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Research Article

Preparation of cocoa flour reference materials and determination of cadmium content using GFAAS

Anna E. Persulessy

Analytical and Environmental Chemistry Laboratory, Research Center for Chemistry, Jl. Cisitu-Sangkuriang, Komp.LIPI, Bandung, Indonesia.

Email: anna_ep@yahoo.com

Abstract

In-house cocoa flour RMs instead of CRMs were prepared and checked for its homogeneity and stability. Due to CRMs limited availability in terms of matrices of concern and high production cost when making in-house cocoa flour, RMs are alternatively used for daily quality control in the laboratory. The analysis method for reliable checking of homogeneity and stability of cadmium content in the in-house cocoa flour RMs was studied. The effects of magnesium nitrate and lanthanum/magnesium nitrate as chemical modifiers in the determination of cadmium in cocoa flour by GFAAS were studied as well. Detection limit of the method was estimated to be 0.07 µg L^{-1}(calculated as $3S_{y/x}$/slope of calibration curve) and calibration curve of working range was linear. The results analysis of candidates for in-house cocoa flour RMs revealed consistent results and no variability between and within-bottle was observed at 95% limit of confidence and stability during the period of time observed.

Keywords: *Theobroma cacao*, confectionery, certified reference materials, contamination, Indonesia.

Introduction

Cocoa (*Theobroma cacao L.*) bean, and its products (cocoa liquor, cocoa powder and chocolate), is an important economic crop in several countries such as Ghana, Ivory Coast, Nigeria, Malaysia and Indonesia. Cocoa products are among the most widely purchased consumer products in the world and are intensely marketed to children. Therefore, concentration of heavy metals, such as lead and cadmium, in these products and raw materials used for their production are a matter of significant public concern. The maximum permissible levels of Cd in chocolate, cocoa powder have been set to 0.4 mg kg^{-1} in Germany, 0.5 mg kg^{-1} in Finland and Central European countries and 1 mg kg^{-1} in Malaysia [1].

Today's global economy depends on reliable measurements and tests, which are trusted and accepted internationally. This is a basic principle to avoid the Technical Barriers to Trade (TBT) that are defined by regulations of regional and international trade organizations. Measurements and testing today are carried out in many scientific and industrial activities so that it becomes a natural and vital part of our everyday life. For instance; coffee, tea, sugar, diet contents of food, blood sample measurements, etc. are regularly tested for safety and health benefits. Issues in accordance with quality of a product or export/import commodity not only from their purity point of view, but also from their impurities point of view, have become the critical points for market acceptability of a product. Briefly, all decisions are taken based on the analytical data of measurement and testing actions.

With respect to a product, inaccurate measurements and testing may lead to an incorrect assessment of the product quality (e.g. with regard to compliance to standards or regulations). Quality has become a priority feature since the beginning of the 1990s and various actions are currently undertaken to ensure the quality of data produced [2].

Reference materials (RMs) and certified reference materials (CRMs) constitute a key tool for laboratories to verify the accuracy of their measurements and testing in the framework of their internal quality control procedure and are widely used for calibrating measuring instruments, for evaluating various methods of analysis or tests and also for long-term quality assurance of measurements. Currently, many kinds of RMs and CRMs are playing an important role in national and international standardizing activities such as in proficiency testing and in accreditation of laboratories. Due to high production cost, CRMs cannot be used for daily quality control in the laboratory, statistical control purpose (control carts), inter-comparative exercises and for evaluation of reproducibility of methods. This requires the laboratory to develop in-house RMs as laboratory reference materials or laboratory control materials which later on act as inexpensive CRM for local laboratories. In this study, in-house cocoa flour RMs were produced and tested its homogeneity and stability. Approaches for the certification of reference materials are provided in ISO Guide 35 [3, 4, 5, 6]. Certification itself includes homogeneity study, stability study and inter-comparative study as a whole processes. Realizing that the inter-comparative study will take much time and money, therefore this study was only done on the homogeneity and stability testing as a first step before proceeding to inter-comparative study. Actually, cocoa RMs are produced by IRMM, IRMM-801, for proximate one [6] and unfortunately not for inorganic elements such as Pb, Cd, etc. Therefore this study is focused on the preparation of candidate RMs for inorganic metals, especially cadmium. Homogeneity within certain limits is an important property of reference materials for quality control purposes in various analytical tasks, therefore the development of analytical methods for reliable checking of homogeneity are also of great importance.

