Introducing an interesting and novel strategy based on exploiting first-order advantage from spectrofluorimetric data for monitoring three toxic metals in living cells

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A R T I C L E   I N F O

Handling Editor: Lawrence Lash-7LLAS

Keywords: Lead, Zinc, Cadmium, Determination, Chemometrics, HeLa Cells

A B S T R A C T

In this work, we did our best to develop a novel and interesting analytical method based on coupling of spectrofluorimetry with first-order multivariate calibration techniques for simultaneous determination of lead (Pb), zinc (Zn) and cadmium (Cd) in HeLa cells. To achieve this goal, quenching of the emission of graphene (GR) was individually investigated in the presence of Pb, Zn and Cd and then, according to the linear ranges obtained from individual calibration graphs, a multivariate calibration model was developed based on modeling of the quenching of the emission of GR in the presence of the mixtures of Pb, Zn and Cd. First-order multivariate calibration models were constructed by partial least squares (PLS), principal component regression (PCR), orthogonal signal correction-PLS (OSC-PLS), continuum power regression (CPR), robust continuum regression (RCR) and partial robust M-regression (PRM) and their performances were evaluated and statistically compared. Finally, the OSC-PLS was chosen as the best model with the best practical performance for analytical purposes.

1. Introduction

Nanomaterials have strange and valuable properties compared with bulk materials and because of that are widely used for different purposes especially for sensing purposes [1–10]. Graphene is a two-dimensional carbon nanomaterial which is not only a flexible structure but also is a robust structure which make it to be very useful for different applications [1]. The graphene can be existed in different structures such as graphene oxide, graphene quantum dots and graphene nanoplatelets [2, 3]. The graphene because of having good electrical, thermal and optical properties, has a great potential for application to developing transistors [2, 4], chemical and electrochemical sensors [5] and biological sensors [6]. The graphene has some extra applications in surface coatings for inhibiting corrosions [7,8] and to reduce wear and friction on sliding metal surfaces [9,10]. The graphene sheets with lateral dimensions less than one hundred nanometers are called graphene quantum dots (GR) which have new chemical and physical properties such as high stability, good solubility, low toxicity, photoluminescence and excellent biocompatibility.

Heavy metals are existed in the earth’s crust but their geochemical cycles and biochemical balance have been significantly affected by human activities. Sometimes, the heavy metals are considered as contaminants which can be hazardous for human health therefore, monitoring of them is important. Lead (Pb) and cadmium (Cd) are heavy metals which are widely and naturally distributed toxic metals. There are some reports on determination of these metals with zinc (Zn) [11]. The Zn is one of the most abundant metals in the human body which is a vital element for growth. There are more than 300 enzymes in human body whose active sites contain the zinc ions and Zn has an important role in synthesis of DNA and RNA and protein and in cell division as well. Therefore, determination of these three metal ions is interesting and so important. Determination of heavy metals is usually performed by atomic absorption spectroscopy (AAS), inductively coupled plasma, atomic emission spectroscopy, X-ray fluorescence spectroscopy and mass spectroscopy which need expensive instruments which can’t be accessible in most of all of laboratories therefore, developing new analytical methods which are fast, low-cost and accessible is sensible.

HeLa is an immortal cell line which is the most commonly used
human cell line in scientific research. The HeLa cell line is durable and prolific which make it to be extremely suitable for scientific research. Therefore in this study, we have used the HeLa cells as a very interesting case for developing a novel analytical method for simultaneous determination of the Pb, Cd and Zn.

Chemometrics combines chemical data with mathematical and statistical methods to extract useful information which can help the chemists to better justify their observations. Chemometricians have performed different projects by the use of instrumental data [12–23]. In this project, we are going to couple first-order chemometric multivariate calibration techniques with spectrofluorimetric data to develop a novel analytical method for simultaneous determination of the Pb, Cd and Zn in HeLa cells. To achieve this goal, the GRs were uptaken by HeLa cells and then, Pb, Cd and Zn were individually uptaken and fluorescence quenching of the GRs was recorded in the presence of the metals to obtain individual calibration graphs. Then, a mixture design was used to multivariate calibration of the quenching of the GRs in the presence of Pb, Cd and Zn simultaneously. The spectrofluorimetric responses of the mixtures were modeled by partial least squares (PLS), principal component regression (PCR), orthogonal signal correction-PLS (OSC-PLS), continuum power regression (CRP), robust continuum regression (RCR) and partial robust M-regression (PRM) to build multivariate calibration models and finally, their performance were compared and the best multivariate calibration model was chosen for practical purposes. Schematic representation of the steps described above are shown in Scheme 1.

