Experimental design for optimizing MALDI-TOF-MS analysis of palladium complexes

Tijana M. Rakić-Kostić[1], Jovana V. Bogojeski[2], Iva A. Popović[1], Maja D. Nešić[1], Boris M. Rajčić[1], Marija R. Nišavić[1], Marijana Ž. Petković[1], Suzana R. Veličković[1]

[1]*University of Belgrade, Institute of Nuclear Sciences "Vinča", Belgrade, Serbia*
[2]*Institute of Chemistry, Faculty of Natural Sciences, University of Kragujevac, Kragujevac, Serbia*

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Abstract

This paper presents optimization of matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer (MS) instrumental parameters for the analysis of chloro(2,2',2"-terpyridine)palladium(II) chloride dihydrate complex applying design of experiments methodology (DoE). This complex is of interest for potential use in the cancer therapy. DoE methodology was proved to succeed in optimization of many complex analytical problems. However, it has been poorly used for MALDI-TOF-MS optimization up to now. The theoretical mathematical relationships which explain the influence of important experimental factors (laser energy, grid voltage and number of laser shots) on the selected responses (signal to noise – *S*/*N* ratio and the resolution – *R* of the leading peak) is established. The optimal instrumental settings providing maximal *S*/*N* and *R* are identified and experimentally verified.

Keywords: MALDI-TOF-MS, design of experiments (DoE), optimization, metallo-drugs.

The quality of the mass spectra obtained by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) is under strong influence of several key factors, such as the type and concentration of both matrix and the analyte, their mole ratio, technique used for the sample deposition (dried droplet, sandwich, double sandwich or thin layer) [1]. However, different spectra quality can be obtained for the fixed combination of the matrix/analyte molar ratio and technique of sample deposition by adjustment of instrument parameters. Experimentally, the influence of instrumental parameters can be studied by varying (increasing/decreasing) one parameter while the remaining settings are on the fixed level. This approach has many disadvantages: the information about the spectra quality is obtained only for a few parameter settings, while there is no data about the system behavior for the settings between investigated points. Moreover, the potential interaction of the factors cannot be seen since they are not varied simultaneously.

Design of experiments (DoE) methodology provides systematic approach for the investigation of instrumental parameters influence on the spectra quality and identification of the settings that provide the most reliable data [2,3]. To date, the DoE methodology has been applied to the optimization of chemical factors during the development of analytical methods, such as, chromatographic [4–7] electroanalytical [8–11], thermogravimetry [12], and liquid–liquid extraction [13,14]. Additionally, the DoE is used for the optimization of instrumental parameters of equipment for analysis at graphite furnace atomic absorption spectrometer (GF AAS) [15,16], inductively coupled plasma optical emission spectrometer (ICP OES) [17,18], inductively coupled plasma mass spectrometer (ICP-MS) [19], and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) [1,20–25]. This methodology involves performing of small number of carefully planned experiments, which allows the establishment of mathematical relationship between investigated factors and important system responses. In that way, the influence of each parameter is quantified, and created relationship allows theoretical examination of experimental space searching for the optimal solutions, without the necessity for performing additional experiments.

In this work, DoE methodology is applied for the optimization of MALDI-TOF instrumental parameters for the analysis of one of the potential metallo-drugs, *i.e.*, $[Pd(terpy)Cl]Cl\cdot 2H_2O$, the structure of which is given in Figure 1.

DoE methodology and related chemometrical strategies are poorly used for MALDI-TOF-MS optimization up to now [1,20–25], and to the best of our knowledge this is the first time that this approach is applied for the MALDI MS investigation of transition metal complexes.

