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Optimization and Validation of Solid-Phase Microextraction of Mercury Species: An Application of Experimental Design

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In this study, determination of mercury species in water by headspace solid-phase microextraction (HS-SPME) has been developed. The extraction procedure consisted of screened using factorial design and followed by optimization using two level central composite designs. An optimum working condition was obtained at 22.5 °C after 20 min, pH 4 and stirring rate (200 rpm) with fixed values of salt addition (8.5 mg L^{-1}) and sample volume (25 mL). Extraction was performed by using 100 µm polydimethylsiloxane (PDMS) fiber and desorption time at 1.2 min. Analytical figure of merit showed good linearity with R^2 in the range of 0.992-0.994. Limit of detection and limit of quantification obtained were from 0.037 to 0.078 µg L^{-1} and from 0.124-0.178 µg L^{-1} for all analytes, respectively. Repeatability and reproducibility were good with relative standard deviation (RSD) < 10 %. Recoveries were obtained in the range of 72-109 % at two levels of concentration.

Keywords: Factorial design, Central composite design, Response surface, Mercury.

INTRODUCTION

Optimization of extraction method can be achieved in the classical trial or known as one-variable-at-a-time, which each factor was studied separately. However, bilateral interactions between multi-variables are difficult to explain and thus being overlooked. Alternatively, an approach based on multivariate statistical technique or known as experimental design was introduced. By applying this technique, data output and their interactions term can be distinguished at optimum conditions, which were not detectable using classical experimental methods[1,2]. The experimental design has three principles; namely randomization, replication and blocking[3]. An advantage of this technique is the number of experiments is reduced without loss of optimum conditions, as well as easy to handle simultaneously[4]. One of the most applied techniques is known as a factorial design. This design is usually used for screening variables, where positive and negative signs in the Pareto chart indicate that the response is enhanced or reduced when passing it on a given factor from the lowest to the highest level[5]. Pareto chart will also help the researchers to visualize the effect of the experimental conditions on the extraction of targeted analytes[6].

After screening, response surface methodology, which is another useful tool applied in the modeling and analysis of processes. This technique discriminates a response of interest in optimum conditions, which are influenced by significant variables[7,8]. One of the most common methods for response surface model is known as a central composite design. The juxtaposition of two-level in this design with star design gives rotation variability and will be a useful tool for estimating a multifactor response surface[1]. To discriminate the significant variable, a combination of mathematical and statistical techniques should be generated to obtain a second order polynomial equation. The quality of the fitted model can be evaluated using analysis of variance, ANOVA. The model with a low standard error was considered as the satisfactory response surface model[9,10].

Solid phase microextraction (SPME) is a simple, sensitive, robust, reliable, low cost sampling technique based on analyte diffusion that combines the advantages of both static and dynamic headspace for qualitative analysis[11]. It has been widely used for volatile compound analysis as combination of pre-concentration, extraction and desorption of one device, which being easy to handle. In this study, a microextraction technique, solid phase microextraction was applied to extract low-level

concentration of mercury species namely methyl, ethyl and inorganic mercury in optimum condition with the aid of experimental design. Method was then applied to the validation test to improve analytical figures of merit, such as the limit of detection, precision and recovery.

EXPERIMENTAL

Mercury standards (methylmercury(II) chloride, ethylmercury(II) chloride, mercury(II) chloride) and sodium tetraphenylborate with grade of purity above 99 % were purchased from Sigma-Aldrich (USA). Sodium chloride, sodium acetate (analytical reagent grade) and methanol of liquid chromatography grade were purchased from Merck (Darmstadt, Germany). Deionized water was obtained by using a Milli-Q EasypureRodi system (Barnstead, USA). Three types of fiber, namely 100 µm polydimethylsiloxane (PDMS), 85 µm polyacrylate (PA), 75 µm carboxen-polydimethylsiloxane (CAR-PDMS) were purchased from Supelco (Bellefonte, USA). Each fiber was conditioned according to instructions provided by the manufacturer before the analysis.

Varian CP3800 gas chromatography-electron captured detector (GC-ECD) which was equipped with DB-5ms capillary column (30 m × 250 µm × 0.2 µm thickness) was used for chromatographic separation of mercury species. The GC oven was programmed as follows: the initial temperature was 100 °C held for 1 min and ramped to 300 °C at a rate 20 °C/min, held for 2 min with an estimated run time of 13.5 min for each sample. The injector port was set to 200 °C. The detector temperature was set up at 300 °C and purified nitrogen was used as the carrier gas at a flow rate of 1.5 mL/min. Desorption time was 1.2 min. No carry over effect was observed in blank runs performed after the sample injections.

