

## MULTIVARIATE OPTIMIZATION AIMING THE DEVELOPMENT AND VALIDATION OF A SELECTIVE ROOM-TEMPERATURE PHOSPHORIMETRIC METHOD FOR THE DETERMINATION OF CHRYSENE

*Alessandra Licursi Maia C.da Cunha<sup>1</sup>, Roberta Lourenço Ziolli<sup>2</sup>, Ricardo Queiroz Aucélio<sup>3</sup>*

<sup>1</sup> Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, Brazil, alicursi@osite.com.br

<sup>2</sup> Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, Brazil, rliolli@rdc.puc-rio.br

<sup>3</sup> Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, Brazil, aucelior@rdc.puc-rio.br

**Abstract:** In this work, a factorial design was applied for the development and validation of a solid surface room-temperature phosphorimetric method aiming the selective determination of chrysene. Data analysis was made using several strategies: 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 aimed the achievement of high accuracy of results and minimization of the time spent for optimization. A statistical program was used as a supporting tool. As the result, the experimental conditions selected for the determination of chrysene were: use of silver (I) as the selective phosphorescence inducer (applied as single 5  $\mu\text{L}$  spike of  $\text{Ag}^+$  solution in the concentration range between 0,03 and 0,05  $\text{mol L}^{-1}$ ); Basic pH of the analyte solution ( $\text{NaOH}$  0,002  $\text{mol L}^{-1}$ ); use of SDS as the substrate surface modifier (employed as single 5  $\mu\text{L}$  of a SDS (Sodium Dodecyl Sulphate) solution between 0,25 and 0,50  $\text{mol L}^{-1}$ ). The optimized method is highly selective towards the presence of pyrene and allowing the detection of an effective mass of chrysene in the ng range (based on the absolute limit detection, ALOD).

**Keywords:** Optimization, Factorial design, Validation, Solid surface room-temperature phosphorimetry, Chrysene.

### 1. INTRODUCTION

The increasing need to obtain accurate chemical measurements has imposed to the analysts the proper evaluation of confidence intervals and traceability and proper comparison of results. In order to guarantee that a new analytical method generates real information about the analyte(s) of interest in a specific matrix, a proper validation process must be made. The validation method is a continue process that begins when the analytical strategy is planned and continues through all the development process. By achieving logical and organized data correlation during the method's development, optimization and validation, the

laboratory can generate results in a very efficient and productive way.

The majority of the experiments involve many variables, therefore, optimization must be designed to verify the effect of each factor as well as to identify mutual interactions among these factors. The use of a proper strategy for optimization generates best experimental conditions in terms of sensitivity and/or selectivity, in a costly and time effectiveness.

The factorial design is a useful technique when there are two and more independent variables (factors), being the only way to predict interactions among these factors. Replication and randomness are two basic principles of such experimental planning. Statistical methods also require that all observations and associated errors are at random and variables to be independently distributed. In addition, the experiments, using authentic replicates, 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 an unvaried experiment) and, in general, the analysis is performed using authentic replicates at a random order. In this case, when considering all the combinations of the n factors (within two determined levels), a  $2^n$  factorial design is used.

In order to develop a phosphorimetric method using a solid substrate, the critical factors are: the pH of the original analyte solution, the phosphorescence inducer and its concentration, and the effect caused by the use of a substrate surface modifier. Depending on the case, other factors can be introduced. These factors are very important in terms of method sensitivity and selectivity and how these two characteristics must be evaluated depending on the matrix to be analyzed. 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.

## 2. OBJECTIVE

The goal of this work is to show a multivariate optimization aiming the achievement of the best experimental conditions (Fig. 1) for the selective determination of chrysenes using solid surface room-temperature phosphorimetry (SSRTP). The analytical method validation has been performed through the analytical figures of merit.

