XIX IMEKO World Congress
Fundamental and Applied Metrology
September 6–11, 2009, Lisbon, Portugal

IMPLEMENTATION, VALIDATION AND APPLICATION OF A METHOD OF EVALUATION OF URINARY 1-HYDROXYPYRENE AS AN INDICATOR OF HUMAN EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS IN RIO DE JANEIRO STATE, BRAZIL

Eliane Cristina Pires do Rego [1], *Annibal Duarte Pereira Netto* [2]

[1] INMETRO, Directorate of Scientific and Industrial Metrology, Chemical Metrology Division, Duque de Caxias, RJ, Brazil, ecrego@inmetro.gov.br
[2] Departament of Analytical Chemistry, Institute of Chemistry, Federal Fluminense University, Niterói, RJ, Brazil, annibal@vm.uff.br

Abstract – Epidemiological studies have shown that the exposure to polycyclic aromatic hydrocarbons (PAHs) increases the risk of several kinds of human cancers. In this way the use of biomarkers to evaluate PAH exposure is of interest. Urinary 1-hydroxypyrene (1-OHPy) that is the major metabolite of pyrene has been considered as a suitable biomarker of exposure to PAHs as a group, but there are few data about urinary 1-OHPy in Brazil. The objectives of this study were the implementation and validation of a method for the evaluation of urinary 1-OHPy and its application to individuals submitted to environmental exposure to PAHs in Rio de Janeiro State, Brazil. Sixty individuals were studied. The study group was composed of 27 smokers and 33 non-smokers individuals. The determination of 1-OHPy was performed by high performance liquid chromatography with fluorescence detection. The concentrations of 1-OHPy were corrected by dividing them with the concentrations of urinary creatinine. Row data were statistically evaluated and invalid data or outliers were discarded. The mean concentration of urinary 1-OHPy for smokers (0,387 µg of 1-OHPy/g of creatinine, n = 25) was more than three times larger than that found for non-smokers (0,116 µg of 1-OHPy/g of creatinine, n = 27).

Keywords: urinary 1-hydroxypyrene, PAH exposure, biomarkers, human exposure, smoking, environmental, PAHs.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants of concern since epidemiological studies have shown that the exposure to PAHs increases the risk of lung, bladder, stomach and skin cancers. PAH exposure may occur by dermal contact, inhalation, ingestion and smoking and it may be of occupational and/or environmental origin. In this way the application of biomarkers to evaluate PAH exposure is a valuable tool and an important task of interest [1].

Several studies have been conducted to develop appropriate indicators of exposure to PAHs in order to implement the monitoring of human exposure. A recent thorough review of biomarkers of PAHs in environmental health suggests that urinary 1-hydroxypyrene, a specific metabolite of pyrene, is the most relevant parameter for estimating individual exposure to PAHs [2].

The abundance of pyrene, a non-carcinogenic PAH, is relatively high in PAHs mixtures found in the atmosphere and in workplaces [3]. PAHs are metabolized in different polyhydroxy-PAHs, with exception of pyrene, which is almost completely (around 90%) metabolised to hydroxypyrene (1-OHPy) that is excreted in human urine either in free form or conjugated as a derivative (glucoronide). As a consequence, 1-OHPy is considered to be a reliable and suitable biological indicator of PAH exposure [4]. The total concentration of 1-OHPy (free and conjugated forms) found in urine thus reflects the overall exposure to PAHs [5].

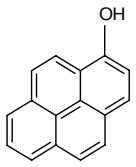

Fig.1. Structure of 1-hydroxypyrene, biomarker of PAH exposure.

There are data about urinary 1-OHPy for several countries but there are few data about it in Brazil. This fact justifies the interest for this work.

2. PURPOSE

The purpose of this work was the implementation, validation and application of a method for determination of urinary 1-OHPy in individuals submitted to environmental exposure to PAHs in Rio de Janeiro State, Brazil and comparison of urinary 1-OHPy levels found in smokers and non-smokers individuals.