Individual mercury standard stock solutions were prepared in methanol at 1000 mg L^{-1} and stored at 4 °C. Stock solutions were then subsequently diluted in deionized water for the optimization and method validation study. The derivative reagent, sodium tetraphenylborate solution (1 % $NaPh_4B$) was prepared daily in deionized water. Buffer solution (sodium acetate + acetic acid) was prepared by dissolving an appropriate amount of sodium acetate in acetic acid, while salt (sodium chloride dissolved in deionized water) was used to adjust pH values and ionic strength of the required solutions.

Selection of fiber: Three different types of fiber were tested to pick up the most suitable fiber for mercury extraction. The sample contained 25 mL of mixture mercury (100 µg L^{-1}) and placed in 40 mL amber vials. The extraction time was 15 min. Fiber type that gave the highest sum of the peak area was chosen for further experiments.

Experimental design headspace-solid phase microextraction procedure: To ascertain the effect of these factors and their possible interactions, 2^5 fractional factorial design was conducted. For this experiment, extraction temperature, time, pH, stirring rate and salt addition were screened; varied at two levels. This design involved 16 experiments which were run in random in order to provide protection against the effect of lurking variables. Main effects were visualized using Pareto charts and data were evaluated by analysis of variance (ANOVA, F test) to distinguish significant levels. The experimental variables and design matrix are shown in Table-1.

TABLE-1
FRACTIONAL FACTORIAL DESIGN MATRIX

Variable	Code	Coded level	
		(-1)	(+1)
pH	A	4	8
Extraction temperature (°C)	B	25	50
Extraction time (min)	C	5	20
Stirring rate (rpm)	D	100	500
Salting (ppm)	E	2	15

A central composite design (CCD) at two-level factorials was chosen to generate second order polynomial equation with the fitted model. Central composite design was generated with $\alpha = \pm 1.414$, calculated to satisfy rotate-ability. In total, the matrix of central composite design involved 30 experiments. Quadruplicate determination at four central points was added to estimate experimental error. Three-dimensional surface plot was applied to visualize the interaction term between two significant variables.

In principle, the targeted analytes were extracted with 100 µm polydimethylsiloxane, by exposing the fiber coating to the sample head-space, according to the experimental factorial design matrix. Sample was prior filled up into 40 mL vials with 25 mL aliquot of samples. 1 mL of $NaPh_4B$ and sodium chloride solution were added into vial, capped and were then left for 5 min to reach the pre-equilibrium phase. After that, the needle of solid phase microextraction was exposed to the headspace of the sample until the end of extraction time. The fiber was then retracted back into the needle and exposed again into the injector port. The immersion depth of the fiber into the headspace area during extraction and desorption into the injector port was always kept inconstant position.

Method validation: To determine the linearity, external calibration curve was determined by preparing a series of mixture concentrations ranging from 1.5 to 50 µg L^{-1}. Triplicate analysis was performed for each level of concentrations. Instrument detection limit (IDL), limit of detection (LOD) and limit of quantification (LOQ) were based on 3:1 and 10:1 signal to noise ratios respectively. Signal to noise was calculated based on 2H/h, where H is the height peak of targeted analytes, measured from maximum of the peak to the extrapolated baseline of signal observed from a distance equal to 20 times the width at half height, while h is the range of background noise obtained through blank injection[12].

Precision of the method was tested based on repeatability and reproducibility performances, which were ascertained by performing five samples extraction on intra-day and continued for the next 3 days (inter-day). Absolute recovery test was performed by preparing a mixture of mercury solutions at two levels of concentration (12.5 and 50 µg L^{-1}) and spiked on four types of water; namely deionized, distilled, salt and wastewater. Uncertainty test was performed to express a combination of bias and precision. It was calculated as $[(|x - x_{true}| + 2s/x_{true}] \times 100$. The symbol of x is the mean concentration of repeated measurement, x_{true} is the actual concentration and s is the standard deviation of measurements[7].

Statistical analysis: Minitab statistical package software version 17 (Minitab Inc., State College, USA) was used for the design of experiment, analysis and data processing. Data

were also tested by using principal components analysis (PCA) in order to visualize grouping tendencies of targeted analytes. Analysis of variance was performed in order to discriminate significant variables.