In the literature, numerous techniques for solid samples digestion have been described, including dry ashing with or without ashing aid and with or without temperature programming and wet digestion with mixtures of mineral acids which use temperature programming. Sample digestions at high pressure in a decomposition vessel have been reported as well [7, 8, 9, 10, 11]. All of these techniques have advantages and limitations. In this study microwave digestion was chosen for treating the sample. Owing to a low concentration and the volatile properties of Cd present in candidate cocoa flour RMs, as well as matrix interference, therefore the GF-AAS technique is most suitable choice for dealing with it. To minimize the interference effects, Lanthanum and Magnesium Nitrate were chosen (among many types of salts) as a mixed chemical modifier to be used for determination of Cd in in-house reference material.

Experimental

Instrumentation

A graphite furnace atomic absorption spectrometer (Hitachi Z-5000, Japan) equipped with Zeeman background corrector was used for measurement of the analytes. Ultra high purity Argon (99.999%) was purchased from Samator Co., Indonesia, which was used as carrier gas. The analytical conditions and temperature program for Cd are presented in Table 1. The reagents and samples were weighed using a Toledo AB 204-S micro analytical balance (METLER) with resolution of 0.1 mg. Dissolution of cocoa flour reference material was achieved using MLS-1200 Milestone microwave (Bergamo, Italy) for three consecutive steps of digestion processes that ensured all samples were dissolved completely with clear appearance of aliquots.

Table 1. Analytical condition for the determination of cadmium using Hitachi Z-5000 GF-AAS with two types of matrix modifier.

Signal Mode	BKG Corr.
Measurement Mode	Peak Height
Slicing Height (%)	-----
Wavelength (nm)	228.8
Determine the W.L	Auto
Slit Width (nm)	1.3
Time Constant (s)	0.1
Lamp Current (mA)	7.0
PMT Voltage (V)	440
Injection Volume (μL)	20
Cuvette Type	Tube A
Temp. Control	Optical
Unknown Volume (μL)	20
Modifier (μL)	10/pre
Injection Speed	5

Reagents

All reagents used were of analytical reagent grade and Milli-Q plus 185, ultra pure water (France) was used throughout this study. Nitric acid (60%) Cica Merck reagent (Japan), hydrogen peroxide (30%) and lanthanum nitrate were purchased from Merck; magnesium nitrate, 99.995% was purchased from Aldrich, USA. Stock cadmium solution (1000 mg l^{-1}) was purchased from Wako, Japan (as Atomic Absorption Standard). Calibration cadmium solution was prepared by diluting stock cadmium solution in 2% (v/v) HNO_3 to give final concentration of 0.5 – 2 μg l^{-1}; Stock Lanthanum solution (10.000 mg l^{-1}) was prepared by diluting approximately 3.12 g of $La(NO_3)_3.6H_2O$ into 100 ml volumetric flask. Matrix modifier [$Mg(NO_3)$], was prepared by diluting 20 mg of $Mg(NO_3).6H_2O$ into 10 ml volumetric flask with 0.05 M HNO_3. To produce matrix modifier [La-$Mg(NO_3)$], 1 ml of stock lanthanum nitrate and 20 mg of $Mg(NO_3).6H_2O$ were diluted to 10 ml volumetric flask with 0.05 M HNO_3. All glassware, polyethylene bottles and other materials were decontaminated with 0.01 M HNO_3 (Soaked 1 day in 0.01 M HNO_3 and rinsed 10 times with deionized water) before use and kept free from contaminants during this study.

Sample pretreatment

Four bottles of samples were taken randomly for analysis of Cadmium and carried out for 0, 1, 3, 6 and 12 month bottling. Four separate portions of each bottle of 200 mg (dry basis) of samples were weighed accurately into Teflon high-pressure vessels for homogeneity and stability study. 5 ml of concentrated HNO_3 and 0.25 ml of 30% H_2O_2 were then added for each of vessel and the vessels were then sealed for 15 minute digestion. The program was set up at program number two according to manufacturer's recommendation. Samples were cooled and then 1 ml of concentrated HNO_3 and 0.25ml of 30% H_2O_2 added for second digestion. The samples were again cooled, 0.25 ml of 30% H_2O_2 added for the last digestion. All aliquots were then diluted to 100 ml with deionized water. This procedure yielded clear sample aliquots. A 10 µl volume of a chemical modifier solution followed by 20 µl of samples was injected into the furnace and the injection of each matrix modifier was carried out separately.