To allow the comparison of values of 1-OHPy obtained in urine samples, a validated methodology for determination

ISBN 978-963-88410-0-1 © 2009 IMEKO

of urinary creatinine was applied to all studied samples and the values of 1-hidroxypyrene were corrected by creatinine value and expressed as μg of 1-OHPy/g of creatinine, as recommended by World Health Organization [6].

3. METHODS

3.1. Method of 1-OHPy determination

The determination of 1-OHPy was performed by high performance liquid chromatography with fluorescence detection (HPLC-Fluo). The method allowed the determination of free and conjugated 1-hidroxypyrene forms in urine. Prior to HPLC analysis, samples were submitted to enzymatic hydrolysis with *β*-Glucuronidase to hydrolyze the glucoronide to form 1-OHPy and to allow the quantification of total 1-OHPy. All steps of the method are represented in Fig. 2.

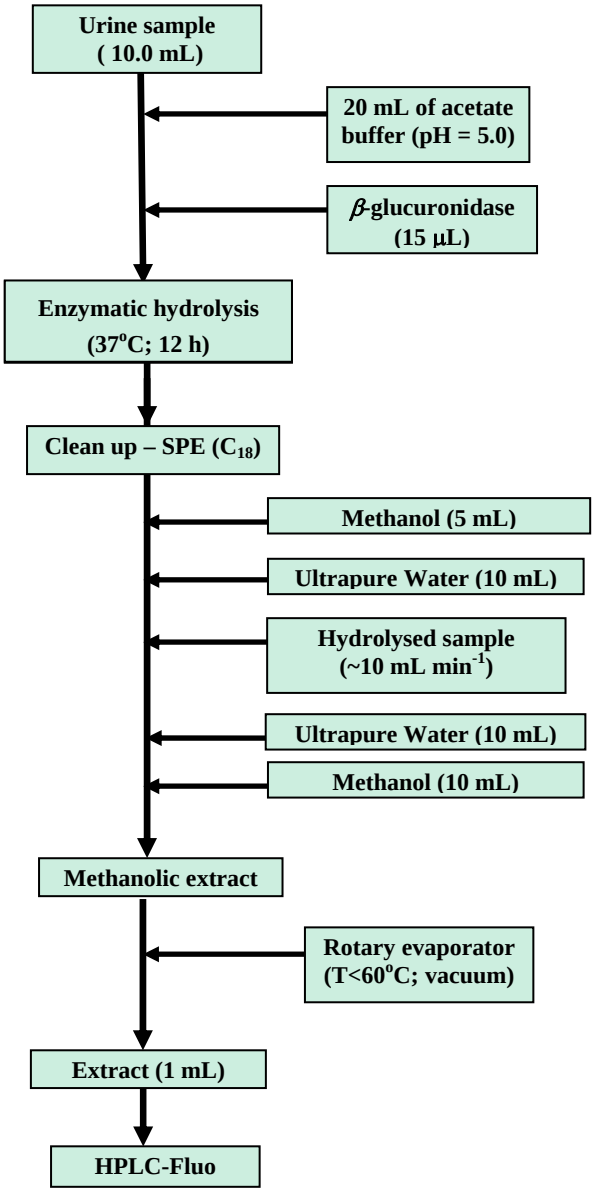

Fig. 2. Adapted method to determination of Urinary 1-OHPy [7].

The analytical procedures were optimized and validated through the determination of the performance parameters of the method [8]. Chromatographic conditions are shown in Table 1.

Table 1. Chromatographic conditions.