Correspondence: S.R. Veličković, University of Belgrade, Institute of Nuclear Sciences "Vinča", Department of Physical Chemistry, Mike Petrovića Alasa 12–14, P.O. Box 522, 11001 Belgrade, Serbia.
E-mail: vsuzana@vinca.rs

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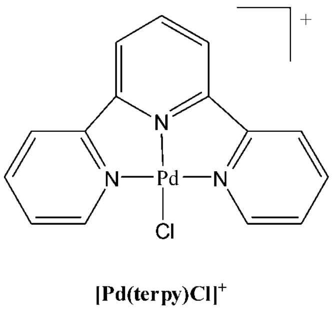

Figure 1.Chemical structure of analyzed Pd metal complex.

EXPERIMENTAL

Chemicals

The complex, chloro(2,2',2"-terpyridine)palladium(II) chloride dihydrate [Pd(terpy)Cl]Cl·2H_2O was prepared by the earlier published procedure [26] and recrystallized from methanol/water (1:1) mixture. The purity of the complex was checked by elemental microanalyses, IR and ^{1}H-NMR spectroscopy.

Matrix for MALDI-TOF-MS was (5,10,15,20-tetrakis-(pentafluorophenyl)porphyrin, MTTP. This matrix, methanol and chloroform were purchased from Sigma Aldrich (Taufkirchen, Germany) and were used without further purification. Working solution of Pd-complex was prepared dissolving in pure methanol in order to obtain the concentration of 1 mg mL^{-1}.

Equipment

All mass spectra were acquired in positive reflector mode on Voyager Biospectrometry DE Pro Workstation (PerSeptive Biosystems, Framingham, MA, USA) MALDI-TOF mass spectrometer. The mass spectrometer was equipped with a pulsed nitrogen laser operated at 337 nm with 3 ns duration pulses, and with 20.0 Hz laser repetition rate. The laser intensity (laser operates in the range from 750 to 3000 W/cm^2) and number of laser shots must be adjusted. Time of flight mass analyzers was operated on the delayed-extraction (DE) mode. Extraction delay time (time between laser flash and ion extraction) may be in the range 25–1000 ns. In this experiment in all cases the delay time of 100 ns was used. An accelerating voltage is applied to the sample plate in the range 15–25 kV. Besides, a grid 1–2 mm above the sample plate exists with an additional voltage to fine-tune ion acceleration; this voltage is called grid voltage. The value of grid voltage is expressed as a certain percentage of the accelerating voltage.

Acquisition of MALDI-TOF spectra

Spectra of the complex was obtained by applying 0.5 μL of working solution onto the MALDI sample plate, followed by the addition of 0.5 μL MTTP solution (prepared dissolving MTTP in chloroform in order to obtain concentration of 10 mg mL^{-1}). Samples were left to evaporate and co-crystallize at room temperature. Spectra were calibrated by setting the peak of the 2,5-dihydroxybenzoic acid to its appropriate value (155.034 Da).

Software

Box–Behnken design (BBD) was selected like an appropriate experimental design in DoE methodology for optimization of MALDI-TOF-MS instrumental parameters. The BBD was performed using Design-Expert® 7.0.0 software (Stat-Ease Inc., Minneapolis, MN, USA). Three-dimensional (3D) response surfaces were obtained by using Statistica 7. For grid search point calculation, MATLAB was used.

RESULTS AND DISCUSSION

In the first phase of the research, preliminary experiments allowed the selection of MTTP matrix as a suitable for the analysis of chloro(2,2',2"-terpyridine)-palladium(II) chloride dihydrate [27]. The leading peak with the most intense signal is identified and its position was *m*/*z* 376.1, which corresponds to the complex with a loss of one Cl^- ion (discussed later in text). Two responses are selected as relevant for the quality of the obtained spectra: signal to noise ratio (*S*/*N* signifies the level of a desired signal to the level of background noise) and resolution (*R* represents the ability of a mass spectrometer to distinguish between the ions of different *m*/*z* ratios). Since palladium metal complexes have higher number of isotopes, achieved resolution is of particular importance. For instance, a signal that arises from tested complex is composed of 9 individual peaks with various intensities [27], which correspond to theoretical presentation of the same spectra (data not shown). Thus, the optimization aim was the simultaneous maximization of both *S*/*N* and *R*. Due to the well-known variability of MALDI-TOF-MS spectra which interfere with adequate quantification, the coefficient of variation (*CV*) obtained for triplicate of identical combination complex-matrix-instrumental parameters was also monitored.

