ALESSANDRA L.M.C. DA CUNHA, ROBERTA L. ZIOLLI, RICARDO Q. AUCÉLIO

Pontifícia Universidade Católica do Rio de Janeiro (PUC-RJ)
Chemistry Department
Brazil, e-mail: alicursi@osite.com.br, aucelior@rdc.puc-rio.br

DEVELOPMENT AND VALIDATION OF A SELECTIVE METHOD FOR THE DETERMINATION OF CHRYSENE USING SILVER-ENHANCED ROOM-TEMPERATURE PHOSPHORIMETRY

A factorial design was applied for the development of a solid surface room-temperature phosphorimetric method aiming the selective determination of chrysene. Data analysis was made using statistical experimental analysis (testing the significance of the factors using the analysis of variation, F-test and t-test), graphic method (Pareto´s chart) and the evaluation of the interactions among all variables. This procedure was used in order to guarantee high accuracy of results and minimization of the time spent for optimization. As the result, the experimental conditions using a selective phosphorescence inducer (silver) and a substrate surface modifier allowed the determination of chrysene in the presence of pyrene. The method allowed the detection of effective masses of chrysene in the ng range. Method validation is presented including a recovery and "t" tests using a Standard Reference Material.

Keywords: Factorial design, validation, solid surface room-temperature phosphorimetry (SSRTP), chrysene, uncertainty

1. INTRODUCTION

The increasing need for accurate chemical measurements requires the proper evaluation of confidence intervals and traceability as well as a proper comparison of results [1, 2]. In order to guarantee that a new analytical method generates real information about the analyte(s) of interest in a specific sample matrix, proper validation must be made. Validation is a continued process that begins when the analytical strategy is planned and continues through all the development process. By achieving logical and organized data correlation during development, optimization and validation, the laboratory can generate results in a very efficient and productive way [1, 2]. The sample matrix is a crucial part to be considered since it will impose on the analyst the search for method variations to allow selective determination of the analyte. Such variations must be evaluated during the validation process in order to understand their impact on the metrological performance of the method.

The majority of the experiments involve many variables (factors), therefore, optimization must be designed to verify the effect of each factor as well as to identify mutual interactions among them. The use of a proper strategy for optimization generates best experimental conditions in terms of sensitivity and selectivity, finally affecting the cost and time of analysis if an alternative simpler analytical method is made to work, replacing analytical methods based on separation of the analyte from other matrix components.

Factorial design is a useful technique when there are two and more independent factors allowing the prediction of interactions among them, requiring that all observations and associated errors are random and variables to be independently distributed. In addition, the experiments, using authentic replicates (analysis of independent sample replicates prepared and measured under the same conditions), must be performed in a way to guarantee equal distribution of all the factors that were not considered. In such design, all the factors are all varied together (contrary to what is done in a univaried experiment). In this case, when

considering all the combinations of the n factors (within two determined levels), a 2^n factorial design is used [2].

The amount of polycyclic aromatic hydrocarbons (PAH´s) indicates the degree of pollution in a sample that can be, for instance, a specific environmental compartment (soil, sediment, air, body of water, etc). The ability to discriminate among different PAH´s is important since they have different degrees of toxicity, carcinogenic and mutagenic actions. The selective determination of PAH´s is also important for the identification of a pollution source or to monitor the extent in a contaminated area [3]. Room-temperature phosphorimetry in solid substrate (SSRTP) has achieved a high degree of maturity and it is a powerful technique for trace-level analysis of organic molecules [4]. The selectivity of SSRTP can be enhanced by choosing among several critical experimental variables that will allow sensitive determination of a specific analyte in samples containing concomitant substances of similar chemical structure.

For the SSRTP, the critical factors are: the pH of the original analyte solution, the nature of the phosphorescence inducer and its concentration, and the effect of the modification of the substrate surface. Depending on the case, other factors can be introduced. The understanding of how these factors affect phosphorescence from the analyte and from concomitant substances is very important in terms of method performance in function of the sample matrix in analysis. The interaction among these factors must be identified in order to promote the correct optimization, allowing the achievement of the best analytical figures of merit and, therefore, taking advantage from the full potential from the analytical method.

The goal of this work is to develop a selective SSRTP method for the determination of chrysene, showing that a multivariate optimization after a previous univaried evaluation of factors is an effective way to achieve best experimental results. The validation of the analytical method has been done through the evaluation of several parameters of performance.