Chromatographic system	Quaternary pump, automatic sampler and fluorescence detector (Agilent 1100 Series)
Fluorescence Detection	Excitation: 232 nm ; Emission: 396 nm ; PMT: 14
Column	Zorbax XDB-C18 (150 x 4.6 mm; 5μm; Agilent)
Mobile phase	Methanol:Water (65:35 *v/v*); 1.0 mL min^{-1}
Column Temperature	25°C
Injected volume	20 μL

The complex urine matrix was shown to influence the determination of 1-OHPy concentrations. As a consequence calibration standards were prepared by appropriate dilutions of a solution prepared form solid 1-OHPy (Sigma) in pooled urine of not occupationally exposed persons. Standard concentrations ranged between 0.1 and 10.0 μg L^{-1}. Blanks, 1-OHPy standards and samples were processed and analyzed in the same way.

3.2. Method of creatinine determination

Urine samples collected at different times over a period of 24 hours have different dilutions, preventing the use of analytical results obtained with one punctual urine sample of urine to be compared. This way, to allow the comparison of analytical results with limits of biological tolerance, with previous reported results or even among workers exposed to similar work conditions, it would be necessary to collect all excreted urine during an interval of 24 hours, which is a very difficult task.

In medical practice, correction for creatinine excretion is preferred to the complicated collection of a 24-hour urine sample. In assurance of analysis quality and/or exposure to toxic substances, proper determination of creatinine concentration is just as important as determination of the analyte (the toxic substance or its metabolite) itself [8]. Creatinine is formed in muscles from creatinine phosphate in amounts that are proportional to the muscle mass and it is minimally reabsorbed in the renal tubular system. Creatinine is excreted from the human body at a relatively stable rate that is little influenced by physical exercise or by stress and its excretion is not affected by diet, temperature, by ingested volume of liquid. Therefore, creatinine is an important value in urine analyses [9] since it allows correction of excreted quantities of different compounds.

Samples of urine that are highly diluted or concentrated in general are not suitable for exposure monitoring. As a consequence, the World Health Organization adopted values between 0.3 and 3.0 g L^{-1} as acceptable limits to urinary creatinine [10].

Urinary creatinine was determined by quantifying the red color Janovsky complex, which is formed in the reaction with picric acid in alkaline medium and measured by

molecular absorption spectrophotometry at 530 nm [11]. This reaction (Fig. 3) is named Jaffé's Reaction because it was observed from Jaffé in 1886 and posteriorly utilized by Folin to measure creatinine in urine in 1904 [12].

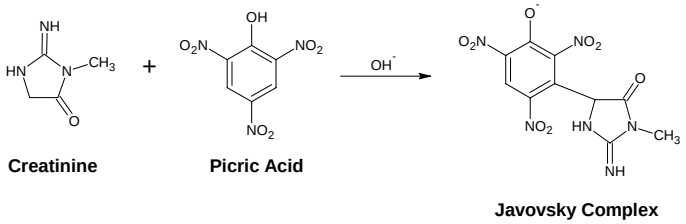

Fig. 3. Jaffé's Reaction for creatinine determination [11].

The calibration curve was prepared with aqueous standards of creatinine (Sigma), in range of 0,1 a 5,0 g L^{-1}.

3.3. Urine samples

Urine samples of 60 volunteers were collected in Rio de Janeiro State, Brazil between June and July 2007. The volunteers received a questionnaire with questions concerning life habits such as nutritional and personal data (age, weight, address, number of smoked cigarettes etc). They were also told about the interest of this study.

4. VALIDATION PROCEDURES

4.1. Selectivity

The selectivity of a method evaluates how the method is indifferent to the presence of species in the sample capable of interfering in the determination of analyte of interest.

A pooled urine sample that represents the complexity of the matrix studied was used to evaluate selectivity for 1-OHPy determination. Analytical curves of 1-OHPy (0.5, 1.0, 2.5, 5.0 and 10.0 μg.L^{-1}) were prepared in triplicates with and without the urine matrix, following the procedure described in Section 3.1. The standards without matrix were prepared in methanol and directly analysed by HPLC-Fluo. Standards and blank prepared in urine were extracted by SPE and analyzed after this stage by HPLC-Fluo. Urine without addition of 1-OHPy was used as reagent blank.