2. Experimental

2.1. Chemicals

Trypsin-EDTA, Dulbecco’s modified Eagle’s medium (DMEM/F-12 (1:1)), fetal bovine serum (FBS, 10%), penicillin-streptomycin (PEN-STREP), zinc nitrate hexahydrate, cadmium nitrate tetrahydrate and Pb (NO$_3$)$_2$ were purchased from Sigma. Commercial Pb, Cd and Zn standards (1 g l$^{-1}$) were prepared from Merck. Graphene quantum dots (blue luminescent) were purchased from Sigma-Aldrich. The other chemicals which were needed for doing this project were available in archive of our laboratory which had been purchased from Sigma or Merck. Doubly distilled water was used wherever water was needed. A phosphate buffer solution (PBS, 0.01 M) was prepared from Na$_2$HPO$_4$ and its pH was adjusted at 7.4 by the use of H$_3$PO$_4$ and NaOH.

2.2. Instruments and software

Spectrofluorimetric data were recorded by a Cary Varian spectrofluorimeter equipped with a quartz cell (1 cm length path). First-order multivariate calibration algorithms including PLS, PCR, OSC-PLS, CPR, RCR, PRM, smoothing of the data and elliptical joint confidence region (EJCR) were run in MATLAB (Version 7.5) by the use of a series of m-files. The first-order multivariate calibration algorithms have been run in MATLAB with the help of PLS-toolbox or TOMCAT. The HeLa cells were prepared from the cell bonk of Kermanshah University of Medical Sciences. Then, the flask was transferred into a culture room where a deep-freezer (–80 °C), a memmert incubator, a JTLV CZS hood and a Motic microscope were existed for cell culturing. pH adjustments were performed by a Jenway pH meter 3510. Performance of the developed
Toxicology Reports 9 (2022) 647–655

methodology was compared with the results of an Agilent atomic absorption spectrometer as reference method (AAS). Operating conditions for the AAS were: PMT voltage (450 V), slit width (0.40 nm), lamp current (9.0 mA), sample volume (20 µl), purging gas (argon), sample injection replicates (2) and measurement (peak height). All the calculations which were needed for data processing were performed on a Dell XPS laptop.

2.3. Procedure

Dispersion of the HeLa cells were performed in DMEM + FBS (10%) + PEN-STREP (1%) and seeded on five confocal dishes and then, they were incubated at an humidified atmosphere (5% CO₂ + 95% air) at 37 °C during a day (24 h). For uptaking the GR, 100 ng mL⁻¹ GR was added to different culture dishes and incubated at different times and then, the cells were washed with PBS (0.01 M, pH 7.4) and left to be in the PBS.

For simultaneous determination of Pb, Cd and Zn in HeLa cells, the seeded cells were allowed to grow during a day (24 h) and 1 mL DMEM having 1300 ng mL⁻¹ GR was used to replacing the culture medium of each dish and for uptaking the GR, the procedure was continued by incubating the dishes in an incubator for 2 h. Afterwards, the extra amounts of GR were removed by washing the dishes with the PBS for three times. Then, 1 mL DMEM having different concentrations of Pb, Cd and Zn (for all the three metals: 700–1600 ng mL⁻¹, with an interval of 100 ng mL⁻¹) were added to the dishes. The cells were further incubated for 2 h and washed with the PBS for three times and kept in the PBS. Spectrofluorimetric monitoring of the Pb, Cd and Zn was performed by excitation at 405 nm. For performing background correction on the data, the control cells which had not been incubated with GR (didn’t

Fig. 1. The images related to: (A) the control cells, (B) the cells which uptook 1300 ng mL⁻¹ GRs, (C) the cells which uptook 1300 ng mL⁻¹ GRs and 500 ng mL⁻¹ Pb, (D) the cells which uptook 1300 ng mL⁻¹ GRs and 500 ng mL⁻¹ Zn, (E) the cells which uptook 1300 ng mL⁻¹ GRs and 500 ng mL⁻¹ Cd, and (F) the cells which uptook 1300 ng mL⁻¹ GRs, 500 ng mL⁻¹ Pb, 500 ng mL⁻¹ Zn and 500 ng mL⁻¹ Cd.
have any GR) was prepared. The procedures described above were continued by digestion of the treated and control cells with trypsin and then, the cells were kept in the PBS. Afterwards, the cells were counted, broken by ultrasonic and centrifuged. Finally, the supernatant of cells were measured spectrofluorimetrically.