Preparation of in-house cocoa flour RMs

Origin of cocoa beans

Cocoa was first cultivated in South America 3,000 years ago and was a favourite food of the Aztecs in Mexico. Christopher Colombus brought cocoa beans back to Europe in the early fifteen hundreds and since then cocoa and chocolate have gone on to become one of the popular food items. Cocoa plants are now grown in many tropical areas in the Americas, Africa and Asia, with West Africa, Malaysia and Indonesia being the main sources of cocoa for the rest of the world. Cocoa fruit were collected from farmers in South Sulawesi (Sulawasi Island), West Java (Java Island) and South Sumatra (Sumatra Island), these regions being well known as suppliers of cocoa beans to the domestic demand and for export. Cocoa fruit were taken randomly without segregation and all of them are representative for Indonesia's cocoa.

Preparation of bulk material and bottling

The pods of cocoa fruit were opened by using a knife and the cocoa beans collected. An amount of 5 kg of cocoa beans were placed on a wooden floor and covered with plastic sheet during 6 days for fermentation process. Fermentation produces an amount of chemicals which are essential in producing the flavours and colours. After fermentation, cocoa beans were dried by spreading these beans out in the sun for 10 days. Roasting and cleaning were carried out after the drying process. Cocoa beans were soaked with 0.01 M HNO_3 free metals during 20 minutes for cleaning and removing the thin white shell covering the bean. Cocoa beans were further rinsed 3 times with aquadest and aerated overnight, then these beans were roasted by increasing the temperature starting from 70-90^{0}C for 1 hour and 110-140 ^{0}C over 15 minutes to 1 hour in the oven.

Grinding of roasted beans was carried out by using a grinder (SK 100 Comfort Rostfrei, RETSCH, Germany) to release the fat and produce a thick cocoa mass which was then pressed in powerful hydraulic presses (at PT Mayora Laboratory) for producing coca solids (press cake). The solids, press cake, were taken back from PT.Mayora Laboratory for further grinding process that was carried out in analytical chemistry and standard laboratory, Indonesian Institute of Science. Approximately 4 kg of press cake was crushed and ground into flour by using a manual mortar instead of a grinder. This flour was further sieved by using a sieving machine (Laboratory Sieve RETSCH, Germany) with diameter 250 µm. This material was then collected and placed in a homogenizer (TECATOR, France).

After preparation of this material, flour was placed in high density 250 ml polyethylene bottles using a plastic spatula and funnel. This resulted in a total of 32 bottles and each bottle contained approximately 120 g of cocoa flour. All of the cocoa flour was stored at room temperature in a

clean room. A flow chart for cocoa flour preparation is shown in Figure1. Proximate Analysis was done according to AOAC methods and results showed that water content is 4,5%, protein is 24% and fat is 35%, respectively.