Analytical curves of creatinine were made at 7 levels of concentrations (0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 g L^{-1}) run in triplicates. Standards were prepared with and without matrix following the method described in Section 3.2. The triplicate results were checked for outliers by Grubbs test.

The analytical curves of both groups of data (1-OHPy and creatinine) were constructed and their slopes were estimated by linear regression. The effect of urine matrix was verified by comparison of angular coefficients of analytical curves with Student t-test The tabulated value of t was obtained from the Student t distribution with (Df1 + Df2) degrees of freedom and confidence level of 95%. This way, a value of $t_{calculated}$ lower than that of $t_{tabulated}$ allows the conclusion that the matrix does not affect the determination. However, if the value of $t_{calculated}$ is greater than $t_{tabulated}$ it is possible to conclude that the matrix has a statistically significant effect on the determination. Thus, standards should be always prepared in the sample matrix.

Although Jaffé´s reaction is the most used method for creatinine determination there are some interferences described in the literature. Interference effects on creatinine method were studied in urine samples that already contain creatinine, with addition of ascorbic acid (1g L^{-1}) and acetone (50 mg L^{-1}). Bilirubin interference was not studied because its interference in creatinine determination only occurs if blood contaminated urine that occurs, for example in case of serious hepatic disorders.

4.2. Limit of detection and limit of quantification

The limit of detection (LOD) is the smallest quantity of analyte present in a sample which can be detected (distinguished from a blank value), but not necessarily quantified, under the experimental conditions established.

The limit of quantification (LOQ) is the smallest quantity of analyte present in a sample can be determined with an acceptable level of precision and accuracy under the experimental conditions established.

To evaluate LOD and LOQ of 1-OHPy method, ten independent standards 0,5 μg L^{-1} of 1-OHPy in urine were quantified as established analytical method. The standard deviation (*s*) of concentrations obtained for the replicates was calculated and LOD and LOQ were established in with Equations 1 and 2.

$$LOD = 3s \quad (1)$$

$$LOQ = 10s \quad (2)$$

To evaluate LOD and LOQ of the creatinine method, the standard deviation (*s*) of the absorbance of ten blanks was calculated and the LOD e LOQ was determined by Equations 3 and 4 were *b* is the slope of the analytical curve.

$$LOD = 3{,}3\ s/b \quad (3)$$

$$LOQ = 3\ LOD = 10\ s/b \quad (4)$$

4.3. Linearity

Linearity is the ability of an analytical method to produce results that are directly proportional to the analyte concentration in samples in a given range of concentrations. In the lower limit of the range of concentrations, the factors limiting values are the limits of detection and quantification. In the higher limit of the range of work, the limiting factors depend on the response system of the measuring equipment.

To study the linearity of 1-OHPy response, a 6 levels calibration curve was run in duplicate. A 7 levels calibration curve, run in triplicates was used to evaluate the linearity of creatinine response. The mean values of peak areas or of absorbances were used to obtain linear regression equations by least squares method.

The correlation coefficient (*r*) is in general, used as a measure of the linearity of the calibration curve and is often used to indicate how much a line can be considered suitable to represent the model. Values of r were calculated with the sets of data and a value of R^2 greater than 0,995 was required to consider a linear model.

Cochran test was used to verify if data variance along the curves were homocedastic (equal variances) or heterocedastic (different variances) the variance. Plots of

residuals versus concentrations were used to to assess the quality of a regression. Random point distributions free of trends were observed in all cases.

4.4. Sensitivity

The sensitivity is reflected in the smallest difference in concentration that can be measured by a method. This parameter shows response variation depending on analyte concentration variation. It is expressed by the inclination of linear regression line and it was estimated by the angular coefficients of the regression lines. Their values were determined simultaneously to linearity evaluation.

4.5. Accuracy

Accuracy is a measurement of closeness of agreement between a measured quantity and a true quantity [13]. Accuracy can be estimated from recovery experiments and expressed as recovery percentage of spikes or by analysis of reference materials.