2.4. Theoretical details in brief

In this work, we are going to develop a novel spectrofluorimetric method assisted by chemometric methods which will enable us to simultaneously determine Pb, Cd and Zn in living cells. Data treatment and development of multivariate calibration models must be very carefully performed to achieve the final goal. Prior to data modeling, all the spectrofluorimetric data were treated according to the following equation [24]:

\[ F_{\text{Cor}} = F_{\text{Obs}} \exp\left(\frac{A_{\text{ex}} + A_{\text{em}}}{2}\right) \]  

All the data used in this work after passing this correction step was used for the next steps. Emission of the control cells was subtracted from the emission of the all of the cells and the corrected emissions were used for developing multivariate calibration models. Background correction was performed on the whole of data by subtracting emission of the control cells from emission of the whole of sets. Performance of the calibration models will be compared by the use of the following equations (RMSEP: root mean square error of prediction and REP: relative error of prediction):

\[ y = A x + B \]

\[ R^2 = 1 - \frac{\text{SSE}}{\text{SST}} \]

\[ \text{RMSEP} = \sqrt{\frac{1}{n} \sum (y_i - \hat{y}_i)^2} \]

\[ \text{REP} = \frac{1}{n} \sum \left| \frac{y_i - \hat{y}_i}{y_i} \right| \]
\[ \text{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n} (y_{\text{pred}} - y_{\text{act}})^2}{n}} \]  

\[ \text{REP}(\%) = 100 \frac{1}{y_{\text{mean}}} \sqrt{\frac{1}{n} \sum_{i=1}^{n} (y_{\text{pred}} - y_{\text{act}})^2} \]

where \( y_{\text{act}} \) and \( y_{\text{pred}} \) are nominal and predicted concentrations, respectively, and \( y_{\text{mean}} \) is the mean of the nominal concentrations. \( n \) are the number of samples in the validation set. Precision and accuracy of the developed calibration models will be compared according to the ellipses of the EJCR as well. Univariate calibrations and multivariate calibration and validation sets were performed in internal medium of the cells and by digestion of the cells with trypsin, the medium was extracted. This is a very important advantage which causes having a same medium for calibration and validation of the method which can help us for exploiting first-order advantage.

### Table 1

| Run | Cd  | Pb  | Zn  |
|-----|-----|-----|-----|
| C₁  | 700 | 700 | 700 |
| C₂  | 700 | 1100| 1500|
| C₃  | 700 | 1500| 700 |
| C₄  | 1500| 700 | 1100|
| C₅  | 1500| 700 | 1500|
| C₆  | 1500| 1500| 1100|
| C₇  | 1500| 1500| 1500|
| C₈  | 1100| 1100| 1100|
| C₉  | 1100| 1100| 1100|

### 3. Results and discussion

#### 3.1. Individual calibration graphs

Generally, developing a novel analytical method needs a calibration step by which an instrumental signal is connected with concentration of the analyte of the interest. Therefore, in this project, at the first step, we must calibrate the spectrofluorimetric response of the GR with concentration of the Pb, Cd and Zn. This goal can be achieved by recording spectrofluorimetric responses of the GR in the presence of Pb, Cd and Zn individually. Building the individual calibration curves needed some complicated steps which will be expanded in this section.

The HeLa cells which had uptaken 1300 ng mL\(^{-1}\) GR, were used to uptaking different concentrations of Pb, Cd and Zn from 700 to 1600 ng mL\(^{-1}\). The images related to the control cells, the cells which uptook GRs, the cells which uptook GRs and Pb, the cells which uptook GRs and Zn, the cells which uptook GRs and Cd and the cells having all of the three metals are shown in Fig. 1 A-F, respectively. As can be seen, obvious variations were observed among the images which confirmed successful uptaking GR and metals in HeLa cells.