2. EXPERIMENTAL

Phosphorescence measurements were performed on a luminescence spectrometer Perkin – Elmer LS-55 (Perkin-Elmer, USA) coupled to a solid surface analysis apparatus modified for purging the sample holder with dry N_2. A delay time of 3 ms and a gate time of 3 ms were applied and found to completely eliminate second order scattering during detection. A laboratory-made photochemical reactor was used to reduce the background of paper substrates. An electronic scale with four decimals (Marte, Brazil) was used.

Experiments were performed with analytical grade chemicals and ultra pure water. Whatman N°42 filter paper was used as solid substrate. Chrysene, pyrene and $TlNO_3$ were from Acros Organics (USA), Ethanol, KI, sodium dodecyl sulfate (SDS) were from Merck (Brazil), $AgNO_3$, Hg_2Cl_2 and $Pb(NO_3)_2$ were from VETEC (Brazil). The reference material, (SRM 1647b, Priority Pollutant Polycyclic Aromatic Hydrocarbons in Acetonitrile) was acquired from NIST.

A $1x10^{-4}$ mol L^{-1} stock solution of chrysene was prepared in ethanol and used to prepare more diluted standard working solutions. More diluted solutions of chrysene were prepared in ethanol/water 50/50% v,v. The stock solutions of SDS (0.25 mol L^{-1}) and heavy atom salt solutions (0.25 mol L^{-1} of $TlNO_3$, 0.2 mol L^{-1} of KI, 0.03 mol L^{-1} of $AgNO_3$, 0.2 mol L_{-1} of $HgCl_2$ and 0.25 mol L^{-1} of $Pb(NO_3)_2$) were prepared in water and used to prepare more diluted solutions. Small volumes of pyrene solutions ($1x10^{-4}$ mol L^{-1}) were used to spike chrysene solutions, simulating a potential interferent substance.

Substrate (filter paper) background reduction consists of washing paper strips with boiling water in a Soxhlet apparatus for 2 h, drying and exposition to ultraviolet irradiation for

another 2 h. These solid substrates were cut in circles (about 0.74 cm in diameter) to be used as substrate during the analysis using a clean sample holder. Each of all employed solutions (the following order was used: 5 µL of SDS, 5 µL of heavy atom solution and 5 µL of the analyte standard, blank or sample) were spotted on the center of the solid substrates using a calibrated adjustable (1–10 µL) automatic pipette. The spotted substrates were vacuum-dried at room temperature for 2 h and then, they were placed in a desiccator until the measurements were carried out. Sample compartment was continuously purged with dry N_2 for 2 min prior and during measurements.

3. RESULTS AND DISCUSSION

In order to optimize unknown analytical systems, a previous univaried study is extremely useful since it establishes solid basis for choosing the experimental range to be used for each of the factors selected for the multivariate study. In this case, the multivariate study is a way to verify interactions between factors, to allow a fine adjustment of experimental conditions to be performed, and to reflect the robustness of each factor.

3.1. Univaried studies

Room-temperature phosphorescence (RTP) in a de-oxygenated environment can be readily obtained by minimizing non-radiative deactivation of the excited triplet state caused by dynamic quenching and vibrational relaxation. In addition, the use of a phosphorescence inducer/amplifier (generally performed by external heavy atom effect- HAE) is almost always required, due to the natural singlet character of the excited state of most molecules [4, 5]. HAE may significantly enhance both the rate of intersystem crossing (excited singlet state – excited triplet state transition) and the phosphorescence rate constant [6, 7]. HAE also has a selective nature due to specific analyte-heavy atom interactions.

Studies were performed in order to find the experimental conditions to induce phosphorescence from chrysene. The effect on the RTP of pyrene was also evaluated since this PAH was chosen as a concomitant substance for interference studies. A phosphorescence signal from chrysene was only observed from the cellulose substrate in the presence of $AgNO_3$, $Pb(NO_3)_2$ and $TlNO_3$. However, the salt of Ag(I) was chosen since it was identified to be selective towards chrysene since it was the only one that did not induce phosphorescence from pyrene under same conditions. The use of SDS on the substrate caused an even further RTP amplification from chrysene (Fig. 1). Such further amplification may be explained by a better approximation between the analyte and the heavy atom in the substrate promoted by the addition of the surfactant [4].