To study the recovery of 1-OHPy, triplicates samples of urine were spiked with levels of 1-OHPy concentration and quantified by the analytical method. The accuracy also was estimated by triplicate analysis of the Reference Material Seronorm Trace Elements Urine FE1114 by the analytical method.

The accuracy of creatinine method was determined by analysing the Reference Material Seronorm Trace Elements Urine NO2525 in independent triplicates.

4.6. Precision

Precision is the closeness of agreement between indications or measured quantity values obtained by replicate measurements of the same or of similar objects under specified conditions. Measurement precision is used to define measurement repeatability, intermediate measurement precision and measurement reproducibility [13]. In this study, the precision was evaluated by repeatability. In the case of 1-OHPy, the repeatability was determined together with the recovery study while for creatinine it was determined with the linearity study.

5. RESULTS AND DISCUSSION

5.1. Selectivity

The comparison of calibration curves of 1-OHPy to evaluate method selectivity led to a $t_{calculated}$ greater than that of $t_{tabulated}$ (5,927 > 2,056) showing that both curves had statistically different angular coefficients (160,2 and 119,6) and a statistically significant effect on the selectivity. Consequently it was shown to be necessary to prepare the calibration curve in sample matrix (urine) and process it in the same way of samples.

With regard to creatinine determination, $t_{calculated}$ was lower than $t_{tabulated}$ (1,787 < 2,024) indicating that the curves had statistically equal (0,447 and 0,440) angular coefficients with no a not a statistically significant matrix effect on the selectivity. As a consequence, calibration curves were prepared with ultrapure water.

The effects of interferences were also are not significant to the creatinine determination, with relative errors below 3% (Table 2).

Table 2. Effects of interferents in creatinine determination.

Sample	Creatinine measured at g L^{-1} (matrix + analyte)	Creatinine measured at g L^{-1} (matrix + analyte + interferents)	Relative error (%)
1	1,628	1,586	2,58
2	1,628	1,600	1,72
3	1,628	1,585	2,64
4	1,620	1,579	2,53
5	1,619	1,587	1,98

5.2. Limit of detection and limit of quantification

LODs and LOQs of both methods are presented in Table 3. In the continuity of this study a calibration level corresponding to the limit of quantification of 1-OHPy (0,10 µg L^{-1}) was included as the first point of calibration curves.

Table 3. Limit of detection and limit of quantification.

Analyte	LOD	LOQ
1-OHPy	0,03 µg L^{-1}	0,10 µg L^{-1}
Creatinine	0,01 g L^{-1}	0,04 g L^{-1}

5.3. Linearity

The coefficient of linear regression (*r*) found to the analytical curve of 1-OHPy was 0,9993 and R^2 was 0,9987 showing a linear range of work between (0,1–10,0 µg L^{-1}) The analytical curve is found in Fig. 4. For the Cochran test, the value of C_{calc} (0,5319) was lower than the C_{tab} (0,7808) indicating that a homocedastic system. The residuals graphic indicated that the system is free of trends.

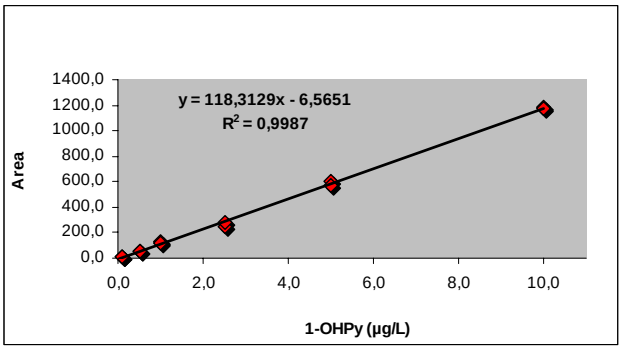

Fig. 4. Analytical curve of 1-hydroxypyrene in urine.