It is well known that variety of instrumental parameters can affect the spectra quality. For this reason, in the second phase, optimal values of input variables, namely, the laser intensity, the grid voltage and the number of laser shots were determined using DoE methodology in order to obtain the best values of *S*/*N* and *R* of the peak of interest within the range defined previously. The values of input variables are defined according to the earlier experience, that is, on the basis of the results obtained in preliminary experiments (Table 1). The DoE methods are often run at different values of input variables (or factors), which are called levels. Each level is placed at one of three equally spaced values usually coded as –1, 0, +1 (Table 1). Each run of a DoE method involves a combination of the levels of the selected factors.

Table 1. Investigated instrumental parameters (three input variables) and their levels

Input variable or factor	Lower level (–1)	Zero level (0)	Higher level (+1)
Laser intensity (x_1)	1100	1300	1500
Grid voltage (x_2)	70	90	80
Number of laser shots (x_3)	300	350	400

After the identification of input factors and responses of interest (*S*/*N* and *R*) the experimental plan is created following Box–Behnken design [28]. This design is based on three-level incomplete factorial designs where two factors are arranged in a full two-level design, while the level of the third factor is set at zero (Figure 2). Points on the diagram represent the experimental runs that are done. It is nearly rotatable and especially useful when the points on one or more corners of the cube represent factor–level combinations that are for any reason unacceptable. Since Box–Behnken design investigates each factor on three levels, the definition of quadratic dependence of responses on investigated factors is enabled. For the investigation of three factors, twelve experiments plus central point replications (in this case three replications are selected) are required. The experimental plan defined by the applied design is presented in Table 2.

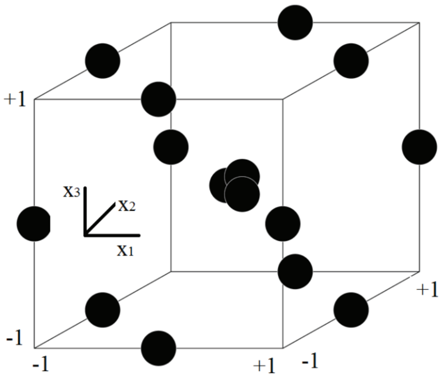

Figure 2. Schematic presentation of Box–Behnken experimental matrix for three factors.

Experiments are performed randomly and *S*/*N* and *R* values are obtained. Each factors combination is investigated in triplicate and the values of coefficient of variation (*CV*) are calculated. Multiple linear regression and least squares methods of approximation are used to create mathematical relationship between investigated the input factors and the responses. In the case of *S*/*N* response, the following equation in terms of coded factor values is obtained:

$$y_1 = 5582.91 - 1390.56x_1 - 426.52x_2 + 405.83x_3 + \\ +734.13x_1x_2 - 2180.43x_1^2 - 518.84x_2^2 \quad (1)$$

where y_1 is the response *S*/*N* and x_i investigated factors (Table 1).

Table 2. Experimental plan according to Box–Behnken experimental design

Experiment	Laser intensity W/cm^2	Grid voltage %	Number of laser shots
1	1100	70	350
2	1500	70	350
3	1100	90	350
4	1500	90	350
5	1100	80	300
6	1500	80	300
7	1100	80	400
8	1500	80	400
9	1200	70	300
10	1300	90	300
11	1300	70	400
12	1300	90	400
13	1300	80	350
14	1300	80	350
15	1300	80	350

The same equation for real factor values has the following form:

$$\frac{S}{N} = -71961.56 + 105.41 \times \text{Laser intensity} \\ + + 310.32 \times \text{Grid voltage} + \\ +8.1 \times \text{No. of laser shots} + \\ +0.37x_1x_2 - 0.05 \times (\text{Laser intensity})^2 - \\ -5.19 \times (\text{Grid voltage})^2 \quad (2)$$

Mathematical equation for real factor values enables easy navigation of experimental space and calculation of the response by simple inserting of factors values. However, coefficients in this equation are influenced by different scales of factor values. Therefore, differences in magnitude of these coefficients cannot be used for factors comparison. On the other hand, equation in terms of coded factor levels enables comparison of factors influence on the response based on coefficient in front of each term. The larger the coefficient is, the greater influence will exhibit the factor.

