



Method validation of heavy metals determination in traditional herbal tablet, capsule and liquid by graphite furnace atomic absorption spectrometer and flow injection for atomic spectroscopy hydride system

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Abstract: The validation on the test method applied showed acceptable limits of detection (LOD), limits of quantification (LOQ), linearity, precision (repeatability and intermediate precision) and accuracy. The LOD and LOQ for cadmium (Cd) were acceptable at below 1.0 ug/L, plumbum (Pb) 10 ug/L, arsenic (As) 5 ug/L, and mercury (Hg) 1.0 ug/L. The working linearity ranges were found as: 1-5 ug/L for Cd, 20-100 ug/L for Pb and As, and 10-30 ug/L for Hg. The correlation coefficient were found to be more than 0.995 each. All concentrations for each heavy metal in the linearity ranges showed repeatability of below 10% RSD value and accuracy in the range of 120-80%. Intermediate precision study within different days of same concentration for each analyts gave similar result with slight different but still in the acceptable range for all studied parameter. Therefore, the test method could be applied in performing routine analytical test in laboratory.

Validasi terhadap kaedah ujian yang dilaksanakan menunjukkan had pengesanan (LOD), had kuantifikasi (LOQ), kelurusan, ketepatan (keboleh ulangan dan ketepatan antara) dan kejituan yang boleh diterima. Nilai LOD dan LOQ untuk cadmium (Cd) telah diterima dibawah 1.0 ug/L, plumbum (Pb) 10 ug/L, arsenic (As) 5 ug/L dan merkuri (Hg) 1.0 ug/L. Julat kelurusan yang boleh digunakan adalah: 1-5 ug/L untuk Cd, 20-100 ug/L untuk Pb dan As dan 10-30 ug/L untuk merkuri. Nilai pemalar korelasi didapati lebih dari 0.995 setiap satu. Semua kepekatan untuk setiap logam berat dalam julat kelurusan menunjukkan kebolehdulangan dibawah 10% nilai RSD dan kejituan dalam julat 80-120%. Kajian ketepatan antara diantara hari berlainan pada kepekatan yang serupa untuk setiap analit memberikan nilai yang sama dengan perbezaan yang sangat kecil tetapi masih didalam julat yang boleh diterima untuk semua parameter yang di kaji. Oleh itu, kaedah ujian ini bolehlah digunakan untuk ujian analitikal rutin di dalam makmal.

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Keywords: Graphite Furnace Atomic Absorption Spectrometer (GFAAS); Flow Injection For Atomic Spectroscopy Hydride System (FIAS MHS); method validation; heavy metals; herbal product matrix.



INTRODUCTION

Heavy metal refers to toxic or poisonous high density metallic chemical element. Due to their high degree of toxicity to human health, cadmium (Cd), plumbum or lead (Pb), arsenic (As) and mercury (Hg) are listed as among the priority metals that should be monitored and only allowed to be present at very low limit in food or water because they could induce toxicity at low level of exposure [1]. Environmental source for heavy metals reported as geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources and the pollution areas pointed into mining, foundries and smelters, and other metal-based industrial operations areas [2]. Heavy metals enter human food chain through consumption on contaminant plants, cattle and their byproducts. Heavy metals are trace elements whereby they present in trace concentrations of part per billion (ppb) range to less than 10 part per million (ppm) in various environmental matrices [3]. Although their absorption after dietary exposure in humans is relatively low, they may retained in human body with a very long biological half-life ranging from 10-30 years [4]. For example, cadmium may bind to protein, other organic molecules and may form salts with organic acids [5], thus bio accumulate in animal or human organ tissue or bone and finally become toxic to the organism.

In order to ensure the safety of food, supplement, herbal or any consume product, The World Health Organization (WHO) recommended continuous monitoring on heavy metals concentrations in food or medicine to be under the maximum permitted concentration [6]. A list of international and national standards for the guideline and list of allowed maximum concentration for each heavy metals such as Codex Alimentarius Commission (CODEX), Mainland, US Food and Drug Administration (FDA) and European Commission (EU), Australia. In Malaysia, The Drug Control Authority (DCA) is the executive body established under the Control of Drugs and Cosmetics Regulations 1984 which has the authority to ensure the safety, quality and efficacy of pharmaceuticals, health and personal care products marketed in Malaysia. Under Drug Registration Guidance Document (DRGD) National Pharmaceutical Control Bureau (NPCB) First Edition, January 2013, they listed the limit concentration for each element as Pb : not more than (NMT) 10.0 mg/kg or mg/litre (10.0ppm), Cd : NMT 0.3 mg/kg or mg/litre (0.3ppm), Hg : NMT 0.5 mg/kg or mg/litre (0.5ppm), As : NMT 5.0 mg/kg or mg/litre (5.0ppm).

