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Research Article

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Development and Validation of a Quantification Method of Cotinine in Urine Using Two Innovative Technologies: Supported Liquid Extraction and QDa Detection

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Abstract

Objective: Cotinine is the best biomarker of tobacco smoke exposure because of its long half-life. Several methods are developed to quantify cotinine in biological fluids, including high performance liquid chromatography. This method requires an extraction step in order to clean-up the sample and concentrate the analyte of interest.

The aim of this study was to optimise the extraction step and to develop an easy, reproducible and specific method to measure cotinine in urine.

Methods: An extraction of neutrals was chosen and performed by Supported Liquid Extraction (SLE). SLE consists in liquid-liquid extraction in the presence of a sorbent enabling efficient extraction with less organic solvent and without any emulsion formation. Urine was basified by treatment with NH_4OH in order to neutralise cotinine before loading on SLE plate. After drying, neutrals were eluted with a mix containing dichloromethane and isopropyl alcohol. Solvent was then evaporated and samples were reconstituted with water.

Detection of cotinine was performed by mass detection using a QDa detector after UHPLC separation with a C18 column at a flow rate of 0.4 ml/min. A gradient elution of H_2O +0.1% NH_4OH and CH_3CN was used. The method was validated based on linearity, precision, recovery and limits of detection and quantification.

Results: The range of linearity was 0.001 µg/ml -5 µg/ml with a determination coefficient of 0.997. The precision was evaluated by the Relative Standard Deviation (RSD) for intra- and inter-assay and was below 5% and 10% respectively. 3 levels of concentration were tested to assess the recovery rate which was consistent and higher than 96%.

Conclusion: Cotinine concentration can be measured in urine by SLE extraction and UHPLC-QDa detection. This method is easy, reproducible and allows quantification of low concentrations. It is a good solution to assess patients' tobacco smoke exposure in medical laboratory.

Keywords: Cotinine; tobacco smoke exposure; mass detection; QDa; supported liquid extraction; ultra-high performance liquid chromatography; quantification method development

Abbreviations

LOD: Limit Of Detection; LOQ: Limit Of Quantification; MS: Mass Spectrometry; RSD: Relative Standard Deviation; SD: Standard Deviation; SLE: Supported Liquid Extraction; SPE: Solid Phase Extraction; UHPLC: Ultra-High Performance Liquid Chromatography

Introduction

With more than 7 million deaths per year in the world, tobacco smoking is the most preventable cause of death [1]. Some biological parameters allow evaluating tobacco smoke exposure, especially nicotine, a specific alkaloid that is the most responsible of the tobacco physical dependence [2].

Nicotine is mainly metabolized into cotinine that is widely used as biomarker in various body fluids due to its longer half-life than the one of nicotine [3]. It allows to distinguish tobacco users from non-users [3,4]. Cotinine can be quantified by different methods, including chromatography, considered the reference method, but that requires pre-treatments such as liquid-liquid extraction, Solid-Phase Extraction (SPE), acid precipitation, centrifugation and filtration. These methods can be time-consuming, expensive or complex [5]. Chromatography is often coupled with ultraviolet detector, single quadrupole mass spectrometry (MS) or tandem MS/MS that is more sensitive and selective [6]. However, it requires expensive laboratory instrumentation.

The aim of this study is to develop an easy, rapid and sensitive method to measure cotinine in urine by ultra-high performance liquid chromatography (UHPLC) connected with a mass detector. The mass detector is more intuitive and less expensive than a mass spectrometer while benefiting from the mass spectral information [7]. The pre-treatment used in this project to clean-up the sample is a Supported Liquid Extraction (SLE), a relatively unknown alternative approach to liquid-liquid extraction. Samples are loaded on a support (highly purified diatomaceous earth or synthetic), the aqueous phase is adsorbed onto the surface, and then neutral analytes are eluted by an organic solvent. The support allows an intimate contact between the aqueous and organic phases, possibly leading to analyte recoveries higher than the ones obtained with liquid-liquid extraction [8,9].

Materials and Methods

Solutions preparation

Cotinine solution: A 10 mg/ml solution of cotinine was prepared by diluting 19.5 mg of cotinine (Sigma-Aldrich, lot 120M4046V, Saint-Louis, USA) in 1.95 ml of water. This solution was then diluted 1:100 by making up 1 ml of solution to 100 ml with water. The final 10 µg/ml working solution was obtained after a 1:10 dilution in water.