The mathematical model in terms of coded factor values for the resolution is created in the following way:

$$y_2 = 1880.08 - 1060.00x_1 - 528.67x_2 + 277.62x_1x_2 \quad (3)$$

Where y_2 is the response R and x_i investigated factors (Table 1).

The same equation for real factor values could be written as:

$$\begin{aligned} &R = 27435.91 - 16.41\times\text{Laser intensity} - \\ &-233.32\times\text{Grid voltage} + \\ &+0.14\times\text{Laser intensity}\times\text{Grid voltage} \end{aligned} \tag{4}$$

The *p*-value is the basic for interpreting statistical results. The response models are created removing model terms whose coefficients had *p* value greater than 0.1, since they are considered unimportant. Statistical adequacy of the obtained relationship is confirmed by high values of coefficient of determination (>0.94), adjusted coefficient of determination (>0.89) and non-significant lack of fit test. The absolute value of the obtained coefficients in coded factor values explains the intensity of particular factor or interaction influence. Generally the factors also might have “interact”, for example, a two-factor interaction occurs when the influence of one factor on the response is different at different levels of the second factor. Taking into account obtained results on basic of p-value, and also, keeping in the mind that certain factors have significant influence if $p < 0.05$, it can be concluded the following: the greatest influence on the signal to noise ratio exhibited laser intensity (both linear and quadratic term had *p* value < 0.0001), followed by the interaction between laser intensity and grid voltage (p = 0.02). Grid voltage by itself exhibited lower influence (*p* value 0.049 for linear and 0.09 for quadratic term), while the impact of number of laser shoots was characterized by *p* value around 0.06. In the case of response *R*, it is shown that number of laser shots does not have significant influence, while the linear terms of the remaining two factors appeared to be significant (*p* value < 0.001). Moreover, the interaction between laser shots and grid voltage had borderline significance (p = 0.1). Response surface plots enable simpler visualization of investigated factors influence and they are presented in Figure 3.

The response, *S*/*N*, exhibits quadratic dependence on laser intensity and number of laser shots. It can be seen on Figure 3 that the saddle point of both quadratic functions was within the investigated region, indicating that increasing these factors up to the certain level, the value of *S*/*N* increases, while the increase of these factors after the saddle point leads to decrease in *S*/*N*. On the other hand, the increase of number of laser shots leads to the moderate linear increase of *S*/*N*. It is important to notice the significance of laser shots and