Generally there are about 10 traditional dosage forms available in the market *i.e* powder/granules, tablet, hard gel capsule, soft gel capsule, pill, oil, liquid (syrup/water), cream, ointment and patch/plaster. Different dosage form is formation of different type of matrix. For example, gelatin capsule is made of partial hydrolysis of collagen which derived from materials rich in collagen such as skin, connective tissue, organ, intestines, bone and leather [7] whereas tablets containing excipients such as diluents, disintegrants, glidant, lubricant and colouring [8]. It is thus we might need different condition for the sample preparation and data acquisition parameter for the analysis method.

The aim of this study was to validate an analytical method to determine the heavy metals cadmium Cd, Pb, As and Hg content in herbal product of tablet, capsule and liquid dosage form by using microwave assisted treatment for sample preparation and atomic absorption spectrometry technique for analysis. For each herbal product matrix, the test method should be validated following guideline such as Eurachem Guide 1998 and ICH Harmonised Tripartite Guideline as one of the reference. According to [9], it is a necessary to validate a method in order to verify its performance parameters are adequate to be use in any analytical problem. The process of validation should be performed with equipment and instrumentation within specification, working correctly and properly



calibrated [10]. In our study, the method validation covers the acid digestion (microwave digester) for sample preparation and analysis method using graphite furnace atomic absorption spectrometer (GFAAS) for Cd, Pb and As and flow injector atomic spectroscopy atomic hydride system (FIAS MHS) for Hg inclusive the instrument parameters and reagents involved in both process.

MATERIAL AND METHODS

Reagents: All reagents shall be recognized analytical reagent quality and water used shall be distilled water or 18 M Ω deionized water. Reagent used was nitric acid (HNO₃), not less than 65% v/v (mass fraction) was purchased from fisher scientific (Waltham, Massachusetts, USA) (batch M020608), hydrogen peroxide solution (H₂O₂: 30% v/v) from Merck (Darmstadt, Germany) (lot no. K46792287525) for digestion. Reagent for preparing matrix modifier solution for cadmium and plumbum was ammonium hydrogen peroxide (NH₄H₂PO₄) and magnesium (Mg) stock solution whereas for arsenic was palladium (Pd) and Mg stock solution. Another chemical used was sodium borohydride (NaBH₄) Acros organics (lot A0348585), sodium hydroxide (NaOH) pellet BDH (lot B899950 634) and acid hydrochloric (HCl: 37% v/v) from Merck (Darmstadt, Germany) (lot no. K46709317524) for mercury analysis. All heavy metal standards was certified standard solution of Cd - 1000 mg/mL (lot 67822177), certified standard solution of Pb - 1000 mg/mL (lot HC56629876), certified standard solution of As - 1000 mg/mL (lot 55536773) and certified standard solution of Hg - 1000 mg/mL (lot 57415826). All standard solutions in HNO₃ 0.5 mol/L was purchased from Merck (Darmstadt, Germany) and traceable to SRM from NIST (National institute of standard and technology).

Samples: Sample of tablet, capsule and liquid was selected from available herbal product that was chosen randomly from market.

Digestion of samples: Each sample was prepared in at least 10 replicates for each studied parameters and concentrations. Briefly, the samples were digested as follows. The 0.5 g samples was added to a Teflon vessel. The known concentration of each analyst was spiked into each sample. Five different concentrations was spike on different individual samples. Then, 5ml acid nitric and 2 ml hydrogen peroxide was added into the sample. The vessels were placed in microwave digestion system by using microwave digester Anton Paar Multiwave 3000, for 50 minutes followed by dilution into 50 ml with deionised water after the digestion. A blank digest was carried out in the same way. The analysis of Cd, Pb and As were performed by GFAAS (graphite furnace atomic absorption spectrometer) AAanalyst 600 Perkin Elmer, USA with Zeeman background correction and equipped with the AS 800 auto sampler. The flow injection for atomic spectroscopy Hydride system (FIAS MHS) 100 Perkin Elmer was used for Hg analyses.