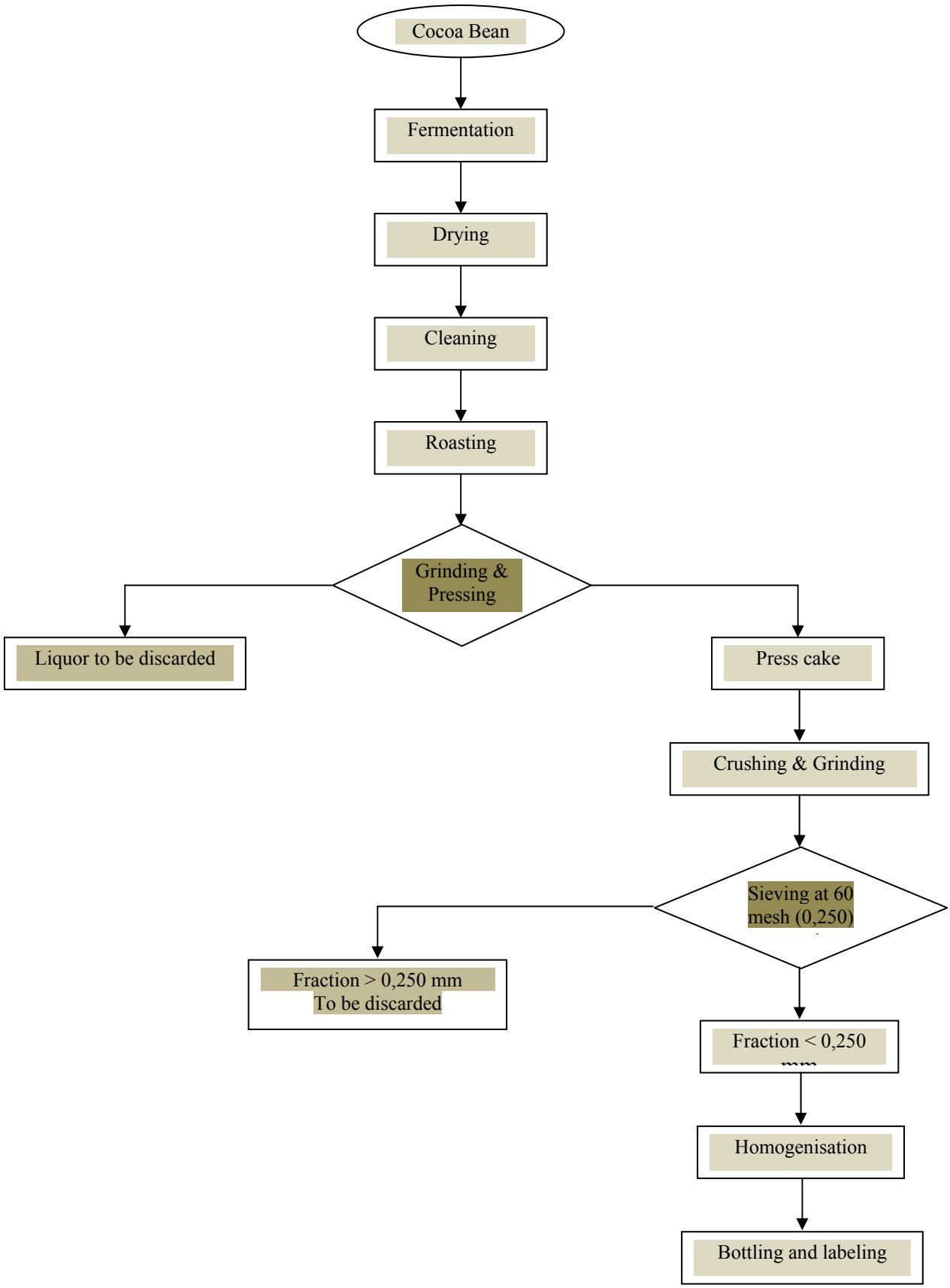

Figure1. Flow chart for the preparation of in-house flour RM

Homogeneity and stability studies

The within-bottle homogeneity test was performed by analyzing four sub-samples taken from one randomly selected bottle. The between-bottle homogeneity test was carried out by analyzing one sub-sample from each of four different bottles randomly selected with three independent replicate determinations of each. The one-way analysis of variance (ANOVA) was applied to compare the variation of within and between bottle homogeneity, using the data analysis tool in Microsoft-Excel program [12]. Four randomly picked bottle samples were also taken and sent to an accredited laboratory for the determination of cadmium content by using ICP-OES. This was performed to ensure the independent methods concept which is mostly accepted by international standard organizations. Further, the stability test of the in-house cocoa flour RMs was checked at 1, 3, 6 and 12 months bottling to make sure that cadmium content did not change significantly.

Results and Discussion

Preliminary experiments were conducted to select a reliable chemical modifier for the measurement of Cd in candidate cocoa flour RMs samples. The main function of chemical modifier is to prevent volatilization of Cd analyte during drying and ashing phases and also to increase the volatility of the matrix in cocoa samples in order to remove it before atomization. Various salts and metallic matrix modifier have been described in many publications [11, 13]. In this study Mg and La salts were chosen as matrix modifier since these metals known not be thermochemically reduced during the ashing and the beginning of the atomization process [14].

Optimization of a furnace temperature program

The most important factor in this method is selecting the optimum temperature program. Cadmium is known as a volatile metal and can be easily lost during the ashing stage. Most suitable chemical modifier must be used to form intermetallic compounds with cadmium, and allow higher ashing temperature to remove as much possible of matrix before the analyte is atomized. The influence of the ashing and atomization temperatures on the determination of Cd in candidate of in-house cocoa flour RMs samples, by GF-AAS without matrix modifier and with $Mg(NO_3)$ as well as $La/Mg(NO_3)$ modifiers were investigated. Working solution containing 2 $\mu g.l^{-1}$ Cd was used to optimize the parameters.

Cadmium determination method by GF-AAS with $Mg(NO_3)$

A 10 μL of 2000 mg l^{-1} magnesium nitrate solution as a matrix modifier was injected into the graphite tube. Two drying steps at 80°C and 140°C were set up to ensure the sample dry completely. Ashing temperatures of 200 up to 400°C were investigated (Fig.2). Optimum ashing temperature was obtained to be 300°C for $Mg(NO_3)$ modifier and the absorbance slowly decreased when the temperature was increased. It has showed that on ashing stage the ability of this matrix modifier to retain Cd analyte was on the 200 – 300°C. And usually on 200 and 250°C organic samples are not ashed completely. It can be explained that on this temperature there is a tiny black colour of charcoal in graphite tube. Nevertheless, at 300°C can be used as ashing stage if this matrix modifier is used.

