



Optimization and validation of microwave digestion method for determination of Copper (Cu) and Iron (Fe) in an classical ayurvedic medicine-Arogyavardhini

Thakur K.S^{*1}, Shivcharan Bidve², Priyanka Patil², Madhuree Gawhankar², Maral A.B¹, Khare R.V²

¹Shree Dhootapapeshwar Limited, Veer Savarkar Chowk, Panvel, Navi Mumbai, Maharashtra, India

²Shree Dhootapapeshwar Ayurvedic Research Foundation, Veer Savarkar Chowk, Panvel, Navi Mumbai, Maharashtra, India

ABSTRACT

The main objective of this study was to optimize and validate an efficient microwave digestion method for determination of Copper (Cu) and Iron (Fe) in Arogyavardhini sample. The samples were digested in Aquaregia (a mixture of HNO₃ and HCl - 1:3) for Copper (Cu) and HCl for Iron (Fe) with closed vessel microwave digestion system. The digested samples were subjected to Atomic absorption spectrophotometer for determination of Copper (Cu) and Iron (Fe). The method validation was carried out as per ICH guidelines and can be adopted for the routine analysis of Copper (Cu) and Iron (Fe). The method was validated for precision, accuracy, linearity, limit of detection and limit of quantification. The optimized method showed good regression ($r^2 = 0.9997$). The limit of detection and limit of quantification were found to be 0.02 PPM and 0.05 PPM for Copper (Cu) and 0.28 PPM and 0.83 PPM for Iron (Fe) respectively. Accuracy of the method was checked by recovery study of three different levels with the average percentage recovery of 99.76% for Copper (Cu) and 104.66% for Iron (Fe).

Keywords: Atomic absorption spectrophotometer (AAS); Herbo-mineral formulation; ICH guideline; Microwave digestion system.

INTRODUCTION

Arogyavardhini a Herbo-mineral formulation consists of Triphala, Shuddha Shilajeet, Shuddha Guggul (*Commiphora wightii*), Chitrakmool (*Plumbago zeylanica*), Katuki (*Picrorrhiza kurroa*), leaf juice of Nimba (*Azadirachta indica*) and metals including Shuddha Parad, Shuddha Gandhak, Loha Bhasma, Abhrak Bhasma, and Tamra Bhasma (Bharat Bhaishajya Ratnakar 1/448). As the name indicates it destroys all diseases and promotes Health. This drug is found effective in Pandu roga as well as in enlargement of Liver and Spleen due to ingredients like Loha bhasma, Tamra Bhasma and Katuki. It also act as Deepak, Pachak, Malashodhak, Hrudya and controls Mansa and Medavruddhi. Tamra bhasma and Loha Bhasma have Lekhan and Sthaulahar properties (Ras-tarangini, Taranga 17 / 46, 2009. p. 420; Ras-tarangini, Taranga 20 / 83, 2009. p.507). Abhraka Bhasma, Shilajeet and Guggul have Lekhan, Rasayan and Pramehahar properties (Ras-tarangini, Taranga 10 / 72, 2009. p.234; Ras-tarangini, Taranga 22/ 25, 2009. p.586). It is the drug of choice in all skin diseases (Kumar G et al., 2012). Arogyavardhini has been

proven safe on liver, kidney, and brain through earlier safety, efficacy and toxicity studies (Kumar G et al., 2012; Dange SV et al., 1987; Patgiri BJ et al., 2001).

Various Instrumental techniques are utilized for estimation of Elements in Herbo-mineral & purely inorganic compounds. Few of the most commonly used methods are AAS (Atomic Absorption Spectrophotometry) by Flame & Graphite, ICP (Inductive Coupled Plasma) Technique by OES (Optical Emission Spectrometry) & MS (Mass Spectrometry); but their efficacy is dependent on the digestion methods applied to that samples.

Sample digestion is the critical step in elemental analysis as it requires total decomposition of sample without loss or contamination of the analytes. Ayurvedic sample of purely organic matrix requires preparation by ashing of sample & then digestion of resulted ash in strong acids over hot plate at a set temperature. Further digested sample is filtered to get a clear transparent sample which is ready for Aspiration. The traditional dry ashing method is time taking & prone to loss of volatile elements. Similarly further treatment with acids on hot plate may cause loss of volatile content & consumes more time to complete the digestion. These methods being manual also require precise analytical hand & thus prone to manual errors & Analyst. Thus the conventional methods have several drawbacks viz. loss of volatiles, fumes of acids, chance of contamination (A. Tanase, et al., 2004). These factors created the

* Corresponding Author

Email: kst@sdlindia.com

Contact: +91-9867199737

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need of closed vessel technique - microwave digestion assembly with closed vessel under controlled temperature & pressure.