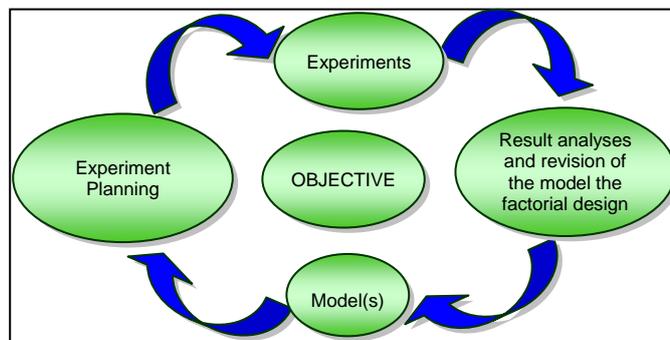


Fig. 1 Initial strategic flow

## 3. METHOD

The development of the phosphorimetric techniques at room temperature has brought higher versatility in terms of analytical routines when compared to the traditional technique at 77 K (labor intensive technique with poor repetitive).

In SSRTP, the solid substrate is used to efficiently immobilize the luminophors, allowing the observation of intense phosphorescence. Cellulose is a widely used substrate for SSRTP since it is a low costly material and compatible with the use of various heavy salt atoms, used as phosphorescence inducers or amplifier.

From the point of view of the spectroscopic methods, phosphorescence is a very attractive phenomenon since it is very dependent on the experimental conditions and therefore very selective. Both sensitivity and selectivity of phosphorimetric methods can be amplified through careful experimental adjustments. In addition, SSRTP is a ultra trace analysis tool (trace determination in micro quantity of samples), which is attractive when small quantity of sample is available.

## 4. GENERAL PROCEDURES

The addition to solutions on the cellulose substrate (quantitative filter paper) followed the following order: 5  $\mu\text{L}$  of SDS (used, when necessary, as surface modifier), 5  $\mu\text{L}$  of the heavy atom solution (when necessary, this addition was repeated several times) and 5  $\mu\text{L}$  of the sample, standard or blank solution. When necessary, the analyte solutions (sample or standards) were treated with ultraviolet radiation before they were placed on the substrate. These substrates were dried in a vacuum desiccator for 2 h. Right before measurement of phosphorescence, each substrate was placed in specific support, attached to the solid surface apparatus in the sample compartment of the spectrofluorimeter.

The general procedure flow chart can be seen in Fig. 2.

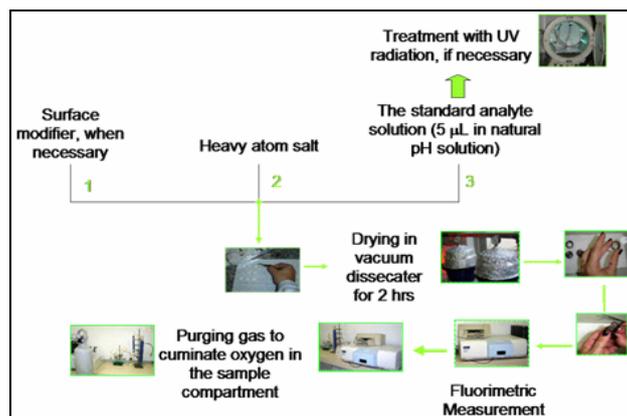


Fig. 2 General procedure for SSRTP

## 5. RESULTS AND DISCUSSION

First, studies were performed in order to find the experimental conditions to induce phosphorescence from chrysenes at room temperature. The effect of several heavy salt atoms (HA) on the analyte signal were tested. Experiments were made using analyte solutions in natural pH as well as at basic and acid pH's. The study described above was also done in substrates treated with SDS (surface modifier). UV pre-irradiated analyte solutions were also tested.

Phosphorescence signal was observed for chrysenes in presence of silver (I), lead (II) and thallium (II). However, silver (I) was identified as a selective HA for chrysenes in samples containing pyrene (used in this study as a potential interferent PAH). It was verified that the use of SDS in the substrate caused a relevant amplification of chrysenes signal. The phosphorescence emission spectrum of the chrysenes in presence of silver (I) can be found in Fig. 3.

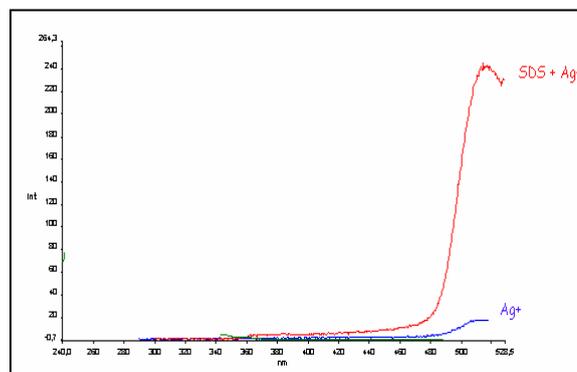


Fig. 3. Silver enhanced room-temperature phosphorescence for chrysenes. Natural pH solution in the presence and absence of SDS.