Analysis without matrix modifier was also performed and the result shows that the relative absorbance at ashing stage was very low even on 300°C. This obviously confirmed that for volatile metals it is recommended to use the matrix modifier.

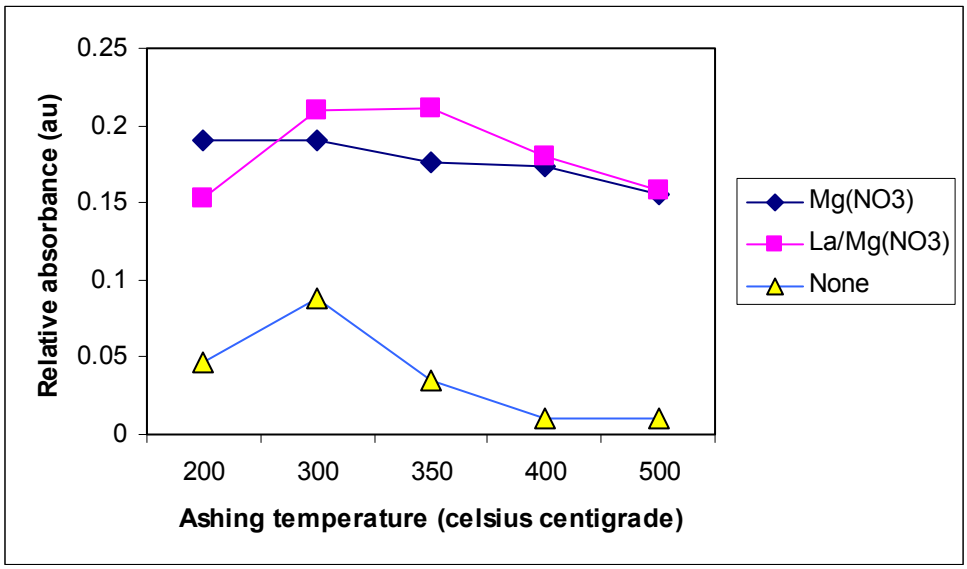

Figure 2. Effect of the ashing temperature (atomization fixed at 1500°C).

Cadmium determination method by GF-AAS with La/Mg(NO$_3$)

A 10 µL volume of a Lanthanum/magnesium nitrate solution (mixture) as a chemical modifier was injected into a graphite tube. Ashing temperatures between 200 up to 400°C were investigated. The optimum of ashing temperatures was obtained to be 300 - 350°C when the atomizing temperature at 1500°C (Fig.2). The maximum absorption was 0.21 for ashing temperatures of 300 and 350°C. It shows the stability of this modifier at a wide range of ashing temperatures stage. This might be caused by intermetallic compounds with Cd shows the thermodynamic activities are lower rather than pure metal Cd analyte itself. And further these analyte elements can be brought to the atomization temperature without loss during the ashing process.

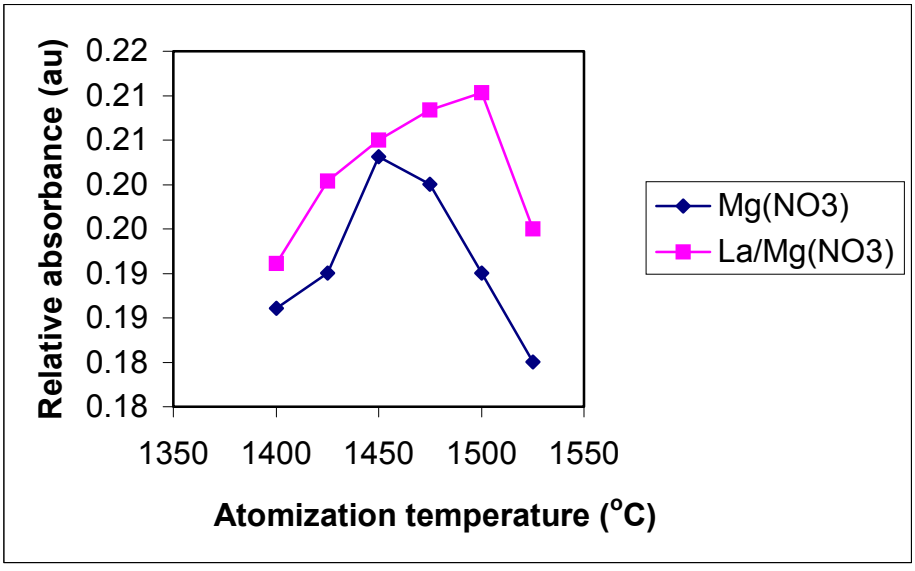

Figure 3. Effect of the atomization temperature (ashing temperature was fixed at 300°C) on the absorption signals Cd.