Internal standard: A 1 g/L solution of 2-phenylimidazole was prepared by diluting 100 mg of 2-phenylimidazole (Acros Organics, lot

A0283801, Molinons, France) in 100 ml of methanol (Biosolve, lot 1328761, Dieuze, France). The solution was then diluted 3:1000 in water to obtain the 3 mg/L working solution.

Calibrators and controls preparation

Five calibrators (0.05, 0.25, 1.25, 2.5 and 5 µg/ml) and two control solutions (0.25 and 2.5 µg/ml) were prepared from the 10 µg/ml working solution of cotinine by dilutions in water. A quadratic calibration curve was performed each day of analysis with a weighting factor 1/X.

Samples pre-treatment

The patients' urines were frozen immediately after collection and centrifuged at 2150 g for 5 min (Heraeus multifuge 1S, Thermo Scientific, USA) after the thawing at room temperature the day of analysis. 200 µl of urine, calibrator or control were mixed with 10 µl of 3 mg/L internal standard, 50 µl NH_4OH 1M (Sigma-Aldrich, lot BCBX5442, Saint-Louis, USA) and 40 µl of water. Samples were then thoroughly vortex-mixed.

Supported Liquid Extraction (SLE)

Pre-treated samples were loaded on a 400 µl Strata DE 96-Well plate (Phenomenex, lot M01129, Torrance, California) and a drying time of 6 min was waited. The elution was performed with 3 x 600 µl of dichloromethane/isopropyl alcohol 95/5 (v/v) (Biosolve, lot 10042731 and lot 1238751 respectively, Dieuze, France). The collected eluate was then evaporated to dryness at 60°C for 75 min (SPD121P-230, Thermo Scientific, USA) and reconstituted in 100 µl of water for 10 min on an orbital shaker.

Chromatographic conditions

Analyses were performed with an ultra-high performance liquid chromatography (UHPLC) system coupled with a QDa detector (Acquity, Waters, Milford, USA). Results were acquired and processed by Empower 3 (Waters). 0.3 µl of extracted samples were injected on a reverse phase column (Acquity UPLC BEH C18 130 Å 1.7 µm 2.1 mm X 100 mm, lot 0327382972, Waters) connected to a pre-column (Acquity UPLC BEH C18 VanGuard Pre-column 130 Å 1.7 µm 2.1 mm X 5 mm, lot 0311380081, Waters) and heated at 40°C. The mobile phase consisted of 0.1% NH_4OH in water (v/v) (Sigma-Aldrich, lot BCBX5442, Saint-Louis, USA) and acetonitrile (Biosolve, lot 1303351, Dieuze, France) and was used in gradient at a flow rate of 0.4 ml/min. The run started with 5% of acetonitrile for 2 min, acetronitrile was then gradually increased to 100% in 4 min. These conditions were kept for 1.5 min before reequilibrating the column for 2.5 min. The QDa detector was configured to positively ionise samples with a capillary voltage of 1.5 kV and a source temperature of 600°C. Cotinine (m/z: 177.3) and the internal standard (m/z: 145.1) were detected by single ion recording with a cone voltage of 15 V.

Method validation

Linearity: The linearity was evaluated from two-fold serial dilutions in water of a 5 µg/ml solution of cotinine. 200 µl of each dilution (n=13) underwent the extraction step and were analysed just as the samples.

Precision: The precision was evaluated by 20 measurements of two cotinine concentration levels (0.25 and 2.5 µg/ml) within-day (repeatability) and between-day (intermediate precision). These solutions corresponded to control solutions used each analysis. The precision was expressed by the Relative Standard Deviation (RSD) between the measurements.

Accuracy: The accuracy was determined by calculating the bias between the mean of results obtained in the inter-assay experiment and the target value. It was calculated for two levels of concentration (0.25 and 2.5 µg/ml).

$$Bias(\%) = \frac{C_{mean} - C_{target}}{C_{target}} * 100$$

Limits of detection and quantification: Limits of detection (LOD) and quantification (LOQ) were estimated from the background noise at the retention time of cotinine in 10 blank injections (purified water). LOD and LOQ were calculated as follows: LOD=mean+3 * standard deviation (SD) and LOQ=mean+10 * SD

Recovery: The percentage of recovery was assessed by spiking urine with small amounts of cotinine (0.2, 0.5 and 1 µg/ml). The samples were extracted and then injected in triplicate. The mean of the injections was calculated and compared to the expected value.