Microwave digestion involves combining the sample matrix and acids in a pressurized container and raising the temperature of solution higher than the boiling point of the acid which significantly accelerates the digestion. Radiation generated by microwave heats the liquid phase only; whilst vapours don't absorb the radiations (Kingston, et al., 1997). The heat from the microwaves speeds up the chemical reaction of the acid with the sample, reducing the digestion time from hours to minutes. Microwaves are an efficient heating method as it transmits the heat directly to the sample, rather than transmitting the heat from a hot plate to the vessel to the sample. This direct method of heating makes it highly controllable. Heating stops as the microwave system stopped, giving instant control over the process (Katriona Scoffin, et al., 2014). Microwave digestion system rapidly digests a wide variety of samples providing a clear solution containing the analytes of interest.

The aim of this study was to develop a rapid, reproducible and reliable analytical procedure for the determination of Copper (Cu) and Iron (Fe) content in Ayurvedic formulation - Arogyavardhani using AAS following closed vessel microwave digestion system.

EXPERIMENTAL

Instrumentation

The samples of Arogyavardhani were procured from stockiest of Shree Dhootapapeshwar Ltd. These samples were digested in a MARS 6 microwave digestion system (CEM corp., USA) equipped with Teflon closed vessels (Easy Prep Plus vessel) for safe operation under 800 psi. The power system (Magnetron) used with a focused microwave apparatus provides continuous microwave emission at each power level. A pressure control system (ESP-1500 Plus) was used for the microwave digestion with maximum pressure of 800 psi which monitors and controls pressure conditions inside sample vessels. The standard temperature control system (MTS-300) monitors and controls temperature conditions inside sample vessels. The instrumental conditions used for digestion of samples are given in Table 1. After completion of digestions the Copper (Cu) and Iron (Fe) content was determined by atomic absorption spectrometry using a Perkin Elmer model - Analyst AA 400 atomic absorption Spectrophotometry. The instrumental conditions of AAS used for Copper (Cu) and Iron (Fe) analysis are given in Table 2

Reagents: All reagents (i.e. Concentrated HNO_3 & HCl) used for analysis were Analytical reagent grade (Merck). Certified reference materials of Copper (1000 PPM) from REGICON and Iron (1000 PPM) from Merck were used for plotting the calibration graph. All sample solutions were diluted with Distilled water.

Standard Preparation : The stock solutions of Copper (Cu) and Iron (Fe) standard (20 PPM) were prepared by diluting 2 ml of Certified Reference Material (CRM) of Copper (1000 PPM) to 100 ml with 2 % HNO_3 and 2 ml of CRM of Iron (1000 PPM) to 100 ml with Distilled water. Working standards were prepared by diluting exact quantity of Copper (Cu) stock solution (20 PPM) with 2 % HNO_3 to get 0.5 PPM, 0.8 PPM, 1.0 PPM, 1.2 PPM, 1.5 PPM, 1.6 PPM, 2.0 PPM and exact quantity of Iron (Fe) stock solution (20 PPM) diluted with Distilled water to get 0.5 PPM, 0.8 PPM, 1.0 PPM, 1.2 PPM, 1.5 PPM, 2.0 PPM and 2.5 PPM.

Sample Preparation: Powdered samples of Arogyavardhani (100 mg for Copper and 300 mg for Iron, accurately weighed) were predigested with 10 ml aquaregia for Copper and with 10 ml HCl for Iron in Teflon vessels (Easy Prep Plus vessel) on water bath for 15 min. Then these predigested samples were subjected to closed digestion in CEM MARS 6 Microwave Digestion System. After completion of sample digestion, the digested samples were filtered through whatman filter paper in 100 ml volumetric flask and volume adjusted with distilled water. Further dilutions were made to get the concentration of the samples within linear range of calibration curve.