Analyses of Cadmium, Plumbum and Arsenic: Stock standard solutions containing 1000mg/L of Cd, Pb, Hg and As were used to prepare working standard solutions; 5 ug/L (Cd), 100 ug/L (Pb) and 100 ug/L (As). The appropriate dilutions were carried out with the addition of 0.2% HNO₃ together with matrix modifier automatically by the AS 800 auto sampler into respective element working range (Pb & As: 20-100 ug/L, Cd: 1-5ug/L). The samples, reagent blank or standard solutions then will be injected into the THGA graphite tube that located at the graphite furnace in the spectrometer. The furnace program for AAanalyst 600 was set up to give the optimum absorbance at the recommended characteristic mass value for each analyst. The data from the serial dilutions was used to develop calibration curve for quantification purpose on the analytes concentration.



Analyses of Mercury: Stock standard solutions containing 1000 mg/L of Hg we used to prepare working solution 10, 20 and 30 ug/L. The graphite furnace at the spectrometer was replaced with the quartz cell which connected to FIAS. The carrier solution of 3 % (v/v) HCL and reductant of 0.2% NaBH₄ in 0.05% NaOH. The reaction of acidified 3 % (v/v) HCL) aqueous sample or standards with a reducing agent (0.2% NaBH₄ in 0.05% NaOH) will generates hydrides that turn into gaseous metal atoms that later will be detected by the detector.

Method validation: Parameters for method validation such as limit of detection (LOD), limit of quantification (LOQ), linearity test, precision (repeatability and intermediate precision), accuracy were investigated for the analytical methods for quantitative determination of toxic metals in capsule, tablet and liquid following the Eurachem Guide 1988 [9] and ICH Guideline Harmonised Tripartite [11].

Linearity: At least five different concentrations for each heavy metal were spiked into blank matrix before digestion. For each concentration, at least 10 individual replicates were prepared. The linearity of analytical response was obtained by plotting the intensity values (y-axis) versus the concentration values (x-axis). The concentration range is consider to give linear analytical response if the regression coefficient, $R^2 > 0.995$.

Limit of detection (LOD) and limit and quantification (LOQ): Limit of detection (LOD) and limit of quantification (LOQ) represent the sensitivity of system and test method used. LOD is the lowest analyte signals that can be distinguished from the noise signal but not necessary could be quantified. Whereas, LOQ is the lowest signal that can be quantified or determined. The LOD and LOQ were analyzed by using the formula $LOD = \text{mean} + 3SD$ and $LOQ = \text{mean} + 10SD$. The data of 20 replicates from blank matrix sample was collected for each analyte.

Accuracy: Accuracy also known as recovery [10]. It represents the closeness between the true value of the analyte concentration and the value from analysis carried out using the analytical or test method [9]. The value could determine any loss of the analytes from the sample or any contamination during the analysis starting from sample preparation. It could be calculated using the formula:
 $\%R = [(CF-CU)/CA] \times 100$

Where CF was the analyte concentration in the spike sample, CU in the unfortified sample and CA in the sample added.

Precision

a) Repeatability

Repeatability shows the closeness between the results which obtained from of the same conditions of measurement such as same laboratory, equipment and operator under short interval time. The value was monitored from the RSD (%) value.

b) Intermediate precision

Precision express the variations within the same laboratory. To determine the precision, the same sample was analyzed under the same laboratory, same equipment, same procedure but different days. The RSD (%) value showed the different of the data obtained from different condition.

Statistical analysis: The study was performed on three different common herbal product matrix i.e. hard gel capsule, tablet and cough syrup (liquid). The analysis was carried out on at least 10 individual replicates and maximum 20 for each concentration of every elements and matrix. The data



for the calculation of validation parameter of linearity, LOD, LOQ, accuracy and precision was presented. The statistical analysis on the data was using Microsoft excel.

RESULTS AND DISCUSSION

Atomic absorption spectrometry is one of the most acceptable analytical method for heavy metals. Two techniques used in our analysis on the heavy metal determination inclusive of graphite furnace atomic absorption spectrometer (GFAAS) and flow injection for atomic spectroscopy Hydride system (FIAS MHS). GFAAS could determine lower elements number compared to flame AAS but the detection limit and sensitivity is 100 to 1000 times better than flame AAS [12]. It is thus applied for determination of specific heavy metals in food and beverage and other product consumers at very trace level. Flow injection for atomic spectroscopy (FIAS) is superb compliment to atomic absorption spectroscopy [13]. For trace element analysis of mercury, it could automated cold vapor and hydride generation for detection limit improvement. Mercury hydride was produce from the reaction between the hydrochloric acid (HCl) and sodium borohydride (NaBH₄) solution under chemical reaction as below [14]:



Where E = analyte of interest and m may or may not equal n

Before method validation, the system condition including the parameter should be optimized to ensure the instrument and analytical method could perform the sample reading. In GFAAS system, heating step at the pyrolytically THGA graphite tube was following the set up furnace temperature program. The temperature program should be optimized following the recommendation by Perkin Elmer with slight modification specifically for the pyrolysis and atomization stage to ensure that the absorption of the element agree with the characteristic mass for each elements. Each element had their own characteristic mass value.