$$\text{Rec}oevry(\%) = 100 - \left(\frac{C_{\text{expected}} - C_{mean}}{C_{\text{expected}}}\right) * 100$$

Carry-over: Carry-over was evaluated by 3 injections of a high concentrated solution of cotinine (H) (5 µg/ml) followed by 3 injections of a low concentrated solution of cotinine (L) (0.05 µg/ml).

$$Carry - over(\%) = \frac{L_1 - L_3}{meanH - L_3} * 100$$

Robustness: Small variations in method parameters were performed in order to test the robustness of the method. The extraction and the injection of 5 calibrators, 2 controls and 3 urine samples were carried out with:

- 3 SLE eluant compositions: dichloromethane/isopropyl alcohol 95.5/4.5, 95/5 and 94.5/5.5 (v/v).

- 3 evaporation durations: 70, 75 and 80 min.

- 3 reconstitution durations: 5, 10 and 15 min.

- 3 mobile phases (variation of the aqueous phase): 0.098, 0.1 and 0.102% NH_4OH

Results

Chromatographic conditions determination

The examination of cotinine and 2-phenylimidazole (internal standard) structures suggested a reverse phase analysis given that molecules were both hydrophobic (log P: cotinine 0.21, 2-phenylimidazole 1.88), thereby a C18 column was chosen. Separation could be achieved according to two approaches: at acidic pH with both molecules positively charged or at alkaline pH with neutral molecules. The first approach involved an attention to the residual silanol groups (pKa: 3.5) to avoid interactions with the positive charge of cotinine and 2-phenylimidazole. A column with a polar modified particle surface (Luna Omega 1.6 µm Polar C18, Phenomenex) was tested to improve the retention of the analytes by interactions with polar functional groups. 10 mM ammonium formate+formic acid (pH 3) was used in

gradient with acetonitrile+0.1% formic acid to elute analytes. Peaks tailing was observed, despite a low pH to avoid secondary interactions (Figure 1A). Therefore, the second approach was chosen to limit tailing and separation was performed on a BEH C18 1.7 µm column (Waters) with an alkaline mobile phase (0.1% NH_4OH in water and acetonitrile in gradient) (Figure 1B).