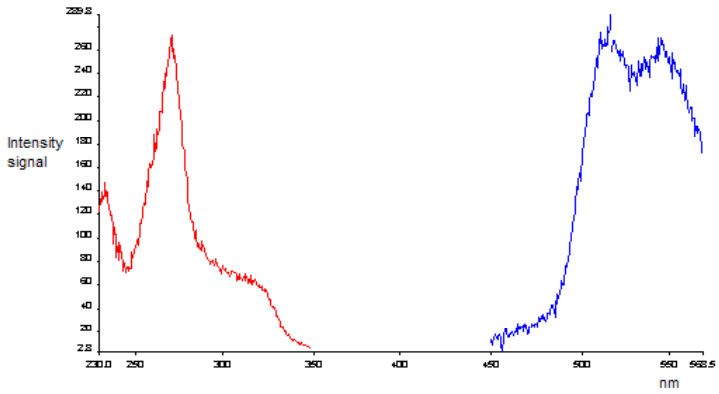

Fig.1. Emission and excitation phosphorescence spectra of chrysene in presence of SDS and a delay time of 3ms.

Univaried studies were then performed in order to evaluate RTP from chrysene in function of the pH of the analyte solution. Based on the previous results, 25 μg of $AgNO_3$ and 360 μg of SDS were added on the substrate on the spot where chrysene is deposited. It was verified that more intense RTP is obtained when chrysene is deposited on the substrate from ethanol/water 50/50, v/v solutions (pH = 6). A small decrease of RTP is observed in a basic solution (0.5 mol L^{-1} sodium hydroxide) while a drastic RTP decrease is observed in acid solutions (0.5 mol L^{-1} nitric acid).

The RTP signal from chrysene was constant over the $AgNO_3$ mass range from 17 to 50 μg. Higher masses of the salt caused formation of a silver oxide dark film, filtering the excitation light beam and therefore decreasing RTP. Such fact may also explain why wider dispersion of results among replicates is observed in the presence of higher amounts of $AgNO_3$. For SDS, the higher employed mass that can be deposited from a single 5 μL solution (360 μg) enabled the maximum signal.

3.2. Multivariate Studies (Factorial design)

From the known behavior of the RTP of chrysene in function of each of the relevant factors, the range of the multivariate study (2^3 factorial design) was selected. A high value (+) and a low value (-) for each factor were chosen in function of the results displayed in the univaried study. The values set for pH were pH = 6.0 (-), the natural one of the ethanol/water 50/50% solution, and pH = 9.0 (+), chosen in order to try to evaluate the signal behavior, through a slight increase of the concentration of NaOH. For the heavy atom effect, $AgNO_3$ mass values of 25 μg (-) and 50 μg (+) were chosen. Since 0.25 mol L^{-1} is the maximum concentration of the SDS solution, the effect of multiple additions of the SDS solution on the substrate was evaluated. Therefore, 360 μg (-), one single 5 μL addition, and 720 μg (+), two sequential 5 μL additions were chosen.

This study emphasized the statistic experimental analysis (the significance of the factors were tested using the analysis of variation, F-test and t-test), the use of the graphic method (Pareto´s chart) and the interpretation of the interactions among variables. Through these results, if necessary, the model was refined, excluding irrelevant variables. Pareto´s chart (Fig. 2) showed that the model needed adjustment only in terms of pH; a variation towards lower pH was found to be necessary (since the pH bar crossed the red pointed line indicating that this result is not within the 95% confidence level). This was performed by adding smaller amounts of NaOH in the analyte solution to achieve a pH = 8.4. The lack of the relevance of interactions among factors and the results below the critical value for the other factors indicated the right choice of the ranges for the experiments and the robustness of these factors within the chosen ranges. The optimum experimental conditions chosen were the addition of chysene from a NaOH aqueous solution/water 50:50% (pH = 8.4) on a substrate containing 34 μg of $AgNO_3$ and 360 μg of SDS. Under such conditions, RTP measurements were made at 272/515 or 272/540 nm.

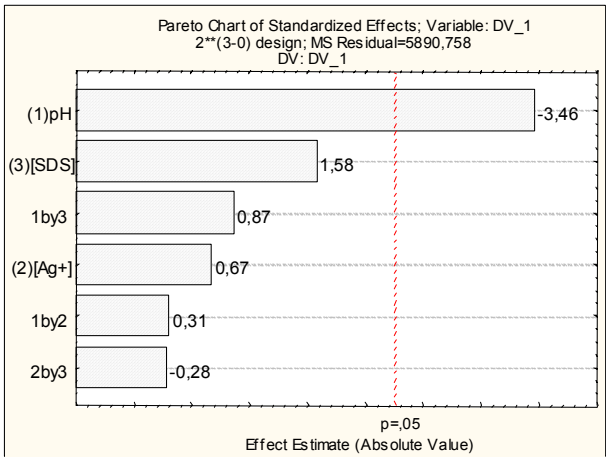

Fig. 2. Pareto´s chart.