The coefficient of linear regression (*r*) found to analytical curve of creatinine was 0,9999 and the R^2 was 0,9998. A linear working range (0,1–5,0 g L^{-1}) was found. For Cochran test, the value of C_{calc} was 0,3514 and C_{tab} was 0,5612. This comparison indicated a homocedastic system. The value of linear coefficient (0,0128) that was very close to zero, indicated also a free trends system.

5.4. Sensitivity

The sensitivity of 1-OHPy method was 118,3 and the sensitivity of creatinine method was 0,447.

5.5. Accuracy and Precision

The data of recovery and repeatability of the 1-OHPy method is shown in Table 4.

Table 4. Recovery and repeatability of 1-OHPy method.

Spiked amount (µg L^{-1})	Measured amount (µg L^{-1})	Recovery (%)	Mean Recovery (%)	Standard Deviation (µg L^{-1})	RSD (%)
0,50	0,460	92,0%	98,5%	0,04	8,0
	0,481	96,2%			
	0,537	107,4%			
1,00	0,986	98,6%	101,9%	0,04	3,6
	1,014	101,4%			
	1,057	105,7%			
2,50	2,255	90,2%	97,7%	0,18	7,1
	2,460	98,4%			
	2,609	104,4%			
5,0	4,898	98,0%	101,3%	0,15	2,9
	5,151	103,0%			
	5,150	103,0%			
10,0	9,541	95,4%	99,8%	0,46	4,6
	9,931	99,3%			
	10,467	104,7%			

A recovery between 90 to 110% was found throughout the studied range. The repeatability throughout this range presented a relative standard deviation < 10%. The results of triplicate analysis of reference material (RM) are presented in Table 5. Although the third replicate had value outside acceptable range of 0,38 to 0,46 µg.L^{-1}, but this value was not considered outlier by Grubbs test. An excellent average result (101%) was obtained for the accuracy, based on the average of these 3 independent replicates. The results are shown on Table 5.

Table 5. Evaluation of accuracy of 1-OHPy determination after analysis of a RM.

Amount in RM (µg L^{-1})	Measured amount (µg L^{-1})	Mean (µg L^{-1})	Standard deviation	Accuracy (%)
0,42 (0,38 - 0,46)	0,448	0,425	0,046	101,1
	0,454			
	0,372			

The results of triplicate analysis of the reference material certified for creatinine are presented in Table 6. They are in the acceptable range of creatinine concentrations for this RM and indicate the very good accuracy of the method.

Table 6. Evaluation of creatinine accuracy after analysis of a RM.

Amount in RM (g L^{-1})	Amount measured (g L^{-1})	Mean (g L^{-1})	Standard deviation	Accuracy (%)
0,814 (0,774 - 0,855)	0,824	0,820	0,004	100,7
	0,818			
	0,817			

The repeatability of creatinine throughout the working range presented a relative standard deviation below 1% (Table 7).

Table 7. Repeatability of creatinine method.

Spiked amount (g L^{-1})	Measured absorvance (u.a.)	Mean (u.a.)	Standard deviation (u.a.)	RSD (%)
0,50	0,236	0,236	0,0006	0,24
	0,237			
	0,236			
2,0	0,916	0,920	0,0032	0,35
	0,921			
	0,922			
4,0	1,805	1,807	0,0029	0,16
	1,805			
	1,810			

5.6. Samples results

The studied group consisted of 27 smokers and 33 non-smokers, with 32 men and 28 women. Samples with 1-OHPy < LOQ or samples with creatinine concentrations outside the acceptable range (0.3 to 3.0 g L^{-1}) were not considered. The results of each group (smokers and non-smokers) were treated and the Grubbs test was applied for outlier exclusion. Normality of data distributions were tested with Shapiro-Wilk test. The hypothesis of normality could not be rejected in both cases. The histograms 1-OHPy levels expressed as µg of urinary 1-OHPy/g of creatinine in non-smokers and smokers are shown respectively in Fig. 5 and 6.