Atomizing temperatures of 1400 – 1525°C were also investigated. When the ashing temperature was set up at 300°C for $Mg(NO_3)$ modifier, the maximum absorption of atomization temperatures increase until 1450°C and drop down when the atomizing temperature was increased. Unlike that has showed by $La/Mg(NO_3)$ modifier, this matrix modifier achieved the maximum absorption at 1500°C if ashing temperature was set up between 300-350°C (Fig.3) this is an advantage of this modifier.

The repeatability from 6 consecutive measurements of 1, 1.5 and 2 μgL^{-1} working cadmium solutions were also calculated by using $La/Mg(NO_3)$ modifier. The repeatability, expressed as a relative standard deviation (RSD), were 1.0, 0.9 and 0.9% respectively and indeed these values are relatively small which exhibited no precipitation of this matrix modifier occurred in graphite tube. When the precipitation of matrix modifier occurred in graphite tube thus it will fluctuate of testing results significantly and consequently the relative standard deviation tends become high and it might be caused by a part of analyte probably retained in graphite tube and the life time of graphite tube will be short in use.

In view of their stabilization and enhancement effect on the Cd signal in standard solutions undoubtedly $La/Mg(NO_3)$ is better rather than $Mg(NO_3)$ modifier, so that $La/Mg(NO_3)$ was selected as a mixed matrix modifier for determination of cadmium on in-house cocoa flour RMs. Further the temperature program was applied in determination of Cd listed in Table 2.

Table 2. Temperature program for the determination of cadmium in cocoa flour with $La/Mg(NO_3)$ as a chemical modifier.

Step	Start/End Temp(°C)	Ramp/Hold Time (sec)	Gas flow rate/ml.min^{-1}
Dry	80/140	40/0	200
Ash	350/350	20/0	200
Atom	1500/1500	0/5	30
Clean	1800/1800	0/4	200
Cool	0/0	0/17	200

Accuracy of method

The accuracy of analytical method was established by determining the cadmium in cocoa flour samples and by quantitative recovery (instead of CRM's) studies of the amount cadmium added to the sample [15]. Different amount of Cd concentration were added to the sample for observing performance of this method. The results showed that recovery range within 99.83 –102%. This is indicated that in the sample pretreatment of cocoa flour was done perfectly and also optimization procedure developed was good so that whole steps (drying, ashing, atomizing and cleaning) in graphite tube running well. Results of recovery studies are presented in Table 3. A series of Cd standard (0, 0.5, 1, 1.5 and 2 μgL^{-1}) was measured for determining linearity and limit of detection and then was evaluated by using linear regression of Excel program. This method is having a limit of detection of 0.07 μgL^{-1}(calculated as $3S_{y/x}$/slope) of calibration curve and showing a good working range linearity of 0.9994

Matrix interferences

The influence of matrix interference on the cadmium determination in cocoa flour was evaluated based on the ratio of the slopes of the calibration graphs (the slope of calibration curve for aqueous standard solution and the slope of calibration curve obtaining by the standard-addition

method). The ratio of slopes was 0.98, which indicated that no matrix effect was observed. This was caused by the developed digestion procedure which was able to decompose and oxidize organic matrix in samples completely.

Table 3. Recovery of cadmium in the candidate of in-house cocoa flour samples.

Sample	Cd (in sample) mg l^{-1}	Cd (added) mg l^{-1}	Cd (found) mg l^{-1}	Recovery %
17A	0.612	0.150	0.765	102.0
8A	0.606	0.323	0.954	101.5
33B	0.607	0.602	1.208	99.83

Analysis results of in-house cocoa flour reference material

The results of determination of cadmium metal in cocoa flour by two methods were carried out only in the first time when this cocoa flour was prepared listed in Table 4. Cd content of cocoa flour was checked with GF-AAS and ICP-OES to compare between two these methods and then evaluated by t-test using Microsoft-Excel program to observe whether the results obtained by the both methods differed. The result shows that critical value t = 3.18 (P = 0.05). Since the calculated value of t (-7) is less than critical value thus the null hypothesis is not rejected: the methods do not give significantly different results for the cadmium concentration. Furthermore, determination of Cd in cocoa flour was only carried out with GF-AAS for 1, 3, 6 and 12 months bottling for stability testing.