### 5.1. Unvaried studies

Unvaried studies were performed in order to study the chrysenes signal in function of the pH of analyte solution, concentration of the heavy atom solutions and concentration of SDS spotted on the substrate. From these studies, the importance of each factor was evaluated and the range to be used in the multivariate study was selected. As silver (I) was chosen as selective HA for chrysenes, only this HA was considered for further studies.

It was verified that maximum phosphorescence was found at the natural pH and using  $0,06 \text{ mol L}^{-1} \text{ Ag}^+$  solution and  $0,25 \text{ mol L}^{-1} \text{ SDS}$  solution. The plots can be seen in the in Fig. 3, Fig. 4 and Fig.5.

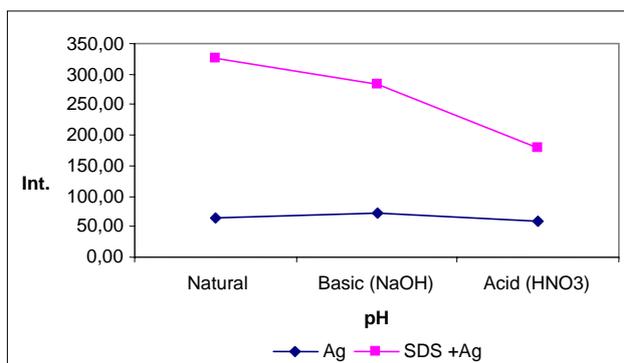


Fig. 3 Chrysenes phosphorescence signal in function of pH, in the presence and in the absence of SDS (average of measurement  $\pm 1$  Std. Deviation).

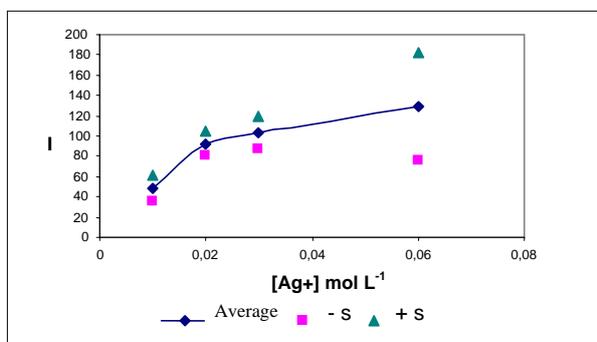


Fig. 4 Chrysenes phosphorescence signal in function of the concentration of silver I solution used to spot the substrate (average of measurement  $\pm 1$  Std. Deviation).

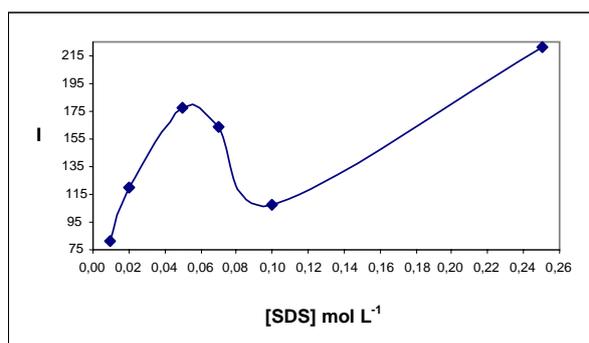


Fig. 5 Silver enhanced chrysenes phosphorescence signal in function of the concentration of SDS solution used to spot the substrate (average of measurement  $\pm 1$  Std. Deviation).

## 5.2. Multivariate Studies (Factorial design)

Since chrysenes phosphorescence is known in function of each of the relevant factors, the range for the multivariate optimization, through a factorial design, was chosen. The generated data by the system (Statistic 6.0 program) and the phosphorescence intensities can be seen on the Table 1.

In this study, it was 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), the interpretation of the interactions among variables. Through these results, if necessary, the model was refined, excluding irrelevant variables.