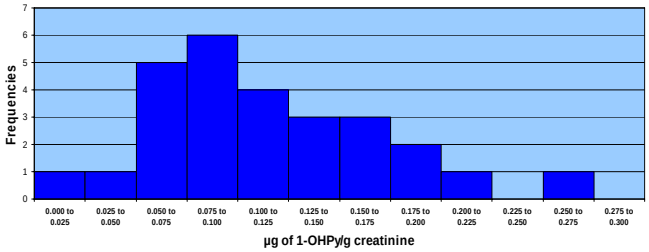

Fig. 5. Histogram of 1–OHPy levels found in non-smokers.

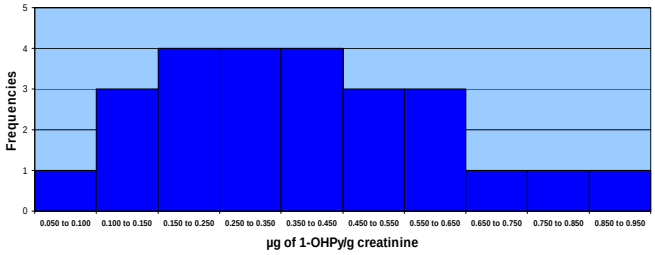

Fig. 6. Histogram of 1–OHPy levels found in smokers.

The concentrations of urinary 1-OHPy found in this study are further summarized in Table 8. The variance and the mean value of smokers and non-smokers were compared with F-Snedecor and t-Student tests (Table 9). The results indicate different variances ($F_{cal} > F_{tab}$) and different mean values ($t_{cal} > t_{tab}$) when both groups were compared. As a consequence smokers and non-smokers represent different populations regarding PAH exposure. These results indicate that smokers are exposed to larger PAH levels than non-smokers showing that as expected and as previously observed, smoking is an important exposure way to PAHs.

Table 8. Summary of urinary 1–OHPy data.

Results	Non-smokers	Smokers
n (number of individuals)	27	25
Mean (µg of 1-OHPy/g creatinine)	0,116	0,387
Std dev (µg of 1-OHPy/g creatinine)	0,058	0,223
Median (µg of 1-OHPy/g creatinine)	0,102	0,353
Range (µg of 1-OHPy/g creatinine)	0,022 to 0,264	0,058 to 0,930

Table 9. Comparison of urinary 1–OHPy levels in smokers and non-smokers individuals by F-Snedecor and t-Student tests.

Smokers and non-smokers		
F_{calc}	14,568	$F_{cal} > F_{tab}$
F_{tab}	1,946	
t_{calc}	5,885	$t_{cal} > t_{tab}$
t_{tab}	1,703	

The concentrations of urinary 1-OHPy found in this study were compared with previous values found in other areas of the world (Table 10). The concentrations of urinary 1-OHPy of smokers and non-smokers levels observed in Rio de Janeiro State, Brazil are comparable to those found in Sweden, Germany and USA. Moreover, in some countries such as China and USA, the difference between urinary 1-OHPy concentrations of smokers and non-smokers are less expressive than in Brazil.

Table 10. Comparison of urinary 1-OHPy concentrations found in this study with previous data [14].

Countries	Urinary 1-OHPy (µmol/mol creatinine) Median (number of individuals) Mean* (number of individuals)	
	Non-smokers	Smokers
Netherlands	0,26 (52) ; 0,17 (14)	0,28 (38) ; 0,51 (28)
Sweeden	0,03 (48)	0,09 (10)
Turkey	0,24* (15)	0,33*(14)
Germany	0,04 (90)	0,12 (49)
Italy	0,08 (19)	0,13 (22)
Canada	0,07 (95)	0,12 (45)
USA	0,27 (10)	0,76 (11)
China	0,68*(74)	0,76*(84)
BRAZIL	0,05 (25) ; 0,06*(25)	0,18(27) ; 0,20*(27)

The influence of certain characteristics of the volunteer in urinary 1-OHPy levels was also evaluated: number of smoked cigarettes, passive smoking, sex, age, nutritional habits and home localization. To smokers, the home localization and age were shown to be factors of importance. The number of smoked cigarettes was compared and there is no statistical difference between results of volunteers that smoke 10 or 20 cigarettes/day. Passive smoking did not influence concentrations of 1-OHPy in non-smokers. Sex and nutritional habits also were not of significant influence in results of this study.