METHOD VALIDATION

Method validation was performed as per standard ICH guidelines (ICH Q2A, 1994; ICH Q2B, 1996; ICH Q2 (R1), 2005) with respect to Linearity, Precision, Accuracy, Limit of Detection (LOD) and Limit of Quantification (LOQ). Linearity of method was performed by plotting calibration curve of seven different concentrations of standards. Precision of method was performed by estimating intraday and interday readings and % RSD (relative standard deviation) was calculated. Accuracy of analytical methods was carried out by recovery study. Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated as per formula. $\text{LOD} = 3.3 \sigma/S$ & $\text{LOQ} = 10 \sigma/S$ Where σ = Standard deviation, S = Slope (ICH Q2A, 1994; ICH Q2B, 1996; ICH Q2 (R1), 2005).

RESULTS AND DISCUSSION

Most important step in optimization of Method for determination of Copper (Cu) and Iron (Fe) is digestion of the sample. Hence, Microwave closed digestion method was developed and validated for complete digestion of the sample. Several parameters have been taken into account and evaluated during the validation of method namely Linearity, Precision, Accuracy, Limit of Detection (LOD) and Limit of Quantification (LOQ).

Linearity

Linearity was determined by analyzing seven different concentrations of Copper (Cu) and Iron (Fe) standard. The accurately measured working standard solutions of Copper (Cu) - 0.5 PPM, 0.8 PPM, 1.0 PPM, 1.2 PPM, 1.5 PPM, 1.6 PPM, 2.0 PPM and Iron (Fe) - 0.5 PPM, 0.8