3.3. Validation of the method

As the best experimental conditions for maximum phosphorescence were obtained, the validation of the method was done by obtaining the parameters of merit (including a selectivity study towards the presence of pyrene). Performing validation is to guarantee, through the experimental studies, that the method follows the requirements of the analytical application, ensuring trustful results [1,8,9,10]. In this work, an in-house validation was done. Such validation consists of all of the validation steps necessary to evaluate all relevant performance characteristics, except the interlaboratorial reproductivity.

The linearity of the method indicates that the range within the analytical signal (phosphorescence) is directly proportional to the amount of the analyte being studied. In order to evaluate the linear response, analytical curves were constructed and the least squares method was used to predict the best straight line passing through the experimental points. Three consecutive analytical curves, using the best experimental conditions for maximum chrysene RTP, were constructed in order to evaluate the confidence of the method and the repeatability of the results. The difference among the sensitivities (angular coefficient) of these curves was under 10 %, indicating good repetitivity. For illustration purposes, the equation of one of the curves is given: $y = 1.12 \times 10^7 x + 24.76$. The average correlation coefficient (r) was 0.99. This value can be considered an excellent one for techniques supported in solid substrate (inhomogeneous media) and it is above the critical value of 0.90 indicated by the Brazilian Metrological Institute (INMETRO) as a linear behavior. It is important to point out that the magnitude of the variance within the points of the analytical curve did not follow any tendency, indicating that there was no need for weighted linear regression. The linear behavior extended up to 114 ng of chrysene deposited in the substrate.

The detectability indicates the capability of the method to discriminate samples that contain similar but not equal amounts of analyte. This parameter depends on the inclination of the analytical curve (sensitivity) and on the magnitude and signal fluctuation of the blank signal. In this work, detectability was evaluated by the limit of detection (LOD) and the limit of quantification (LOQ) which indicates the smallest signal from the analyte amount that can be identified (LOD) or quantified (LOQ), using given statistical criteria. Depending on the criteria, the blank signal magnitude is not considered, only its standard deviation, then such parameters are only useful for comparison of different analytical methods. For ultra-trace techniques (analysis of trace amounts of analyte in micro samples) such as SSRTP, these parameters are better expressed in terms of the effective mass deposited in the substrate, the absolute limit of detection (ALOD) and absolute limit of quantification (ALOQ) considering

the sample volume used. In this work, LOD and LOQ are expressed respectively as $3s_b/m$ and $10s_b/m$, where s_b is the standard deviation of ten replicates of the blank signal and m is the sensitivity of the curve which was the average value of the three analytical curves. In terms of absolute mass values, the LOD and the LOQ are multiplied by the volume of the sample (5μL) and by the molar mass of the analyte. LOD and LOQ for chrysene using the optimized experimental conditions were 6.2 x 10^{-7} mol L^{-1} and 2.1 x 10^{-6} mol L^{-1} respectively. In terms of absolute mass value, ALOD was calculated to be 0.7 ng and ALOQ was 2.4 ng.

According to INMETRO, robustness is a measurement of the sensibility that a method presents facing small variations of factors, being robust when it is not affected by a small and deliberated modification of a given parameter [9]. In this work, the robustness of the method was evaluated through the multivariate study previously performed. Taking into consideration the influence of the pH and the mass of $AgNO_3$ the method was considered robust if no significant signal variation is observed when the parameter was varied by at least 10% of the optimized value. The pH values were varied from 6.0 to 8.4 (average value of 7.2) and the mass of $AgNO_3$ was varied from 26 to 43 μg (average value of 34 μg). Based on the insignificant signal variation observed, the method can be considered robust in terms of these two factors.

Repeatability s_r is a way to express precision (dispersion of the results), in other words, it is the degree of agreement among consecutive measurements of same samples under the same experimental conditions [11]. In this study, the precision was estimated through the relative standard deviation (%RSD) based on 10 measurements of the same sample. In general, for the SSRTP technique, a RSD as high as 15% is considered adequate since this is an analytical technique based on measurements from a non-homogeneous substrate. In addition, in the case of PAH´s that can be degraded by the incidence of the excitation UV radiation, a 20% RSD value will be accepted. The result of repeatability was 19% for 10 ng of chrysene.