6. CONCLUSION

Our results indicate that the implemented method allows the determination of urinary 1-OHPy and creatinine with sufficient sensitivity, repeatability and accuracy. Adequate LOQs were found for both parameters. With regard to the studied samples non-smokers individuals presented lower levels of urinary 1-OHPy than smokers individuals. Home localization and age also influenced urinary 1-OHPy concentrations. Nutritional habits and number of smoked cigarettes did not influence the results of this study.

REFERENCES

[1] IPCS - International Programme on Chemical Safety. "Selected Non-Heterocyclic Polycyclic Aromatic Hydrocarbons", *World Health Organization*, Geneva, 1998.

[2] F. Dor, W. Dab, P. Empereur-Bissonet and D. Zmirou, "Validity of biomarkers in environmental health studies; the case of PAHs and benzene", *Critical Reviews in Toxicology*, Vol. 29, pp.129-168, 1999.

[3] J. P. Buchet. J. P. Gennart. F. Mercado-Calderon. J. P. Delavignette. L. Cupers and R. Lauwerys, "Evaluation of exposure to PAH in a coke production and graphite electrode manufacturing plant", *British Journal of Industrial Medicine.* Vol.49, pp.761–768, 1992.

[4] S. S. Hecht, "Human urinary carcicogen metabolities: biomarkers for investigating tobacco and câncer", *Carcinogenesis*. Oxford University Press, vol. 23, n° 6, pp. 907-922, United-Kingdom, 2002.

[5] P. Apostoli, C. Minoia, L. Alessio, "Polycyclic aromatic hydrocarbons in the sphere of life and work: exposure and effects - Idrocarburi Policiclici aromática negli ambienti di vita e di lavoro: esposizione ed effetti", *Gargnano*, pp.27-29, March 1996.

[6] WHO, "Biological Monitoring of Chemical Exposure in the workplace", *World Health Organization*, vol. 02, Geneva, Switzerand, 1996.

[7] F. Jongeneelen, R. B. M. Anzion, "1-Hydroxypyrene. In: Analyses of Hazardous Substances in Biological Materials", *VCH*, 2ª edition, vol. 3, pp.151-169, Germany, 1991.

[8] DOQ-CGCRE-008. "Guidance on validation of methods for chemical testing - Orientação sobre validação de métodos de ensaios químicos", *INMETRO*, rev.02, Brazil, 2007.

[9] J. Kratochvíla, B. Friedecký, M. Budina, I. Sperlingová, "Creatinine determination in urine fromthe point of view of reference values", *Accred Quali Assur*, vol.12, pp.146-150, 2007.

[10] ACGIH. "Documentation of TLVs and BEI". *ABHO*, Brazilian edition, 2008.

[11] IRSST: Institut Recherche em Santé et em Sécurité du Travail du Québec, "Método 163-2: Determination of urinary creatinine - Détermination de la créatinine urinaire", *Biblioteca Nacional de Quebec*, 12ª edição, Canadá, 1996.

[12] S. Narayanan, H. D. Appleton, "Creatinine: A Review", *Clinical Chemistry*, Vol.26, n° 8, pp.1119-1126, 1980.

[13] VIM. "International vocabulary of metrology - Basic and general concepts and associated terms", *JCGM*, pp. 21-22, 2008.

[14] F. J. Jongeneelen, "Benchmark Guideline for urinary 1-hydroxypyrene as biomarker as occupational exposure to Polycyclic Aromatic Hydrocarbons", *Annals of occupational Hygiene.* Vol.45, n° 1, pp. 3-13, 2001.