The Pareto's chart (Fig. 6) shows that the model needs adjustment in terms of pH. This adjustment was done using a univariate curve (Fig. 7).

Table 1 Generated data by the system

Standard Run	Design: 2**(3-0) design (Spreadsheet)				
	Replicat	pH	[Ag+]	[SDS]	DV_1
14	2	9,0	0,03	0,50	269,14
9	2	6,0	0,03	0,25	440,57
13	2	6,0	0,03	0,50	460,76
6	1	9,0	0,03	0,50	433,70
15	2	6,0	0,05	0,50	503,09
11	2	6,0	0,05	0,25	408,92
10	2	9,0	0,03	0,25	318,92
1	1	6,0	0,03	0,25	408,23
8	1	9,0	0,05	0,50	400,85
5	1	6,0	0,03	0,50	398,99
4	1	9,0	0,05	0,25	269,00
3	1	6,0	0,05	0,25	423,44
7	1	6,0	0,05	0,50	428,63
16	2	9,0	0,05	0,50	289,51
2	1	9,0	0,03	0,25	107,93
12	2	9,0	0,05	0,25	321,03

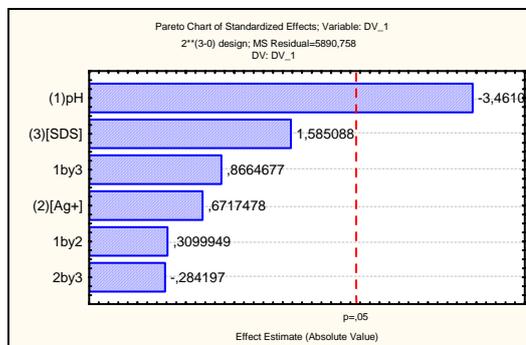


Fig. 6 Pareto's chart showing that it is necessary to refine the model in terms of pH.

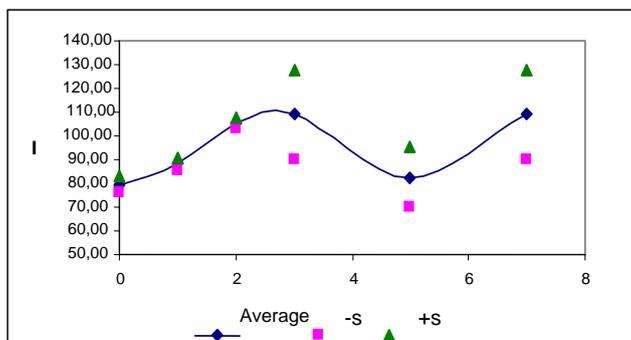


Fig. 7 Univariate plot of pH (NaOH  $0,002 \text{ mol L}^{-1}$  drops versus phosphorescence signal (average of the measurements  $\pm 1$  Std. deviation).

## 5.3. Analytical figures of merit

As the best experimental conditions were obtained, the validation of the method was done by obtaining the analytical figures of merit, which guaranteed best performance or method.

The concept of validation evolves continuously and it is defined by several authors as following:

“The validation must be guaranteed, through the experimental studies, that the method attends the requirements of the analytical application, ensuring trustful results” (ANVISA).

“Validation is a process to define an analytical requirement and to confirm that the method has the capability and consistency for a required application” (EURACHEM).

“Validation is confirmed through testing and by the presentation of objective evidence which ensures that the method has the proper requirements that fulfils its application for given intentional use” (Standard ISO/IEC 17025).

Inside of the general scope of method validation, it is possible to distinguish two types:

First, the validation in the laboratory (“in house validation”) that consists of validation steps made inside a laboratory validating a new method. This validation must be used in the preliminary stage of the method development, when all relevant performance characteristics are evaluated, except the interlaboratorial reproducibility.

The second type, the “full validation”, involves evaluation of performance characteristics and a interlaboratorial study, aiming to verify how the methodology behave through the analysis of a determinate matrix in various laboratories. This procedure establishes the reproducibility of the methodology and the associated expanded uncertainty.

The analytical parameters for method validation is being defined nationwide and abroad and they must be based on the intended application for the method. In this work, the following analytical figures of merit (performance parameters or performance characteristics) will be considered: detectability (through the limit of detection, LD, and limit of qualification, LQ), robustness, repetitivity (in the same day and in different days), selectivity and confidence intervals - uncertain measurement.