The formula of characteristic mass is as follow [15]:

$$\text{Mo (pg)} = [\text{volume (injected sample)} \times \text{concentration} \times 0.0044] / \text{Abs of corrected value}$$

The furnace programme for GFAAS method was as in Table 1

Table 1: Instrument data acquisition parameter

Element	Temperature (°C)	Wavelengths (nm)
Pb	110, 130, 850, 1600, 2450	283.3
Cd	130, 400, 1300, 2500	228.8
As	110, 130, 1200, 2000, 2450	193.7

In the FIAS MHS system, the flow of the hydride from sample to the quartz cell should be controlled through the pump speed to get the optimal absorbance value. The wavelength for Hg analysis was set up at 253.7 nm and cell quartz temperature at 100 °C. After reaching all the optimized condition for GFAAS and FIAS MHS system, the calibration range preparation using standard heavy metal could be proceed using heavy metal standard solution. The calibration coefficient R² determination for each element should be ensure to be under R² ≥ 0.99 (ICH Harmonised Tripartite Guideline). The achievement of this R² value would indicate that the instrument and the analytical method gave the consistent concentration value of elements present within the determined and



selected working range. It is thus allowed validation on the optimized methods to proceed to ensure that the result obtained is true and acceptable.

Linearity study was performed on data from different matrix sample spiked with 5 different concentrations of each element and matrix. The concentration data for each sample then plotted against theory value to get linear correlation. From the linear regression, the correlation coefficient R2 will be derived. Table 2 shows that all elements of all matrix perform R2 > 0.99.

Table 2. Linearity data of heavy metal from three different herbal product matrix

Element	Cd			Pb			Hg			As		
	1-5ug/L			20-100 ug/L			10-30 ug/L			20-100 ug/L		
Linear range	capsule	tablet	liquid	capsule	tablet	liquid	capsule	tablet	liquid	capsule	tablet	liquid
R2	0.996	0.995	0.995	0.999	0.995	0.997	0.999	0.995	0.998 0	0.992	0.998	0.997
Slope	0.978	1.022 8	1.0215	1.040	1.158	1.206	0.953	1.031	0.948 8	0.994	0.964	1.073
intercept	0.0385	0.138	-0.140	4.140	- 8.588	- 6.049 1	0.346	-0.415	0.700	0.526	- 3.379	-11.177

The value of limit of detection (LOD) and limit of quantification (LOQ) was generated from the readings of blank matrix of hard gel capsule, tablet and cough syrup which represent liquid.

The LOD and LOQ value represent the signal strength or sensitivity and signal stability of the analytical test method and instrument [16]. From the result in **TABLE 3** can be seen that the LOD and LOQ value for cadmium is below 1.0 ppb, arsenic 5 ppb, plumbum 10 ppb dan mercury 1.0 ppb. During the sample preparation, the possibility of analyte or heavy metal loose could happened. Factors affecting change of analyte concentration in the sample could be derived from either human, apparatus, machine or test method involved. It could be monitored from the difference between the experimental and theoretical concentration value called accuracy. From the result, it shows that the accuracy or recovery for all concentration range in all three matrices was in the range of 80% to 120% which is required under ICH Harmonised Tripartite Guideline [11]. The readings between different replicates of individual preparations from the same batch of sample gave very close repeatability. It can be observed from the results that the %RSD is below 10% for all studied concentrations.

In order to investigate the effect of any changes in the analysis result due to the single factors variation such as different days but with same analytical method, chemicals, instruments, environment and operator, the analysis was carried out in different days. Different sample preparation was carried out on different days. The difference was presented in the RSD% (2) value between the different days. All elements and matrix showed the RSD% is below 10% except mercury capsule which gave 10.566%.