The reproductivity s_R can be also estimated through the %RSD based on 10 measurements of one sample under the same experimental conditions [11], but evaluating the effect of different analysts. In this work, the reproductivity was calculated based on the variable analyses (ANOVA) according to the equations below. The result of reproductivity was 20.9% (equivalent mass of ± 2.1 ng for a 10 ng of chrysene in the substrate).

$$s_r = \sqrt{SqAv_{inside}}\ , \quad s_R = \sqrt{s^2{}_r + s^2{}_{between_{analysts}}}\ , \quad s_{between_{analysts}} = \sqrt{(SqAv_{between} - SqAv_{inside})} / n\ ,$$

where: $SqAv_{inside}$ is the square average of each analyst and $SqAv_{between}$ is the square average between analysts.

The selectivity of a method is the capability to detect the analyte in an unequivocal way, even if it is mixed with other components in a complex matrix. The selectivity study evaluates how potentially interferent species (impurities, degradation products and other compounds similar to the analyte) affect the determination of the analyte. The selectivity is a crucial validation parameter for instrumental methods and it must be evaluated during the validation procedure for a specific sample type and periodically during subsequent use of the method.

A simulated sample solution containing chrysene (2.0 x 10^{-5} mol L^{-1}) and the equivalent quantity of pyrene (used as the interferent) was used to evaluate the selectivity of the method. It was observed that the method is very selective towards pyrene since no effect in the chrysene signal was found. In the samples containing higher proportions of pyrene, a matrix effect was observed, decreasing the chrysene signal by 33 and 89 % respectively for sample solutions containing 10 and 25 times higher concentrations of pyrene. However, this interference can be properly corrected by the use of analyte addition technique.

The accuracy of the method was evaluated through a recovery test, which also indicates some associated bias. The determination of the overall bias in respect to the appropriate reference values is important in establishing the traceability to recognized standards. Bias should be negligible or able to be corrected for, but in either case, the uncertainty associated with the determination of the bias remains as essential component of overall uncertainty [12].

The recovery (or recovery factor) is defined as the proportion of the analyte, present or added in the material tested, "extracted" through its quantification [1]. In this work, the accuracy was evaluated using a Standard Reference Material (SRM 1647d – Priority Pollutant Polycyclic Aromatic Hydrocarbons in Acetonitrile).

Bias was expressed as analytical recovery (value observed - average of the 6 measurements - divided by the value expected) [1] in percentage and it was found to be 110% (average value). In this work the t-test was applied to verify, in terms of accuracy, if the measurement process is satisfactory. The value of the statistic test found (0.706) is smaller than the listed value "t student" distribution (2.571), considering the effective degree of freedom (n – 1 = 5). Therefore, the overall process was acceptable.

3.4. Uncertainty

A crucial point in chemical measurement is the estimation of the uncertainty, because analytical results may be used for several purposes allowing important decisions to be made. It is incorrect to declare that a measurement process enables results classified as true before the quantification of the variation sources (uncertainty) associated to the measurement. A measured result cannot be characterized by one single value since the whole process is dominated by sources of uncertainty. In fact it is the analyst task to perform such calculation and determine if such uncertainty is tolerable for a given application. Normally, the experiment itself involving chemical measurements has many associated sources of uncertainty, for instance, imperfections in the measurement instrument, imperfections in the apparatuses employed for sample preparation, bias associated with the analyst procedure of sampling, sample preparation and measurement, and so on. The uncertainty is a parameter associated to the result of a measurement and it is characterized by a standard deviation that can be reasonably attributed to the measured value and it is classified as type A or type B, according to how the evaluation is made [12]. Type A is evaluated by the statistical analysis of the experimental observations. Type B evaluation of measurement uncertainty requires knowledge based on the experience and general know-how of procedures and techniques that are employed. In this case, instead of the general statistics associated with a series of observations, the source information comes from experience or general knowledge of the properties and behavior of both the tested material and relevant instruments, specification from material suppliers, data provided by calibration and from certificates and/or uncertainties attributed to reference data provided by manuals or publications [12].