### 5.3.1. Linearity

In the linear range, the analytical signal is directly proportional to the concentration of the analyte being studied. In order to perform such study, a calibration or analytical curve must be constructed. For this analytical curve, the equation is defined by  $y = ax + b$ , where:  $a$  is the angular coefficient (the inclination of the curve or its sensitivity) and  $b$  is the linear coefficient (the interception of the curve to the  $y$  axis).

The determination of this equation is based on the study of the behavior of the two variables (analytical signal and analyte concentration) looking to identify if there is a defined relation between them.

The best known technique for this is as the linear regression using the minimum squares method, that it is a form to predict the best straight line passing through the

experimental points of the plot. The calculation of the regression coefficient is very important to prove that there is a linear relationship between the two variables under study. The closest to 1 is the value of this coefficient, the highest is the probability of a defined linear relationship. The correlation coefficient under 0,999 is considered as evidence of an ideal adjustment of the data to regression line. The ANVISA recommended a value equal or superior to 0,99 and the INMETRO consider 0,90 as the critical value to consider a linear relationship. In this work, the INMETRO critical value is considered.

Two consecutive analytical curves, made in the best experimental conditions for maximum chrysene phosphorescence, were made in order to evaluate the confidence of the method and the repetitivity of the results. A difference between the sensitivity of these curves under 10% indicated good repetitivity. In Fig. 8, one of the analytical curves is shown. The first curve, presented the following linear equation:  $y = 1,3 E+07x + 110,5$  and  $r^2 = 0,9526$ . For the second curve, the linear equation was  $y = 1,5 E+07x + 90,2$  and  $r^2 = 0,9424$ . It is important to point out that the magnitude of the standard deviation does not follow any tendency, indicating that there is no need for the use of weighted linear regression.

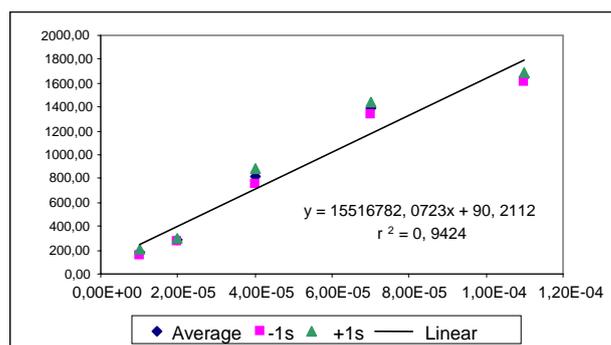


Fig. 8 Analytical curve showing the linear behavior between the chrysene phosphorescence signal in function of the amount of chrysene deposited on substrate (average  $\pm 1$  Std. deviation).

### 5.3.2. Detectability

Detectability indicates the capability of the method to discriminate samples containing similar but not equal amounts of analyte. This parameter depends on the inclination analytical curve (sensitivity), the angular coefficients obtained in the previous section. In this work, detectability is evaluated by the limit of detection and quantification (LD and LQ) which indicates the smallest signal from the analyte that can be identified, using a given statistical criteria. Depending on the criteria used, the blank signal magnitude is not considered, only its standard deviation. Then, such parameters are only useful to compare of different analytical methods

The limit of detection is the most commonly used criteria for detectability. According to the INMETRO, the LD represents the minor concentration of the substance in test that can be detected, but not necessarily quantified, using a specific analytical method. In this work, the LD was calculated using parameters of the analytical curves. This

parameter can also be expressed in terms of the effective mass deposited in the substrate (absolute limit of detection, ALOD) considering 5  $\mu\text{L}$  of sample

The LD can be expressed as:  $LD = 3 \times Std. Dev./\alpha$ , where: *Std. Dev.* is the standard deviation of ten replicates of the blank and  $\alpha$  is the inclination of the curve (sensitivity). In this work, the value of  $\alpha$  applied is the average of the two analytical curves described in the previous section.

The ALOD can be expressed as:  $ALOD = (3 \times Std. Dev. / \alpha) \times V \times MM$ , where: *V* is the deposited volume in the substrate ( $5\mu\text{L} \times 10^{-6}$ ) and *MM* is the analyte molar mass in  $\text{g mol}^{-1}$ .