After observation of the appearance of the cells microscopically, the broken cells were monitored spectrofluorimetrically. It should be noted that prior to selection of the optimum concentration of the GR for having the best emission, its concentration was varied and its emission was recorded as the data shown by Fig. 2 A. Variation of the emission of the GR versus concentration of the GR is shown by Fig. 2 B, and as can be seen, the graph is increased and leveling off which helped us to choose 1300 ng mL\(^{-1}\) as the optimum concentration of the GR. Afterwards, the broken cells having GR and Pb, Cd and Zn were monitored individually to build the individual calibration graphs which are shown in Fig. 2 C-H. The calibration graphs gave us the linear ranges where emission of the...
GR was linearly correlated with concentration of the Pb, Cd and Zn which will be used for developing multivariate calibration models.

### 3.2. Multivariate calibrations

In order to multivariate calibrate the emission of the GR with concentration of Pb, Cd and Zn, a central composite design was developed based on linear ranges obtained from individual calibration graphs. Composition of the calibration set is shown in Table 1. All the cells related to the calibration set had 1300 ng mL\(^{-1}\) GR as its optimum concentration where each run had different concentrations of the Pb, Cd and Zn chosen according to the linear ranges obtained from individual calibration graphs. The images taken from the cells related to the calibration set are shown by Fig. 3A-J. The work was continued by the application of PLS, PCR, OSC-PLS, CPR, RCR and PRM to the spectrofluorimetric data recorded for the calibration set which are shown by Fig. 4A. Wherever number of latent variables (LVs) was required, it was determined by leave one out cross validation (LOOCV). Different algorithms used in this study needed some parameters which were optimized as follows: PLS: number of LVs = 3, OSC-PLS: number of LVs = 3, CPR: number of LVs = 3 and power = 1, RCR: number of LVs = 3, percentage of data contamination = 0.05 (δ), and PDC = 0.12. After application of the algorithms and optimization of their parameters and constructing multivariate calibration models, their performance was verified by their application to a validation set having cells with different concentrations of Pb, Cd and Zn whose composition is shown by Table 2. The images taken from the cells related to the validation set are shown by Fig. 3K-T and their spectrofluorimetric responses are shown by Fig. 4A.

### Table 2
Concentrations (ng/mL) of the metals in the validation set.

| Run | Cd  | Pb  | Zn  |
|-----|-----|-----|-----|
| 1   | 800 | 1000| 700 |
| 2   | 1000| 1200| 1000|
| 3   | 900 | 900 | 1200|
| 4   | 1200| 800 | 800 |
| 5   | 1600| 700 | 850 |
| 6   | 750 | 1400| 1200|
| 7   | 800 | 1300| 1300|
| 8   | 900 | 950 | 700 |
| 9   | 1400| 1000| 950 |
| 10  | 800 | 1050| 900 |

Application of the constructed multivariate calibration models to the validation set for examination of their performance was performed and the predicted concentrations by different algorithms have been collected in Table 3. By calculating REPs and RMSEPs which are collected in Table 4, it can be clearly seen that OSC-PLS had the best performance among the tested algorithms and their performance obeys from the following order: OSC-PLS > PLS > PRM > RCR > PCR > CPR. For further comparison of different algorithms, their accuracy and precision were compared by the use of EJCR and the results are shown in Fig. 5. The outputs of the EJCR are ellipses whose size is proportional to the precision of the method and falling the ideal point within the ellipse.
confirms the accuracy of the method. The ellipses related to the application of different algorithms for prediction of Pb, Zn and Cd in the validation set are shown in Fig. 5 A, B and C, respectively. Blue ellipse, pink ellipse, green ellipse, yellow ellipse, black ellipse and red ellipse are related to OSC-PLS, PLS, PRM, CPR, RCR and RCR, respectively, and the black point shows the ideal point. According to the results of EJCR, the blue ellipse which was related to OSC-PLS confirmed the best performance which motivated us to select it as the best model for simultaneous determination of the Pb, Zn and Cd.