RESULTS AND DISCUSSION

Fiber screening: The results of fiber screening in Fig. 1 showed that extraction efficiency of mercury analytes in all species was the best for PDMS fiber. Extraction efficiency of selected fibers for the sum total of the peak area is following the order of PDMS > PDMS-DVB > PA. ANOVA test was performed to determine statistical significance differences between selected fibers. Results of ANOVA test explicated that with 95 % probability all tested fibers can be considered statistically significant different ($p < 0.049$). Findings of the results presented in this study were in line with a similar trend of a past study reported by Mishra *et al.*[13]. Therefore, PDMS type was chosen for further experimental study.

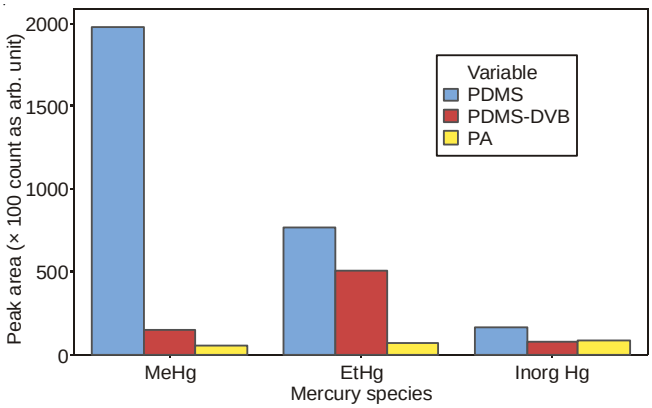

Fig. 1. Signal response on fiber screening

Screening experiment: In this work, a 2^5 factorial design was studied to screen the analytical variables that affect all analytes' response signals. The influence of variable interactions on the experimental study was as illustrated in the Pareto chart (Fig. 2). In this study, the technique of SPME was largely influenced by the values of extraction time, stirring rate, extraction temperature, pH as an individual component or their behaviour of interactions. Only extraction time and temperature showed enhancement when it passed from the lowest to the highest level design set. High interaction can be observed between extraction times and stirring rate, which explicated that the kinetic effect was found to be significant for the partition coefficient between the sample and headspace area, as well as attached to fiber. Good agitated aqueous phase simply means that the mass transport of analytes in the aqueous phase is much faster than other two phases, not acting as a limiting step in the whole diffusion process[7].

Variable of extraction time and temperature can be seen in Fig. 2, as they interacted closely to each other (BC). According to Welke *et al.*[14], increased samples temperature is able to reduce exposure times. Thus, accelerating the extraction time of analysis. This phenomenon was clearly explained through a positive sign when it passed through from the lowest to the highest level design set. To be certain that a significant

Fig. 2. Pareto chart of standardized effects for the selected variables

level of every interaction term can be distinguished, ANOVA test was performed and descriptive analysis is shown in Table-2.

TABLE-2
DESCRIPTIVE STATISTIC OF ANOVA TEST FOR FACTORIAL DESIGN

Variables	DF	Sum of square	Mean square	F value	P value
Model	6	13.177×10^3	2.196×10^3	10.84	**0.001**
B	1	0.659×10^3	0.659×10^3	3.36	0.105
C	1	5.481×10^3	5.481×10^3	27.06	**0.001**
AC	1	2.189×10^3	2.189×10^3	10.81	**0.009**
AE	1	0.582×10^3	0.582×10^3	2.87	0.124
BC	1	1.776×10^3	1.776×10^3	8.77	0.016
CD	1	2.488×10^3	2.488×10^3	12.28	**0.007**
Error	9	1.823×10^3	0.202×10^3	–	–
Total	15	15.000×10^3	–	–	–

Bold value is significant at $p < 0.01$.

Principal component analysis was performed to visualize the grouping tendency between multispecies toward interaction of variables. The first two principal components were able to explain 76.20 % out of the total variance, indicating that the proposed experimental design could explain most of the characteristic of targeted analytes. First component was dominated by inorganic Hg with a factor loading of 0.634. The score plot of principal component analysis is illustrated in Fig. 3.