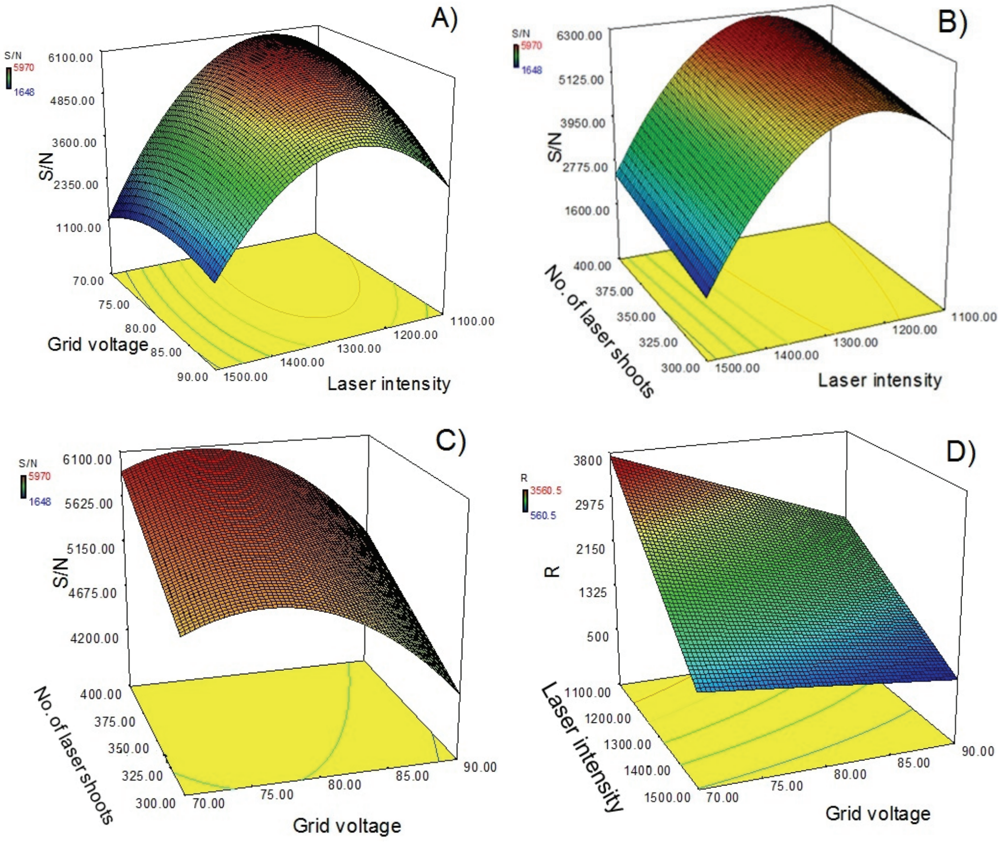

Figure 3. Response surface plots presenting the dependence A) $S/N = f(x_1, x_2)$; B) $S/N = f(x_1, x_3)$; C) $S/N = f(x_2, x_3)$; D) $R = f(x_1, x_2)$, where x_i are the input variables or factors: x_1 is laser intensity, x_2 is grid voltage, and x_3 is number of laser shoots.

grid voltage interaction since the influence of simultaneous change in these two factors is different than their individual influences.

As far as resolution is concerned, the linear influence of laser intensity and grid voltage and their interaction can be seen in Figure 3, as well.

As far as the coefficient of variation (*CV*, a statistical measure of the dispersion of data points in a data series around the mean) is concerned, it is estimated for both *S*/*N* and *R*. However, statistical analysis has shown that the overall mean is better prediction of both *CV* than the mathematical models involving examined factors. Therefore, the system variability was not under control of investigated instrument parameters.

Response surface plots for *S*/*N* and *R* presented in Figure 3 can be used for identification of the optimal instrumental settings where maximal *S*/*N* and maximal resolution could be achieved. However, it can be seen that settings providing maximal *S*/*N* are different from those providing maximal *R*. Therefore, in order to resolve such multiobjective optimization problem the priority is given to *S*/*N* and the solution is searched as the point where maximal *S*/*N* value with satisfactory *R* value could be found. In this study, among several potentially adequate solutions, the following settings are chosen: laser intensity – 1220 W/cm^2, grid voltage – 79% and number of laser shots – 360.

The theoretical value of responses in this point is estimated by created mathematical models to be 5938 for *S*/*N* and 2368 for *R*. The experimental verification of this settings is performed in five replicates and one of the obtained spectra is presented in Figure 4.

Positive ion MALDI TOF mass spectrum of [Pd(terpy)Cl]Cl$\cdot$2H$_2$O complex is dominated by the signal at *m*/*z* 376.1, that corresponds to the cation [Pd(terpy)Cl]$^+$. Much smaller signal that arises from [Pd(terpy)]$^+$ can be detected at *m*/*z* 340.7. Other signals, arising from matrix are indicated by an asterisk. Inset of the Figure 4 represents the expanded region of the signal at *m*/*z* 376.1, which is, in fact, composed of several isotopes (*m*/*z* indicated in the Figure 4).