The optimized test method for cadmium, plumbum, arsenic and mercury was developed using GFAAS and FIAS MHS system. The sample preparation on three different matrices of hard gel capsule, tablet and liquid was also optimized using nitric acid and hydrogen peroxide and digested using microwave digester with suitable temperature program. The credibility of the test method was successfully evaluated and validated on their sensitivity, range of analysis, accuracy and some ruggedness aspect. The value for all validation parameters was discussed and falls under the acceptable criteria regarding to AOAC Official Methods of Analysis, Guidelines for Standard Method



Table 3: Validation parameters and result

Analyte		Spike value (µg/L)	% recovery (n=50 or n=100)	Mean (µg/L) (n=50 or n=100)	%RSD (n=50 or n=100)	%RSD (2) (n=20 or n=40)	LOD (n=50 or n=30)	LOQ (n=50 or n=30)
Tablet	Cd	1	108.780	1.0878	2.288	0.986	0.450	0.581
		2	111.692	2.234	1.968			
		3	106.300	3.189	1.933			
		4	109.509	4.380	2.742			
		5	102.300	5.115	1.467			
	Pb	20	114.767	22.953	3.599	8.664	4.107	6.876
		40	107.053	42.821	3.673			
		60	103.057	61.834	3.326			
		80	95.440	76.352	1.520			
		100	92.151	92.151	3.150			
	As	20	118.284	23.657	2.377	3.991	2.050	4.107
		40	110.962	44.385	2.429			
		60	112.224	67.335	1.239			
		80	110.012	88.009	1.113			
	Hg	10	103.150	10.315	1.632	3.665	0.149	0.340
		15	102.466	15.370	4.018			
20		94.941	18.988	2.81				
25		97.556	24.389	0.748				
30		99.779	29.933	0.590				
capsule	Cd	1	85.610	0.764	6.978	7.168	0.509	0.661
		2	107.730	2.0626	1.892			
		3	102.853	2.993	1.429			
		4	101.235	3.957	2.378			
		5	99.996	4.907	1.256			
	Pb	20	116.828	23.231	0.850	0.548	5.600	9.716
		40	104.104	42.599	2.454			
		60	102.314	61.659	1.105			
		80	99.1907	80.118	2.302			
		100	101.641	100.616	0.851			
	As	20	96.371	20.403	3.638	5.147	2.050	4.107
		40	90.152	37.189	4.715			
		60	94.614	57.897	7.662			
		80	102.729	83.312	3.0439			
	Hg	10	100.746	10.068	4.821	10.566	0.126	0.276
		15	101.461	15.213	4.709			
20		105.401	21.074	3.957				
25		102.576	25.638	3.643				
30		103.525	31.0516	2.273				
liquid	Cd	1	107.05	1.071	3.188	7.654	0.476	0.638
		2	105.661	2.113	3.312			
		3	102.757	3.082	0.577			
		4	105.015	4.201	14.731			
		5	98.002	4.900	0.626			
	Pb	20	101.257	20.251	8.247	2.192	4.072	6.609
		40	97.136	38.854	0.869			
		60	94.009	56.405	2.701			
		80	89.832	71.866	0.752			
		100	86.430	86.430	0.615			
	As	20	103.688	20.7376	4.703	0.909	1.942	4.274
		40	107.057	42.823	2.655			
		60	111.227	66.736	1.261			
		80	109.631	87.705	1.326			
	Hg	10	104.997	104.997	2.898	2.006	0.286	0.625
		15	96.333	9.677	1.384			
20		103.385	15.490	1.633				
25		90.953	18.355	3.984				
30		102.848	25.647	0.935				
		102.818	30.769	0.960				



Performance Requirements, Appendix F [17]. Thus, the proposed test method is suitable and ready to analyze heavy metal of Cd, Pb, Hg and As in tablet, capsule and liquid.

CONCLUSION

The optimized test method for cadmium, plumbum, arsenic and mercury was developed using GFAAS and FIAS MHS system. The sample preparation on three different matrices of hard gel capsule, tablet and liquid was also optimized using nitric acid and hydrogen peroxide and digested using microwave digester with suitable temperature program. The credibility of the test method was successfully evaluated and validated on their sensitivity, range of analysis, accuracy and some ruggedness aspect. The value for all validation parameters was discussed and falls under the acceptable criteria regarding to AOAC Official Methods of Analysis, Guidelines for Standard Method Performance Requirements, Appendix F [17]. Thus, the proposed test method is suitable and ready to analyze heavy metal of Cd, Pb, Hg and As in tablet, capsule and liquid.

DECLARATION OF CONFLICTS OF INTERESTS

No conflicts of interests to declare.

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