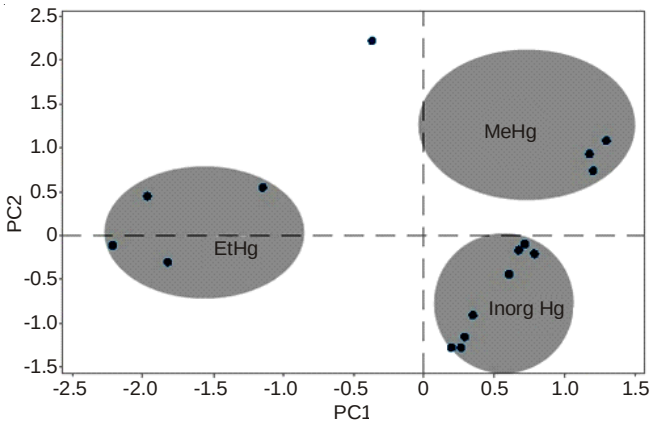

Fig. 3. Score plot of principal components analysis

Optimization of solid phase microextraction: A central composite design, 2^4 was performed to determine the influential factors under optimized condition, in order to build response surface models. The second order polynomial equation obtained using coded values for the optimized variables is given below:

$$\text{Response surface} = 271900 + 23500A - 20800B + 29000C - 7100D - 67400B^2 + 182000AB - 89900AC + 81400BC - 43300CD$$

In this equation, only variable A, C and interactions AB, BC had positive linearity of the fitted model. The significant of each variable was determined using ANOVA test and the p value as presented in Table-3. In this case, variables namely extraction time and interaction terms behaviour (AB, AC, BC) contribute significantly ($p < 0.05$). A good agreement was presented by the coefficient of determination between R^2 (0.794) and R^2 adjusted (0.803) from the fitted model. Four additional experiments were carried out under optimal conditions, in which a good agreement between calculated and experimental responses was obtained with 3.2 % RSD value.

TABLE-3
DESCRIPTIVE STATISTIC OF ANOVA TEST FOR CENTRAL COMPOSITE DESIGN

Variables	DF	Sum of square	Mean square	F value	P value
Model	9	69.105×10^6	7.678×10^6	9.08	0.001
A	1	3.310×10^6	3.310×10^6	3.91	0.062
B	1	2.601×10^6	2.601×10^6	3.08	0.095
C	1	5.029×10^6	5.029×10^6	5.95	**0.024**
D	1	0.301×10^6	0.301×10^6	0.36	0.577
B^2	1	8.168×10^6	8.168×10^6	9.66	**0.006**
AB	1	33.107×10^6	33.107×10^6	39.14	**0.001**
AC	1	0.808×10^6	0.808×10^6	9.56	**0.006**
BC	1	6.628×10^6	6.628×10^6	7.84	**0.011**
CD	1	1.871×10^6	1.871×10^6	2.21	0.153
Lack-of-Fit	15	14.127×10^6	0.941×10^6	1.69	0.294
Pure Error	5	2.790×10^6	0.558×10^6	–	–
Total	29	86.023×10^6	–	–	–

Bold value is significant at $p < 0.05$

An increase in the extraction temperature theoretically will enhance the diffusion of analytes through the fiber coating. Nevertheless, an increase in the high level of extraction temperature would lead to a reduction in the partition coefficient, consequently reducing the amount of extracted analytes[15,16]. The phenomenon was not observed in this study due to two reasons. Firstly, the highest level of temperature was still low (50 °C) and detrimental effect of temperature on fiber was not shown. Secondly, mercury compounds are volatile thus increasing too much temperature would lead to loss through volatilities. Furthermore, extracted analytes required enough time to reach equilibrium phase without an excessive heating of the fiber and this interaction was visualized in Fig. 4a.

Adjustment of pH can either improve or diminish the sensitivity of the method performance toward analytical signal response. In this study, the role of pH in conversion of analytes to non-ionic form (neutral) was not clear. When the sample was exposed to low pH value (4), the signal response seemed to be favourable but in the meantime, peak area in high pH value (8) also yielded high signal response surface. Therefore, we may presume based on theory that the dominant in low pH were the soluble mercury compounds, such as $HgCl_2$ and MeHg, meanwhile in mild alkaline conditions Hg^0 and EtHg were favourable to be dominant[17]. By using response optimizer in Minitab software, optimized condition on pH value was set up at pH 4. Another variable, agitation effect had efficiently enhanced the adsorption of analytes to fiber phase. This interactions term was illustrated in Figs. 4b and 4c.