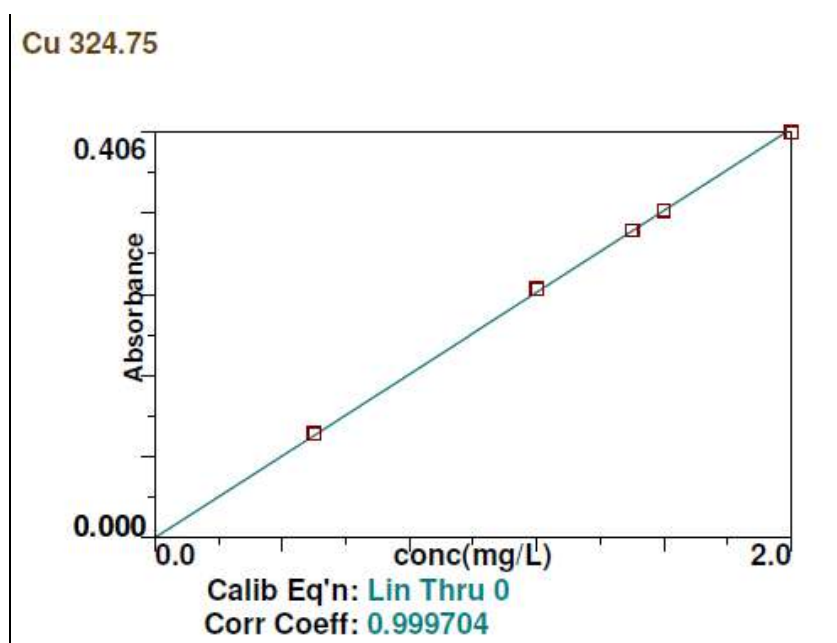


Figure 1: Calibration curve of Copper (Cu) Standard

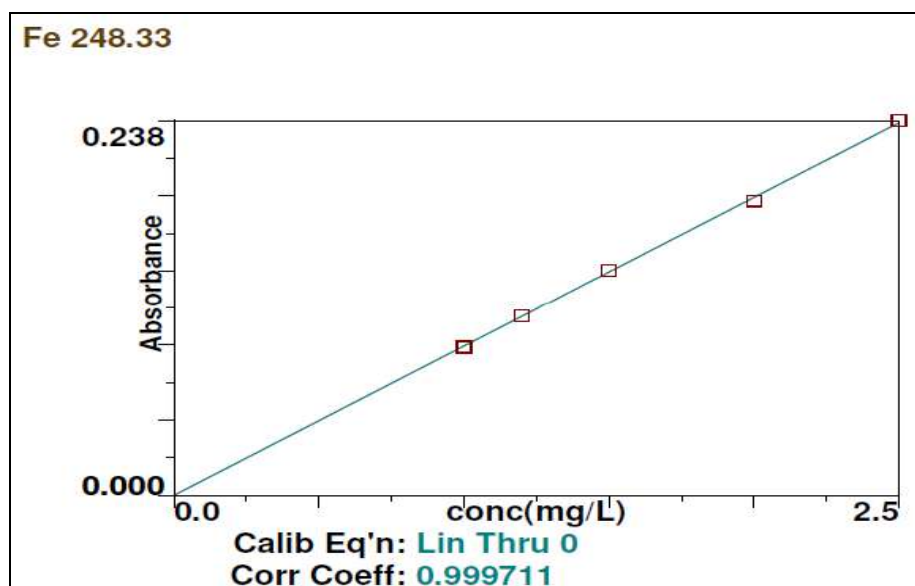


Figure 2: Calibration curve of Iron (Fe) Standard

Table 1: CEM MARS 6 Microwave Digestion System Operating Parameters

Instrumental Parameters	Iron (Fe)	Copper (Cu)
Acid used for Digestion	HCl	Aquaregia (HCl : HNO ₃ :: 3 :1)
Method	Fe	Cu
Temperature	170°C	190°C
Pressure	650 psi	650 psi
Ramp Time	20 min.	20 min.
Hold Time	10 min.	15 min.
Cooling Time	15 min.	15 min.

PPM, 1.0 PPM, 1.2 PPM, 1.5 PPM, 2.0 PPM, 2.5 PPM were used for evaluation of linearity. The coefficient correlation (r^2) values obtained were 0.999704 and 0.999711 for Copper and Iron respectively (Table 3, Figure 1 & Figure 2). According to Eurachem, if Coefficient Correlation (r^2) value obtained is higher than

0.995 then the analytical method is said to be linear over certain concentration ranges (Eurachem, 1998).

Precision

Precision of the analytical method for analysis of Copper (Cu) and Iron (Fe) was checked in order to show the Repeatability (Method Precision) and Reproducibil-

Table 2: AAS Operating Parameter

Instrumental Parameters	Iron (Fe)	Copper (Cu)
Lamp	Fe	Cu
Lamp Current	30 mA	15 mA
Wavelength	248.33 nm	324.75 nm
Slit	1.35 nm	0.8 nm
Burner position	Normal	Normal
Flame	Air-Acetylene	Air-Acetylene

Table 3: Linear Regression data for Copper (Cu) and Iron (Fe)

Sl.No	Parameters	Observed Values	
1	Element	Copper (Cu)	Iron (Fe)
2	Linearity range (PPM)	Linear through zero	Linear through zero
3	Correlation coefficient	0.9997	0.9997
4	Slope \pm SD	0.19 \pm 0.001	0.12 \pm 0.01
5	LOD (PPM)	0.02	0.28
6	LOQ (PPM)	0.05	0.83

Table 4: Repeatability (Method Precision) Data for Copper (Cu)

Sample	Wt. of sample (gm)	Copper (Cu) (%)
Arogyavardhini	0.1001	1.27
	0.1003	1.30
	0.1004	1.32
	0.1003	1.31
	0.1003	1.31
	0.1001	1.27
	0.1001	1.27
	0.1003	1.27
	0.1090	1.30
	0.1003	1.32
Mean		1.29
SD		0.0217
% RSD		1.68

Table 5: Repeatability (Method Precision) Data for Iron (Fe)

Sample	Wt. of sample (gm)	Iron (Fe) (%)
Arogyavardhini	0.3146	1.71
	0.3365	1.73
	0.3144	1.75
	0.2960	1.70
	0.3149	1.77
	0.3144	1.73
	0.2877	1.76
	0.3149	1.79
	0.3144	1.74
	0.2835	1.72
Mean		1.74
SD		0.0279
% RSD		1.60

ity (Intermediate precision) of the results. Repeatability of the method was evaluated from the analysis of 10 sample solutions with same concentrations under the similar conditions (day, analyst, instrument, and sample). The RSD value obtained was < 2.0 % for repeatability (Table 4). Furthermore, the Intermediate precision was evaluated by performing Interday & Intraday

measurements of the sample solutions. The RSD value obtained during the intermediate precision was < 2.5 % (Table 5 & 6). According to Horwitz, the maximum RSD value acceptable for the sample concentration of 1.0% is 4.0% (Horwitz, W, 1982). Therefore, it can be stated that the developed method exhibited a good precision.