Table 4. Determination of cadmium in the candidate of in-house reference materials after digestion and using La/Mg(NO_3) as a chemical modifier (0 month).

Sample	GF-AAS	Sample	ICP-AES (accredited laboratory)
	Mean ± %RSD(mg l^{-1})		Mean ± %RSD (mg l^{-1})
17A	0.61 ± 0.9	34A	0.62 ± 0.9
8 A	0.61 ± 2.0	19A	0.63 ±0.8
33 B	0.61 ± 2.0	41B	0.63 ±1.6
18 B	0.60 ± 1.6	28B	0.62 ±2.1

Homogeneity and stability testing of in-house cocoa flour RMs

Homogeneity and stability testing is another important component of certification process of reference materials, which is indicating the validity of the certified values and their uncertainties in the analysis of individual units. In relate to the state-of-art in reference materials certification, as indicated implicitly in ISO Guide 35 concerning the homogeneity study, ANOVA and F-test are normally used to determine the discrepancy between the variance of within and between–bottle homogeneity. In this work, variability of Cd content in cocoa flour was calculated by one-way analysis of variance (ANOVA) using the data analysis tool in Microsoft-Excel program at significant level of α = 0.05. The result shows that critical value F = 3.49 (P=0.05). Since the calculated value of F (0.91) is less than this the null hypothesis is not rejected: which is indicating that no variability of the within bottle and the between bottle. Therefore the in-house cocoa flour reference material can be considered homogeneous. Stability testing was carried out

also during 1, 3, 6 and 12 months bottling in room temperature. Data of analyses are processed by using Minitab 15 Program. Between and within bottle stability testing was done to ensure that this candidate of in-house RMs is stabile within certain time. The interval plot illustrates the time (1, 3, 6 and 12 months bottling) and variation in the data. The plot shows the mean for each bottle group and displays bars around the mean. The mean value is relatively the same and confidence interval is overlapping the other neither for between-bottle nor within bottle stability studies so that it can be concluded that no concentration changes were found for cadmium metal over period of time (1, 3, 6 and 12 months bottling) with significant level used ($\alpha = 0.05$). The results of stability testing are summarized in (Figs. 4 and 5).