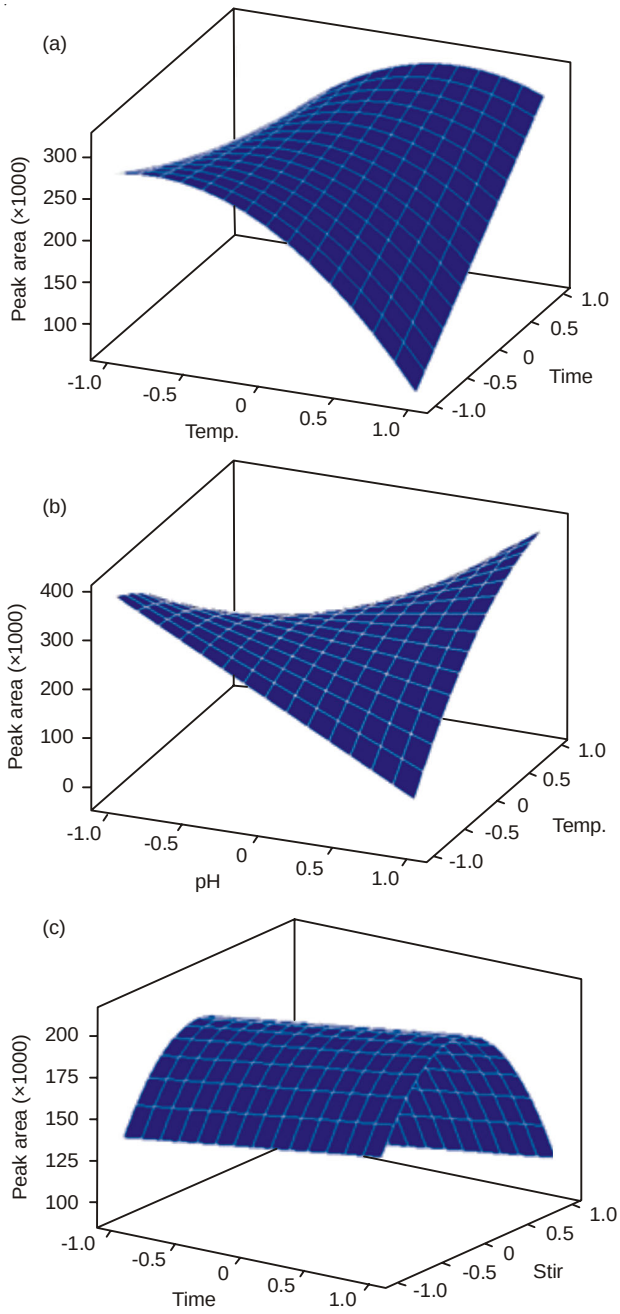

Fig. 4. Plots of the response surface for the interaction term between (a) temp *vs.* time (b) pH *vs.* temp and (c) time *vs.* stir

Optimum working condition was obtained at temperature (22.5 °C), time (20 min), pH (4), stirring rate (200 rpm) with fixed values of salt addition (8.5 mg L^{-1}) and sample volume (25 mL).

TABLE-4
FIGURE OF MERIT ON SPME METHOD PERFORMANCE

	MeHg	EtHg	Inorg Hg
Linear range (n = 7)	1.5-50	1.5-50	1.5-50
Regression equation	y =7.682x – 3.924	y = 7.428x + 2.510	y = 1.246x – 0.176
R^2	0.992	0.994	0.992
LOD (S/N = 3) (ppb)	0.078	0.050	0.037
LOQ (S/N = 10) (ppb)	0.125	0.178	0.124
Instrument detection limit (ppb)	4.12	5.87	4.07

Analytical performances: Fig. 5 shows the chromatogram under optimized condition, obtaining a good separation of mercury species with relatively short run time. Carry-over effects were tested by desorbing fiber twice into the injector port and results indicated only less than 0.7 % residual. The calibration curve of a series of standard mixture solution was obtained with linear regression correlation coefficients in the range of R^2 = 0.992-0.994 (Table-4).The limits of detection for mercury species were in the range 0.037-0.078 μg L^{-1}. Overall, the detection limit obtained is lower than the maximum contaminant level (MCL) for Hg^{2+} in drinking water, namely 2 μg L^{-1} as regulated by the USEPA[18].

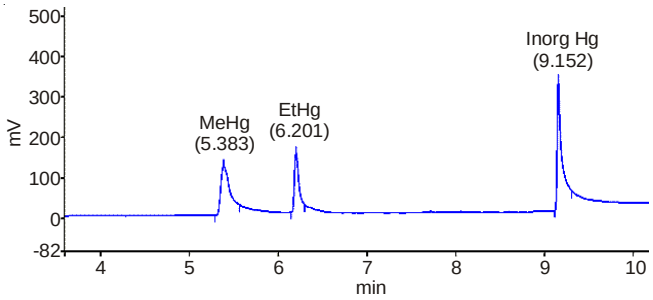

Fig. 5. Chromatogram of extracted spiked analytes (75 μg L^{-1}) in water

The optimized method shown in Table-5 had a good precision, calculated as the relative standard deviation (intra-day, n = 5) and (inter-day, n = 15) using two levels of concentrations, 12.5 and 50 μg L^{-1} for each analyte. The inter-day accuracy tests indicated that the value of RSD is slightly higher than intra-day, which can be related to the stability of analytes in water sample. According to Guevara and Horvat[19], the degradation of mercury analytes especially Hg^{2+} can be varied depending on the types of water and storage condition over after 10 days. This phenomenon would suggest that the sample should be analyzed quickly especially for routine work.