The limit of quantification (LQ) represents the lowest analyte concentration that can be measured, using a specific analytical method. The LQ, as well as ALOQ (absolute limit quantification) can be calculated using the same criteria described for LD and ALOD but using the relation of 10:1 instead of 3:1. The LQ can be expressed as:  $LQ = 10 \times Std. Dev./\alpha$ . In a similar way to ALOD, the ALOQ can be expressed as:  $ALOQ = (10 \times Std. Dev. / \alpha) \times V \times MM$ .

The results of LD/ALOD and LQ/ALOQ in the use of the optimized experimental conditions are shown in Table 2.

**Table 2: Values of LD, LDA, LQ and LQA for chrysene using the optimized phosphorimetric method.**

		Ag <sup>+</sup>	
Blank average (Intensity sign)		24,73	
Std.deviation		3,02	
$\alpha$ (average)		1,46E+07	
LD	6,2E-07	LQ	2,06E-06
ALOD (1E-1ng)	7,07E-10	ALOQ (ng)	2,36E-09

### 5.3.3. Robustness

According to INMETRO, robustness is a measure of the sensibility that a method presents facing small variations of factors. Let's say that a method is robustness when it is not affected by a small and deliberated modification of a given parameter. In order to determine the method robustness the INMETRO recommends the Youden test. This test permits to evaluate the robustness and ranking of the factors in function of the influence in the final result. In this work, robustness of the method was evaluated through a similar statistical treatment: the multivariate study (section 5.2.), in other words, through a factorial design previously performed.

Taking into consideration the influence of the pH, 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 (comparison between tolerance and average band). Using this criteria, the method can be considered robust ( see Table 3).

**Table 3: Robustness evaluation in function of pH.**

[Ag <sup>+</sup> ] mol L <sup>-1</sup>	Range average	Tolerance	Variation (%)
0,03 - 0,05			
0,04 +/- 0,01	0,04	0,01	25

### 5.3.4. Repetitivity

The term repetitivity is adopted by International Metrology Vocabulary (VIM), with the approval of the INMETRO. As according to VIM, the repetitivity is an expression of precision (dispersion of the results), in order words, it is the degree of agreement among consecutive measurements of a same sample under the same experimental conditions. According to what is recommended by the INMETRO, at least 7 repetitions must be done in order to get a estimate of the standard deviation. In this study, the precision was estimated through the relative deviation standard (RSD %), also known as variation coefficient (CV), based on 10 measurements of the same sample.

In general, for the SSRTP technique 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 PAHs that can be degraded by the incidence of the excitation radiation, a 20% RSD value will be accepted. The result can be found on table 4.

**Table 4: Repetitivity study for the SSRTP method for chrysene.**

		Ag <sup>+</sup>
[Analyte] mol L <sup>-1</sup>		10 <sup>-5</sup>
Average (Intensity sign)		467,25
Std. deviation		90,69
RSD(%)		19

### 5.3.5. Selectivity

The selectivity of a method is its capability to detect, in an unequivocal way, the analyte of interest even if it is mixed with other components in a complex matrix. The selectivity study evaluates how potential interferent species (impurities, degradations 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 reevaluated continuously during the validation procedure and the subsequent use of the method.

A simulated sample solution containing chrysene ( $2\text{E}-05 \text{ mol L}^{-1}$ ) and an equivalent quantity of pyrene 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 samples containing higher proportions of pyrene, a matrix effect was observed, however, this interference can be properly corrected by the use of analyte addition technique (Fig. 9). In the Fig. 10 and Fig. 11 the absorption and the phosphorescence excitation and phosphorescence emission spectra of chrysene were shown.

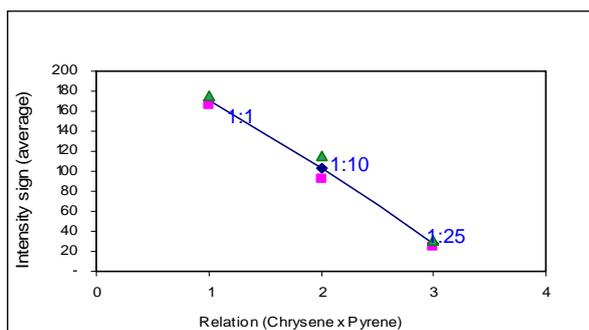


Fig. 9 Effect of increasing amounts of pyrene in the phosphorescence of chrysene (chrysene:pyrene 1:1, 1:10 and 1:25).