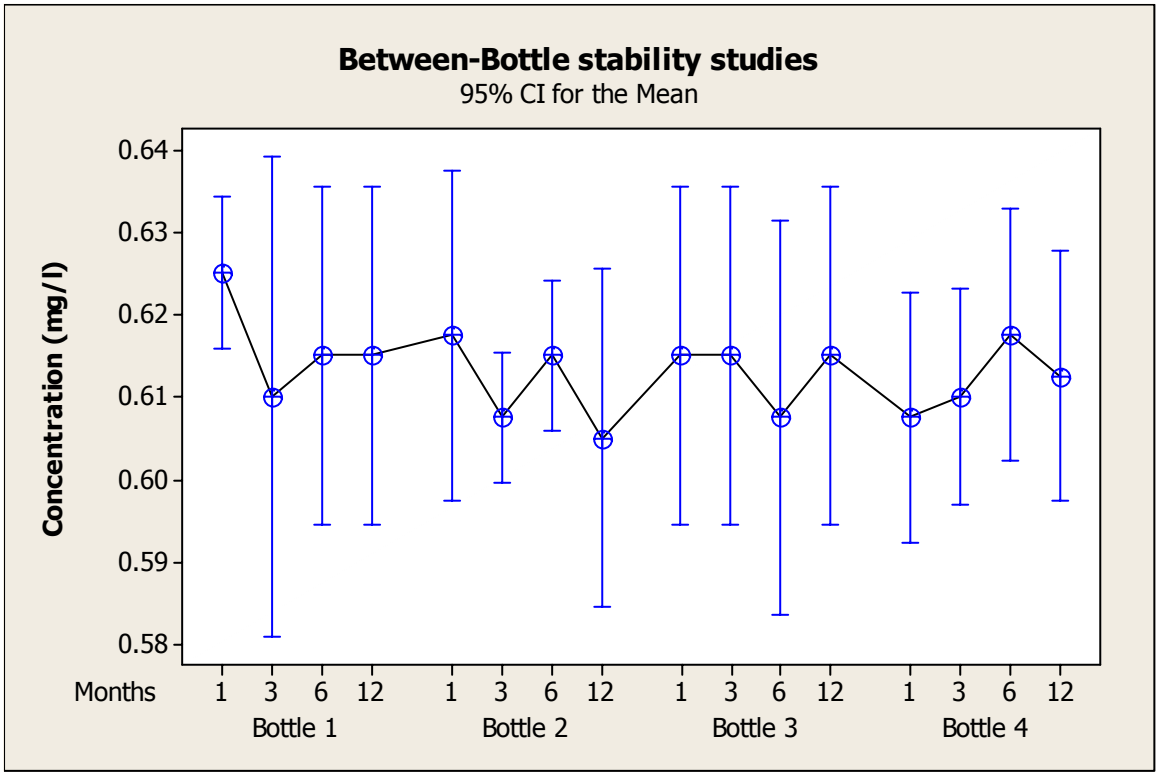

Figure 4. Between-bottle stability test of Cd metals on in-house cocoa RMs stored at room temperature.

(bars are confidence interval of four sub-sample analyses)

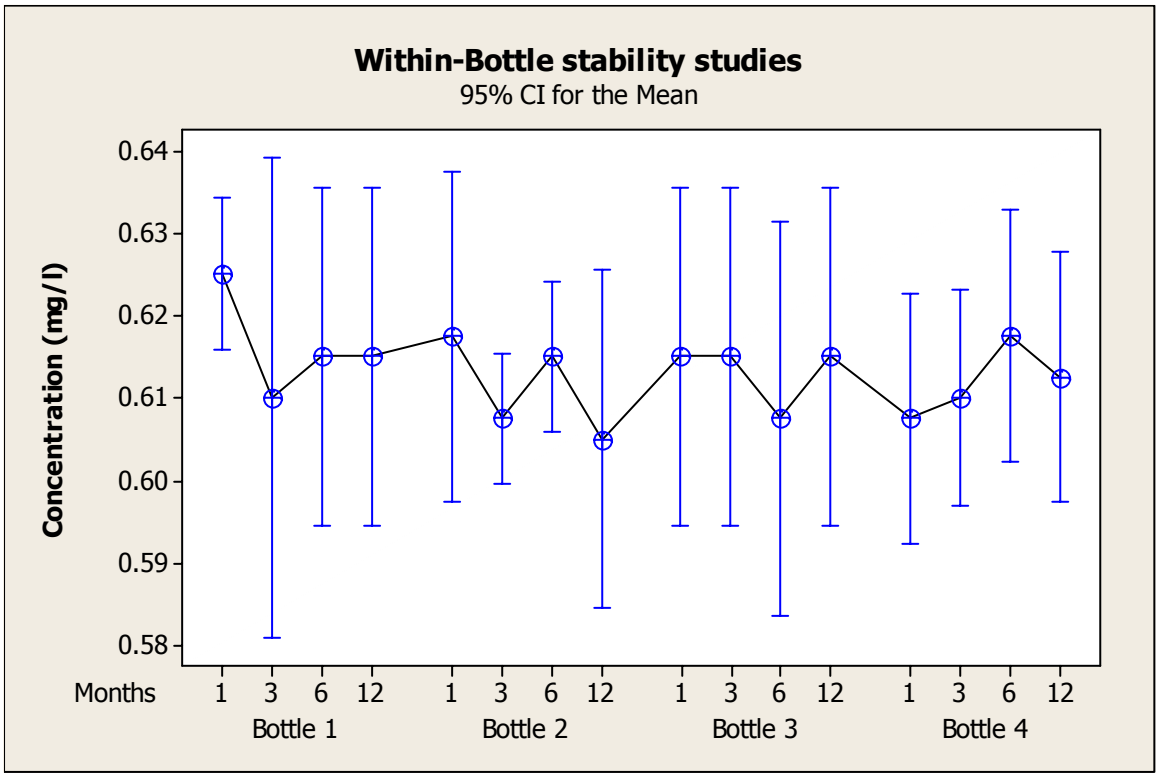

Figure 5. Within-bottle stability test of Cd metals on in-house cocoa RMs stored at room temperature.
(bars are confidence interval of four sub-sample analyses)

Conclusion

It has been described that the method based on application of La-Mg(NO_3) modifier can be applied successfully for determining cadmium in cocoa flour. The maximum ashing temperature was found to be 350°C and the maximum atomizing temperature was 1500°C. Homogeneity and stability testing of candidate in-house cocoa RMs also shows no variability within-bottle and between-bottle at significant level of $\alpha = 0.05$. This method can be proposed as a precise and accurate method for determining cadmium and also for certification purposes of in-house cocoa flour reference materials.

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