Table 6: Interday Precision Data for Copper (Cu)

Sample	Arogyavardhini (Sample-1)			Arogyavardhini (Sample-2)			Arogyavardhini (Sample-3)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Copper (Cu) (%)	1.30	1.27	1.32	1.30	1.34	1.34	1.30	1.27	1.30
Mean	1.30			1.33			1.29		
Standard deviation (SD)	0.03			0.02			0.02		
% RSD	1.94			1.74			1.34		

Table 7: Interday Precision Data for Iron (Fe)

Sample	Arogyavardhini (Sample-1)			Arogyavardhini (Sample-2)			Arogyavardhini (Sample-3)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Iron (Fe) (%)	1.77	1.81	1.77	1.62	1.66	1.67	1.74	1.79	1.73
Mean	1.78			1.65			1.75		
Standard deviation (SD)	0.02			0.03			0.03		
% RSD	1.29			1.60			1.83		

Table 8: Intraday Precision Data for Copper (Cu)

Sample	Arogyavardhini (Sample-1)			Arogyavardhini (Sample-2)			Arogyavardhini (Sample-3)		
	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
Copper (Cu) (%)	1.27	1.30	1.31	1.27	1.27	1.32	1.27	1.31	1.32
Mean	1.29			1.29			1.30		
Standard deviation (SD)	0.02			0.03			0.03		
% RSD	1.61			2.24			2.04		

Table 9: Intraday Precision Data for Iron (Fe)

Sample	Arogyavardhini (Sample-1)			Arogyavardhini (Sample-2)			Arogyavardhini (Sample-3)		
	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
Copper (Cu) (%)	1.57	1.63	1.59	1.76	1.75	1.73	1.71	1.69	1.73
Mean	1.60			1.75			1.71		
Standard deviation (SD)	0.03			0.02			0.02		
% RSD	1.91			0.87			1.17		

Table 10: Percentage Recovery of Copper (Cu)

Amount of Copper (Cu) in sample (%)	Amount of standard Added (%)	Theoretical amount of Copper (Cu) in mixture (%)	Analysed amount of Copper (Cu) in mixture (%)	RSD %	% Recovery
1.30	2.20	3.50	3.53	0.57	100.86
1.30	4.40	5.70	5.81	0.34	101.93
1.30	6.60	7.90	7.61	0.26	96.33

Table 11: Percentage Recovery of Iron (Fe)

Amount of Iron (Fe) in sample (%)	Amount of standard Added (%)	Theoretical amount of Iron (Fe) in mixture (%)	Analysed amount of Iron (Fe) in mixture (%)	RSD %	% Recovery
1.77	0.70	2.47	2.76	0.96	111.74
1.77	1.44	3.17	3.38	2.58	106.73
1.77	2.10	3.87	3.70	0.83	95.52

Accuracy

The accuracy of the method was determined by recovery studies. These studies were carried out at three different levels using standard addition method. The known amounts of standards were added in the sample by spiking. Then the percentage recovery was calculated which was found to be in range from 90 – 110% (Table 7) indicated method to be accurate (TrAC Trends Anal. Chem., 23: 535-552). This indicated that digestion procedure used had no significant loss during Microwave digestion and AAS analysis.

Limit of Detection and Limit of Quantification

The Limit of Detection (LOD) is the lowest concentration of analyte that can be detected and reliably distinguished from zero, but not necessarily quantified and the Limit of Quantification (LOQ) is the lowest concentration of analyte that can be determined quantitatively with an acceptable level of precision (Gonzalez, et al., 2007). The Limit of Detection (LOD) and Limit of Quantification (LOQ) for Copper (Cu) were 0.02 PPM and 0.05 PPM respectively and for Iron (Fe) were 0.28 PPM and 0.83 PPM respectively which indicate the adequate sensitivity of the method (Table 3).

The content of Copper (Cu) quantified using the optimized method were found to be 1.30 %, 1.27 % and 1.31 % in Arogyavardhini sample-1, sample-2 and sample-3 respectively. Also the Iron (Fe) contents were found to be 1.65 %, 1.60 % and 1.71 % in Arogyavardhini sample-1, sample-2 and sample-3 respectively.

CONCLUSION

Recovery studies show that the digestion method used in this study is satisfactory and reproducible for analysis of Ayurvedic formulation - Arogyavardhini. The use of the closed-vessel microwave digestion system for Ayurvedic samples provides a good, safe and clean method of sample preparation. None of the element tested was lost in the digestion. Hence, this method can be successfully used for determination of Copper (Cu) and Iron (Fe) content in Ayurvedic formulations using AAS following closed vessel microwave digestion system.

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