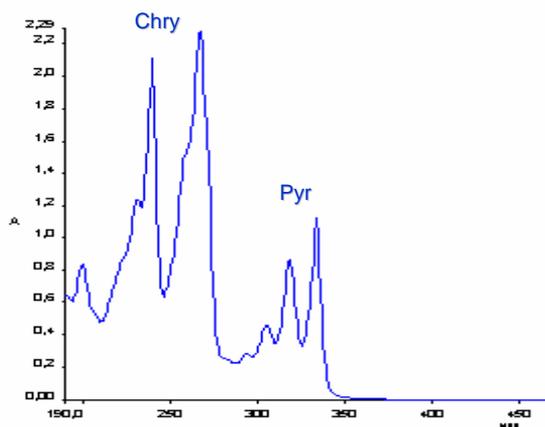


Fig. 10 Absorption spectrum of chrysene (Chry) and pyrene (Pyr)

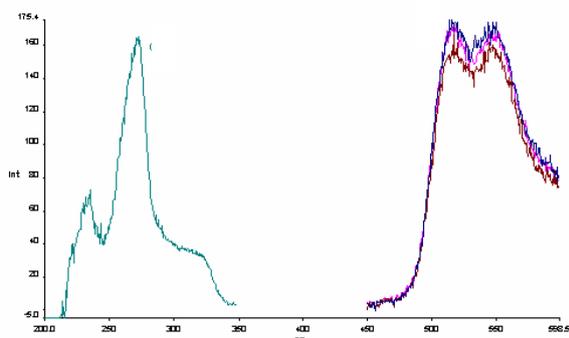


Fig. 11 Emission and excitation phosphorescence spectra of chrysene in presence of pyrene.

### 5.3.6. The average confidence intervals (Uncertainty Measurement - U)

The average confidence intervals (other expression of “precision”), is the range value where there is a given probability to find a true value of a variable, considering a specific confidence level (normally 95%). In this work, 10 repetitions of the same sample under the same experimental conditions were done. The U is calculated by equation:

$$U = \text{average} \pm t_{(95\%, n-1)} \times \frac{s}{\sqrt{n}}$$

where: *average* is the average sample’s reading,  $t_{(95\%, n-1)}$  is the critical value of the Student distribution for a level of 95% confidence and  $n-1$  degrees of freedom ( $t$  table  $(95\%, n-1)$

$= 9) = 2,26$ ),  $s$  is the estimation of the standard deviation of ten replicates ( $n = 10$ )

The results of  $U_{(95\%, n-1=9)}$  can be found in the Table 5.

Table 5: Uncertainty calculation for the chrysene using the SSRTP method.

	Ag <sup>+</sup>
Average (Intensity sign)	467,25
Std. deviation	90,69
$t_{(95\%, n-1)} \times \frac{s}{\sqrt{n}}$	64,81
U	467,25 +/- 64,81

## 6. CONCLUSION

The validation strategy must be planned taking into consideration the analytical technique to be applied, the specific matrix to be analyzed, the concentration (or amount) of the analyte of interest and the relative concentration (or amount) of potential interferent species.

The multivariate optimization has shown to be very useful for the evaluation of the effect of each variable as well as the interactions among variables on the phosphorescence signal of chrysene. Such study allowed an effective optimization of the analytical method and the achievement of best analytical performance (evaluated by the validation parameters).

According to the obtained results (section 5.3.5), silver (I) can be used as selective HA for the determination of chrysene in presence of pyrene, indicating the successful application of the optimized SSRTP method.

The sequence of this work will include the use of the Certificate Reference Material (CRM) to establish the traceability to acquire complete accuracy of the method in the determination of PHAs. Further studies including proficiency test schemes will also be included in order to get a full evaluation not only of the repetitivity and reproductivity among labs, but also systematic errors (tendency).

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