Recovery test has showed varied percentage among types of water used in this study. An analysis result has showed good recovery was obtained in deionized water which percentage more than 75 %. Meanwhile, recovery value was obtained more

TABLE-5
REPEATABILITY AND REPRODUCIBILITY
AT TWO LEVEL CONCENTRATIONS

	Concentration (μg L^{-1})	% RSD Intra-day (n = 5)	% RSD Inter-day (n = 15)	Uncertainty
MeHg	50	3.01	3.63	8.3
	12.5	7.13	8.13	
EtHg	50	7.19	8.90	12.4
	12.5	9.61	9.66	
Inorg Hg	50	7.16	9.12	15.4
	12.5	7.61	9.20	

than 100 %, at least for one species when it was tested in wastewater and salt water samples. The % RSD value showed in Table-6 ranged in fortified wastewater sample (WW) was higher than other samples. This is probably due to the presence of suspended solids in the water samples, since the sample was not filtered prior to extraction. This matrix had little effect on the proposed method and can be eliminated. Nevertheless, recoveries are still in good agreement because recovery ranging from 70-120 % is considered as acceptable in any method development[20]. These performances indicated that the proposed method was reliable for determining mercury species in water samples.

Application on real samples: In demonstrating the performance of the developed method, it was applied to marine water samples. Surface water sample was collected from 13 stations along Johor Strait, Malaysia in the month of April-May 2014. Each species was present in the marine water sample, in concentrations ranging between (<LOD-2.10 μg L^{-1}) for MeHg, (<LOD-1.12 μg L^{-1}) for EtHg and (<LOD-7.08 μg L^{-1}) inorganic mercury respectively. The level of concentration was quantified by external calibration method and is illustrated in Fig. 6.

Conclusion

By applying experimental design namely factorial and central composite, the developed solid phase microextraction method can be optimized through latent variables. Central

TABLE-6
RECOVERIES OF FORTIFIED WATER SAMPLES

Analyte	Concentration (μg L^{-1})	Distilled water (%)		Deionized water (%)		Wastewater (%)		Salt water (%)	
		Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
MeHg	50.0	86.2	0.48	97.4	0.98	102.0	1.96	85.1	1.96
	12.5	84.0	0.66	95.1	0.90	101.3	1.86	82.8	2.02
EtHg	50.0	89.3	0.68	96.7	0.30	104.9	3.50	88.2	3.50
	12.5	83.2	0.46	93.4	0.52	101.5	3.83	84.9	3.73
Inorg Hg	50.0	76.2	1.01	81.3	1.61	105.0	8.84	109.8	1.72
	12.5	72.9	1.22	78.9	1.06	102.2	8.32	106.4	1.54

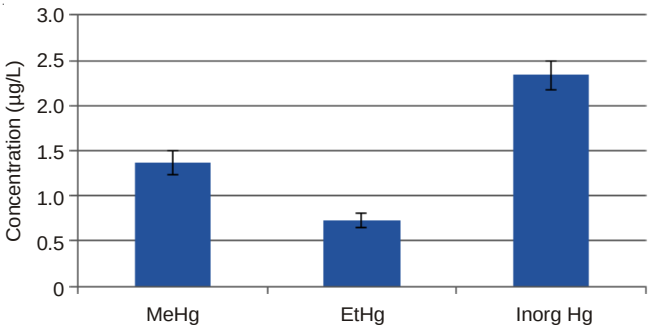

Fig. 6. Level of mean concentrations of mercury species in marine waters

composite design was successfully used discriminate behaviour interaction terms between variables. Under optimized condition, method validation was performed in order to improve analytical figures of merit, such as the limit of detection, precision and accuracy. Method performance gives a good agreement, represented by low < 10 % RSD. Despite the limit of detection had only reached µg L^{-1}, the developed method is still reliable to be applied for mercury determination in environmental analysis especially in water.

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