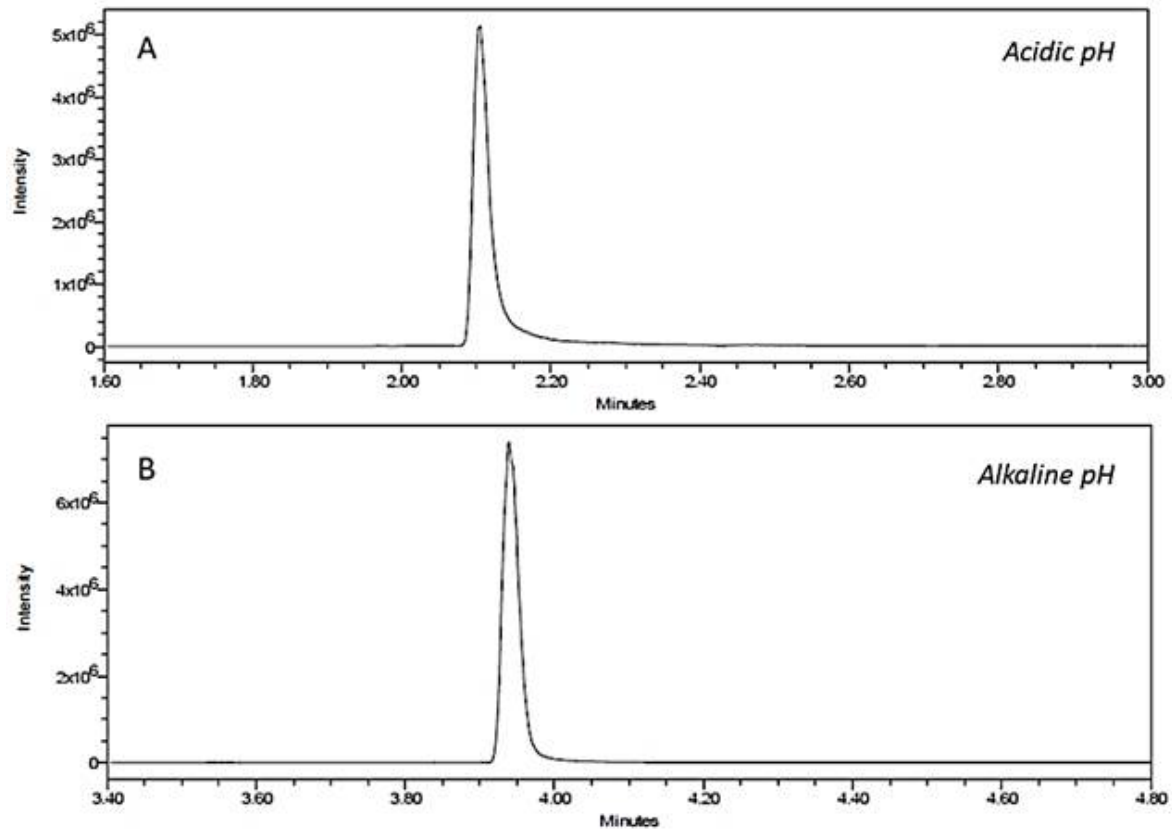

Figure 1: Cotinine chromatogram obtained with an (A) acidic mobile phase or with an (B) alkaline mobile phase.

QDa settings were optimised by varying the cone voltage (10, 15, 20 and 30 V), the positive capillary voltage (0.8, 1 and 1.5 kV) and the source temperature (400, 500 and 600°C). Parameters that gave the greatest peak area for both cotinine and internal standard were chosen: cone voltage=15 V, capillary voltage=1.5 kV and source temperature=600°C.

Extraction method selection

Different extraction methods were tested: liquid-liquid extraction with chloroform, Solid Phase Extraction (SPE) (Strata-X-C, Phenomenex and Oasis MCX, Waters) and Supported Liquid Extraction (SLE) (Novum and Strata DE, Phenomenex). The liquid-liquid extraction showed a cotinine recovery varying from 70% to 88% that was not consistent for all tested concentrations (Table 1). Moreover, this method required the use of important volumes of toxic solvents and involved results variation due to the phases separation difficulty. The other methods were tested on 96 well plates allowing the easy treatment of many samples with smaller volumes of toxic solvents and without any delicate step as the phases separation. The two SPE plates tested showed poor recovery rates (Table 1), requiring a better optimisation of the extraction steps. However, SLE was prefered thanks to its easy and quick use: no conditionning, equilibrating and washing steps are required. Strata-DE plate was finally chosen and allowed a good recovery rate and the quantification of low concentrations (Table 1).

	Liquid-liquid extraction	SPE 96 Well Plate		SLE 96 Well Plate	
		Strata-X-C	Oasis MCX	Novum	Strata DE
Recovery (%)	70-88	18	15	66	96
Lower limit of the linearity range (µg/ml)	-	-	-	0.039	0.001

Table 1: Cotinine recovery and lower limit of the linearity range for the different tested extraction methods.

SLE optimization

SLE does not required a lot of optimisation, only the sample pre-treatment and the elution solvent can be modified. The sample dilution before the loading on the plate was tested with water and with NH_4OH 0.5 M and cotinine recovery was 2 times bigger when diluting with NH_4OH. The volume of diluent was chosen to comply with the maximal loading volume (300 µl). Elution was also performed with

ethyl acetate before selecting dichloromethane/isopropyl alcohol 95/5 as eluent to achieve the best recovery rate.

Validation of the method

The final developed method allowed to measure cotinine corrected by an internal standard (Figure 2). A linear relationship was observed between the measured cotinine concentration and the expected cotinine concentration over a range from 0.001 µg/ml to 5 µg/ml (Figure 3). The regression equation was y=0.876x+0.0416 with r^2=0.997. The within-day variation was <3.3% and the between-day variation was <8.8% (Table 2). The bias between the mean of between-day results and the target value was <6.3%. LOD and LOQ were both calculated at 0.000 µg/ml based on the background noise. Therefore the LOQ was defined as the lower limit of the linearity range (0.001 µg/ml). The recovery was consistent for all concentrations tested and was >96%. No carry-over (0%) was observed for the injection of 3 low concentration samples after 3 high concentration samples. All variations tested in method parameters showed the robustness of the method given that results variation was <7.4% which is lower than the between-day variation.