In order to further verification of the performance of the spectrofluorimetric method assisted by OSC-PLS, the AAS was applied to the prediction of the concentrations of the validation set as reference method and the results are shown in Table 5. The REPs and RMSEPs are presented in Table 5 as well, and as can be seen, the method showed a good performance. For graphical comparison of the AAS and OSC-PLS by the use of EJCR, their results were fed to MATLAB and the EJCR was run on them and the results are shown in Fig. 6. As can be seen, the AAS (black ellipse) showed better accuracy and precision than OSC-PLS (red ellipse) but, by tacking to account that the OSC-PLS is low-cost, simple and fast method in comparison with the AAS which motivated us to suggest it for practical applications.

The intra-day precision of the assay was estimated by calculating the relative standard deviation (RSD) for the analysis of 800 ng mL\(^{-1}\) Pb, Zn

Table 4

| Algorithm | Pb | Zn | Cd |
|-----------|----|----|----|
| REP(%)    | 6.1612 | 7.2473 | 7.5837 |
| RMSEP (Cd) | 62.5364 | 74.6472 | 72.9032 |
| REP(%)    | 0.0623 | 0.0434 | 0.0988 |
| RMSEP (Pb) | 0.6325 | 0.4472 | 0.9487 |
| REP(%)    | 17.5396 | 17.3320 | 194.3698 |
| RMSEP (Cd) | 178.0267 | 178.5195 | 194.3698 |
| REP(%)    | 17.2087 | 17.0729 | 195.3735 |
| RMSEP (Cd) | 174.6680 | 175.8505 | 194.2395 |
| REP(%)    | 17.6132 | 17.3180 | 112.5438 |
| RMSEP (Cd) | 178.7736 | 178.3752 | 241.6752 |
| REP(%)    | 11.0881 | 11.7868 | 25.1745 |
| RMSEP (Cd) | 112.5438 | 121.4043 | 241.6752 |

Table 5

Predicted concentrations of the validation set by the reference method.

| Nr | Cd | Pb | Zn |
|----|----|----|----|
| 1  | 800 | 999.5 | 699 |
| 2  | 999 | 1199 | 1000 |
| 3  | 900 | 900 | 1199 |
| 4  | 1201 | 799 | 801 |
| 5  | 1601 | 700 | 851 |
| 6  | 749 | 1400 | 1202 |
| 7  | 799 | 1300 | 1300 |
| 8  | 900 | 951 | 699 |
| 9  | 1399 | 1000 | 949 |
| 10 | 800 | 1051 | 899 |

Fig. 5. (A), (B) and (C) Ellipses obtained by EJCR related to the prediction of the concentration of Pb, Zn and Cd, respectively. Blue ellipse, pink ellipse, green ellipse, yellow ellipse, black ellipse and red ellipse are related to OSC-PLS, PLS, PRM, CPR, RCR and RCR, respectively. The black point shows the ideal point.
and Cd in six replicates which gave us RSDs of 2.08%, 2.15% and 2.11% for Pb, Zn and Cd, respectively. Inter-day precision was determined by the analysis of six replicates 800 ng mL\(^{-1}\) Pb, Zn and Cd on three consecutive days which gave us RSDs of 2.34%, 2.28% and 2.21% for Pb, Zn and Cd, respectively. The results obtained for examination of intra-day and inter-day precision confirmed acceptable precisions for the developed methodology.

4. Conclusion

In this work, a novel and interesting analytical methodology based on coupling of spectrofluorimetry and chemometrics was developed for simultaneous determination of Pb, Cd and Zn in Hela cells. Among the tested chemometric algorithms, the OSC-PLS showed the best performance for simultaneous monitoring of Pb, Cd and Zn whose performance was comparable with AAS as reference method. The results of this work showed that chemometrics has a great potential for assisting instrumental techniques to develop accurate novel methods which have very better performance than those instrumental alone. As a new research field for our research group, we are going to continue coupling of chemometric method with instrumental techniques for bioanalytical purposes and definitely, this work will be a bridge to connect the world of chemometricians with the world of bioanalysts.

Statement

The main idea of this project belongs to Dr. Ali R. Jalalvand and the other authors contributed equally in this project.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The financial supports of this project by Kermanshah University of Medical Sciences, Kermanshah, Iran are acknowledged.

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