The average experimentally obtained value of signal to noise ratio was 5850, while the average value of resolution was 2456. The deviation of experimental *S*/*N* value from theoretical was 1.5%, while the deviation of *R* was calculated to be 3.7%. Moreover, the obtained signal to noise ratio in located optimal point is significantlly greater than all *S*/*N* values obtained for instrument settings combinations presented in Table 2. Therefore, the DoE methodology proved to be valuable assistance in optimization of MALDI-TOF-MS instrumental settings for the analysis of Pd metal complex.

CONCLUSION

In presented work, we investigated the influence of several MALDI-TOF-MS instrumental settings on the quality of obtained spectra of Pd metal complex by experimental design methodology. It is shown that signal to noise ratio is under strong quadratic influence of laser intensity, and moderate influence of grid voltage and number of laser shots. Resolution appeared to be linearly dependent only on laser intensity and grid voltage. Coefficients of variation of both *S*/*N* and *R* were not affected by the examined instrumental settings. Analyzing response surfaces created for *S*/*N* and *R* optimal instrumental settings with maximal *S*/*N* and

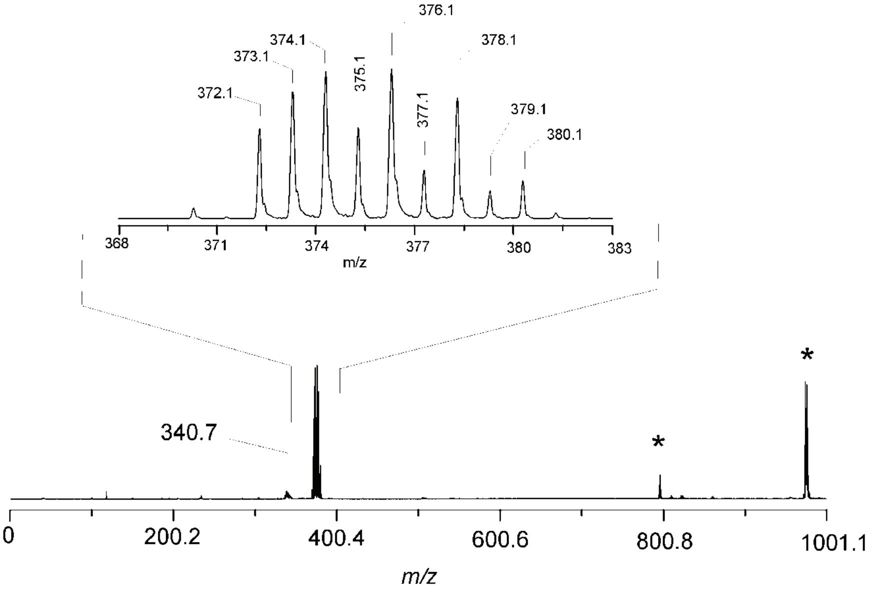

Figure 4. Positive ion MALDI-TOF mass spectrum of Pd metal complex obtained for optimal instrumental settings. Signals are indicated by their position, whereas matrix signals are indicated by an asterisk. Inset represents expanded m/z region from 368 to 380. To illustrate mass resolution achieved under experimental conditions.

satisfactory *R* are located. Experimental verification proved high agreement of theoretically predicted and experimentally obtained response values.