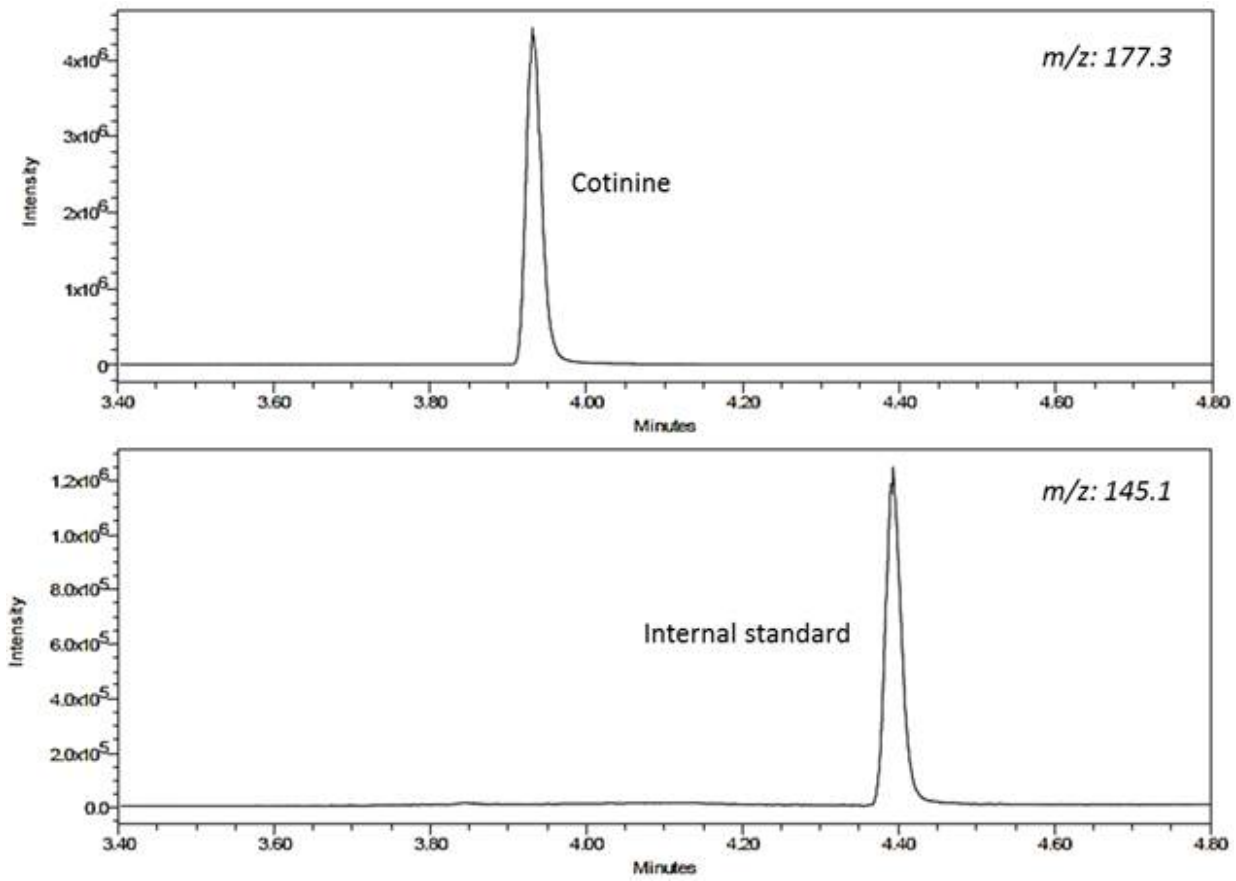

Figure 2: Chromatograms of cotinine and internal standard.

	Within-day precision (n=20)			Between-day precision (n=20)		
	Mean (µg/ml)	SD (µg/ml)	RSD (%)	Mean (µg/ml)	SD (µg/ml)	RSD (%)
Low concentration (0.25 µg/ml)	0.294	0.005	1.561	0.266	0.023	8.755
High concentration	2.739	0.091	3.31	2.503	0.128	5.102

Table 2: Within-day and between-day precision of the method.

