values obtained by the proposed method (nominal parameters). Variations of more that two standard deviations from the result obtained using the proposed method (nominal parameters) were considered to indicate that a parameter caused alteration in the method. Ruggedness was demonstrated, since no statistically significant difference was observed between the nominal parameters and the values obtained with the variation described in Table II.

The values for cotinine concentrations observed in urine were 2,980 ± 2,160 ng mL^{-1}, for active smokers and 132.00 ± 80 ng mL^{-1}, for passive smokers.

DISCUSSION

Urine is the preferred specimen over plasma and saliva because it is much easier to obtain, particularly in epidemic studies (Hariharan, Vannoord, 1991). The advantages of using urine in the investigations included lower viscosity and ease of handling compared with saliva or blood, as well as constituting a relatively nonintrusive sample collection/ donation method without an occupational health risk (Tuomi, Johnsson, Reijula, 1999; Hagan, Ramos Jr., Jacob III, 2002). Since urinary cotinine is considered a biomarker of exposure to tobacco, the objective of this study was to develop a single method able to determine urinary cotinine in active and passive smokers. The literature describes this determination but the sample preparation required a high urine volume (25 mL) (Vacchino *et al.*, 2006). The present study used 5.0 mL urine.

Among available sample-preparation techniques, liquid-liquid extraction is an efficient clean-up procedure

for removing unwanted substances from urine matrix and can also be used to concentrate the analyte (Kowalski *et al.*, 2007).

Due to its pKa (4.5), cotinine is found in greater quantity in non-ionized form in the blood (pH 7.4) and the free base form is poorly soluble in lipids, showing a low rate of distribution to tissues, which partly explains their prolonged existence in the blood. Another factor that contributes to its prolonged half-life is the low rate of renal excretion in relation to nicotine (Feyerabend, Russell, 1980). Therefore, because of its longer half-life, cotinine is often the marker of choice to demonstrate exposure to cigarette smoke (Chadwick, Keevil, 2007).

A high pH was used in order to co-extract cotinine and lidocaine, with satisfactory extraction efficiency, since the p*K*a of cotinine was reported to be < 5.0 (Beckett, Gorrod, 1972) with basic characteristics. In this study, COT was extracted efficiently in chloroform, after alkalization with sodium hydroxide 5 mol L^{-1}.

Linearity was determined for COT using a pool of blank urine that was spiked with the analyte and the internal standard. Peak area ratios (COT/IS) and analyte concentrations were found to be linear in the range evaluated. The least-squares linear regression was used to determine the slope and intercept. Man *et al.* (2006), using gas chromatography and mass spectrometry, obtained a linear range from 0.5 to 5000 ng mL^{-1}.

The present study was proposed for determination of urinary cotinine of active, passive and non-smokers. The limit of quantification obtained (10 ng mL^{-1}) was sufficient for monitoring passive smoking, because according to Kolonen & Pahukainen (1991), urinary cotinine levels in passive smokers are typically less than 100 ng mL^{-1}, serving as a cut-off point to verify tobacco-free status. Other authors have suggested a cut-point of 50 ng mL^{-1} for urinary cotinine as a means to distinguish smokers from non-smokers (Haufroid, Lison, 1998; Song *et al*., 2005). Besides, zero cotinine concentration is generally observed in urine of non-smokers not exposed to environmental tobacco smoke.

In the present study with a nitrogen phosphorus detector, the method developed was able to distinguish different groups exposed to tobacco smoke, which can be considered an advantage in routine analysis.

Intra- and inter-assay precision were less than 8.5% and 14.2%, respectively, and this is considered satisfactory according to FDA guidelines (2001). This parameter should not exceed 15% of the RSD, except for the LOQ, where it should not exceed 20% of the RSD.

Relative recovery of COT was in the range of 88.5% and 108.2% with an accuracy in the range of -1.7 % and +11.9%. The guidance of validation for bioanalytical methods of the FDA (2001) establishes that recovery of the analyte need not be 100%, where lower values are acceptable provided the recovery offers precision and accuracy. Other studies (Voncken, Schepers, Schafer, 1989; Hagan *et al*., 1997; Ji Jr *et al*., 1999; Shin *et al*., 2002; Chadwick, Keevil, 2007) using liquid-liquid extraction as the sample preparation technique obtained recoveries of between 81 and 112%.

Ruggedness was evaluated through the Youden approach and allowed the conclusion that small variations did not affect the method under tested conditions, since the results did not differ significantly across the conditions evaluated.

Cotinine concentrations observed in this study were 2,980 ± 2,160 ng mL^{-1}, for active smokers and 132.00 ± 80 ng mL^{-1}, for passive smokers, in line with urinary cotinine concentrations described in the literature (Voncken, Schepers, Schafer, 1989; Oddoze, Pauli, Pastor, 1998; Moyer *et al*., 2002; Ji Jr *et al.*, 1999; Kuo, Yang, Chiu, 2002; Man *et al.*, 2006; Chadwick, Keevil, 2007) of 1,560 to 6,680 ng mL^{-1}, for active smokers and approximately 50 ng mL^{-1}, for passive smokers.

CONCLUSION

A fast and simple GC-NPD method was developed and validated for urinary cotinine analysis using a low sample volume. The results revealed that satisfactory chromatographic separation between the analyte and interferents was obtained with a ZB-1 column. This method is reliable, precise, linear and presented ruggedness over the range evaluated. The results suggest that it can be applied in routine analysis to passive and active smokers, since it is able to quantify a wide range of cotinine concentrations in urine.

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