The mensurand is the object or a specific quantity subjected to measurement and the uncertainty can be evaluated using a cause and effect diagram ("fish spine") such as the one shown in Fig. 3, where the sources of variation (uncertainty) associated to the SSRTP measurement and based on the know-how and from the literature taken to develop this work.

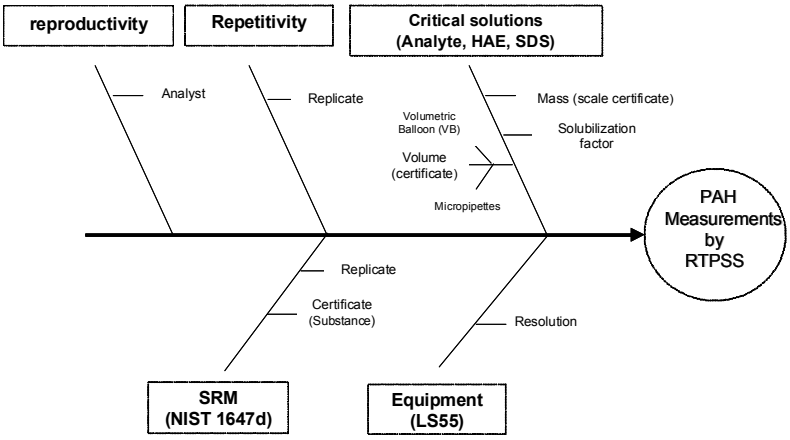

Fig. 3. Cause and effect diagram ("fish spine") indicating uncertainty sources associated to the SSRTP measurement.

Uncertainty type A is the experimental standard deviation of the average value ($u = s/\sqrt{n}$, where *s* is the calculated standard deviation and *n* is the replicate number). The uncertainty type B is based on the rectangular ($c/\sqrt{3}$) or triangular ($c/\sqrt{6}$) distribution, where $c = u_{certificate} = U_{declared}/k$. In the equation, c is the estimation from the equipment or from the reference standard, $U_{declared}$ is the standard uncertainty that must be extracted from the certificate, and *k* is the enhanced coefficient, normally used for 95% confidence level. The coefficient of the sensitivity must be used when it wants to transform the entranced quantity into uncertainty.

The last step, the components of the uncertainty are defined through the ranking of the listed uncertainties according to the percent contribution of each one in order to perform a critical analysis of the components and identify the most relevant variables in the measurement process. Then, the combined measurement uncertainty (u_c), is obtained through the square root from the quadratic sum of the estimated uncertainties. The effective degree of freedom (ν_{eff}), must be calculated using the "t student" distribution and the expanded measurement uncertainty (+/-U) must be estimated through the product of combined uncertainty (u_c) and a wide factor *k*. The expanded uncertainty attributed to a broad probability of about 95% [12]. Normally, the *k* (effective degree of freedom) probably used will be 2. The measurement result will be expressed according the equation below. In this work, the expanded measurement uncertainty found was equivalent to 0.7 ng of chrysene.

$$U_{Expanded(95\%;k=2)}(\pm) = 2U_{Combined}$$

4. CONCLUSION

The method's development must be planned taking into consideration the analytical technique to be applied, the specific sample matrix, including the relative amount of potential interferent species, and the amount of analyte that must be determined. It was verified that performing a univaried study is very important before the application of the factorial design when the behavior of the signal of the substance of interest is not known. The multivariate optimization has shown to be very useful for the evaluation of the principal effect of each factor as well as to identify interactions among the factors responsible for the RTP from chrysene. Such study allowed an effective optimization of the method and the achievement of the best analytical performance. Ag(I) was used as selective RTP enhancer for chrysene in the presence of pyrene, indicating the successful selective application of the method.

Analytical results using the Standard Reference Material indicated that the proposed method was adequate for the quantification of chrysene and pyrene in samples containing a

whole myriad of PAH´s. Both validation and uncertainty estimation were very important to ensure trustful results and allow traceability to be made, attesting, in the end, the quality control in the laboratory. It can be said that the performed measurements provide traceability to International System (SI) units, because the value of the SRM is traceable to the NIST (SI).

Following this work, the method will be applied in different matrices (fish bile, marine sediment, Brazilian spirit drink and others) and comparing the method based on SSRTP to a reference method based on liquid chromatography (HPLC-Fluorescence). Further studies will include a proficiency test in order to get a full evaluation not only of the repeatability and reproductivity among laboratories, but also to evaluate systematic errors (tendency).

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