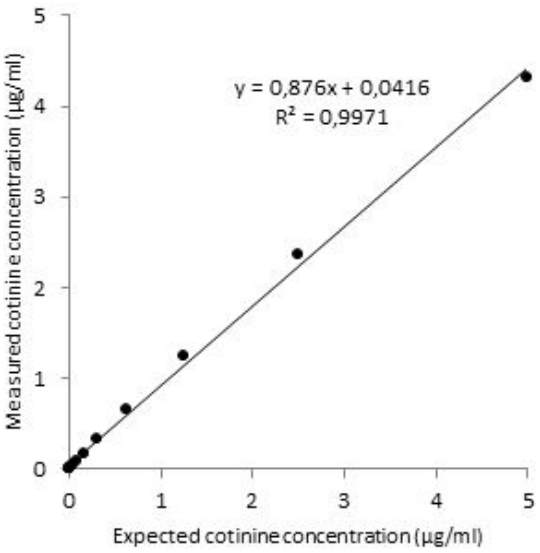

Figure 3: Linearity of the method over a range from 0.001µg/ml to 5 µg/ml.

Discussion

The method developed in this study allows cotinine quantification in urine by a rapid and easy extraction step following by a mass detection with a QDa detector. Cotinine is a biomarker commonly used to distinguish tobacco users from non users and evaluate the extent of tobacco smoke exposure [3]. This analyte can be measured in several biological matrices (urine, blood, saliva, hair, etc.) but urine was selected in this study because its collection is non-invasive and most of all, the cotinine concentration is four to six times higher in urine than in blood or saliva. It allows to detect low concentration exposure [10].

Various techniques are showed in the literature to quantify cotinine including immunoassays, gas chromatography and high performance liquid chromatography [2,10-15]. Chromatography is usually prefered because of its good specificity. Currently, chromatography is increasingly coupled with mass spectrometry to achieve high specificity and sensitivity of analysis [16]. However, the cost of this equipment and the required training can curb laboratories. An alternative is the use of a mass detector (QDa, Waters) that is intuitive and considerably less expensive while benefiting from the specifity of the mass [7].

The quantification range obtained with this described method are satisfactory compared with the one obtained by mass spectrometry in the literature. The linear range for this method was 0.001-5 µg/ml, allowing the quantification of high concentrations as well as low concentrations as in the passive exposure case (0.005-0.02 µg/ml [17]). The linearity range obtained with HPLC-tandem mass spectrometry in urine is varying depending of the studies, for example, 0.002-6 µg/ml [16], 0.01-3 µg/ml [18] or 0.0002-4 µg/ml [19]. The other chromatographic specifications were also suitable to validate the developed method: the precision is <15%, the bias is <15%, the recovery is >70% and the carry-over is <0.05% [20,21].

Several extraction techniques were tested before choosing the SLE which consists in liquid-liquid extraction in the presence of a sorbent enabling an efficient extraction with less organic solvent and without any emulsion formation [8,9]. This method is easier and faster to implement than SPE and provided a good cotinine recovery in this study. Urine samples were basified before the loading on the SLE plate in order to neutralise cotinine that is then eluted with the organic solvent. For this reason, the recovery rate was better when pre-diluting samples with NH_4OH rather than with water.

The extraction step implies the use of an internal standard to take the cotinine loss into account. Usually, analyses by mass spectrometry resort to deuterated internal standards that have the same extraction recovery, ionisation rate and retention time as the molecule of interest [22]. In the case of cotinine extraction, cotinine-d3 can be used as internal standard [16,18,19]. Nevertheless, the cost of this molecule is high. This study used 2-phenylimidazol as internal standard that has similarities of structure with cotinine and whose the price is approximately 3 times lower than the one of cotinine-d3. The intermediate precision of the cotinine quantification with this developed method showed a maximal variation of 8.8% while the variation observed for the cotinine quantification by HPLC-tandem mass spectrometry is for example 3.6% [16], 4.4% [18] or 7.9% [19]. The variation obtained with this developed method is possibly lightly higher, but is anyway <15%, the maximal recommended intermediate precision in HPLC methods [20], indicating that 2-phenylimidazol is suitable as internal standard.

This study offers the prospect of developing quantification methods of other tobacco biomarkers by UHPLC analysis, such as trans-3'-hydroxycotinine, anabasine and anatabine, with mass detection after an SLE step [23,24].

Conclusion

A quantification method of cotinine in urine was developed by ultra-high performance liquid chromatography coupled with a mass detector. This method is a good alternative to the detection by a mass spectrometer and allows laboratories to assess easily patients' tobacco smoke exposure without investing in expensive instrumentation. The pre-treatment was performed by Supported Liquid Extraction that is easy and quick. The method described is reproducible and allows quantification of low and high concentrations of cotinine. Therefore, it can be used both for assessing passive and active smoking.

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