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IZVOD

PRIMENA EKSPERIMENTALNOG DIZAJNA ZA OPTIMIZACIJU MALDI-TOF-MS ANALIZE METALNIH KOMPLEKSA PALADIJUMA

Tijana Rakić-Kostić[1], Jovana Bogojeski[2], Iva A. Popović[1], Maja D. Nešić[1], Boris M. Rajčić[1], Marija Nišavić[1], Marijana Ž. Petković[1], Suzana R. Veličković[1]

[1]Univerzitet u Beogradu, Institut za nuklearne nauke „Vinča”, Laboratorija za fizičku hemiju, Beograd, Srbija,
[2]Univerzitet u Kragujevcu, Prirodno-matematički fakultet, Institut za hemiju, Kragujevac, Srbija

(Naučni rad)

U okviru ovog rada ispitane su mogućnosti korišćenja matematičke metodologije dizajniranja eksperimenta (Design of Experiments (DoE) methodology) za optimizaciju instrumentalnih parametara kod spektroskopije masa sa laserskom desorpcijom i jonizacijom uz pomoć “matrice” (MALDI-MS*)*, za analizu [Pd(terpy)Cl]Cl$\cdot$2H_2O kompleksa. Navedeni metalni kompleks predstavlja potencijalni lek u antikancer terapiji, tako da povećanje efikasnosti razvoja metoda njegove analize/karakterizacije, kao i uspostavljanje uslova za analizu proizvoda njegove interakcije sa biomolekulima, predstavljaju važan zadatak. DoE metodologija obezbeđuje sistematsko ispitivanje uticaja izdvojenih instrumentalnih parametara (predstavljaju ulazne promenljive ili faktore) na ključne karakteristike kvalitetnog spektra (tzv. “odgovor”) u cilju dobijanja pouzdane identifikacije. Eksperimentalno, spektar je poznat samo na tačno odabranim vrednostima instrumentalnih parametra (u određenom rangu), ali se ne dobijaju informacije o spektru za vrednosti između pomenutih parametra. Ispitivanje uticaja vrednosti instrumentalnih parametara, između onih već ispitanih, na kvalitet spektra, eksperimentalno predstavlja izuzetno komplikovan postupak, zbog velikog broja kombinacija. DoE metodologija uspostavlja matematičke relacije između odabranih faktora i “odgovora”, na osnovu malog broja pažljivo isplaniranih eksperimenata (*Box-Behnken design*-pristup). U analitičkoj hemiji, ova metodologija uspešno je primenjena za optimizaciju hemijskih faktora kod hromatografskih, elektroanalitičkih, termogravimetrijskih i drugih metoda. DoE pristup, do sada, primenjen je za optimizaciju instrumentalnih parametara u slučaju, atomske apsorpcione spektrometrije sa grafitnom kivetom, indukovano kuplovane plazme optičke emisione spektrometrije, indukovano kuplovane plazme spektrometrije masa, kod MALDI spektrometrije masa, ali ne za detekciju kompleksnih jedinjenja. Analiza kompleksa prelaznih metala uz pomoć MALDI-TOF-MS je poseban izazov zbog velikog broja signala koji potiču od izotopa prelaznih metala, tako da je rezolucija koju treba postići od velikog značaja. Ulazni faktori (promenljive) u ovom radu bili su: intenzitet lasera, broj udaraca lasera i napon na “gridu”, a važne karakteristike spektara, kao što su odnos signal/šum i rezolucija, predstavljaju traženi “odgovor”. Raspon vrednosti ulaznih faktora određen je na osnovu preliminarnih eksperimentalnih podataka. Rezultati su pokazali da vrednosti odnosa signal/šum pokazuju jaku kvadratnu zavisnost od intenziteta lasera i umereno zavise od napona na “gridu” i broja udaraca lasera. Rezolucija linearno zavisi od intenziteta lasera i napona na “gridu”. Među nekoliko potencijalnih rešenja izabrani inetenzitet lasera od 1220, napon na “gridu” od 79% i 360 udaraca lasera predstavljaju optimalne uslove za dobijanje kvalitetnog spektra [Pd(terpy)Cl]Cl$\cdot$2H_2O kompleksa, što je kasnije i eksperimentalno potvrđeno.

Ključne reči: MALDI-TOF-MS • DoE metodologija • Optimizacija • Lekovi na bazi metala