CHAPTER III

RESEARCH METHODOLOGY

Key words

Oxidative stress, lipid hydroperoxide, malondialdehyde (MDA), total antioxidant capacity, NAG, Graphite-AAS, Cadmium

Scope of the study

Total participants in this study are the people in the Mae-Sot district, Tak province. All participants are both male, female, age over 35 years and lived in Mae-Sot district over 10 years ago. The diabetes mellitus participants are excluded from the study.

Research methodology

All data are collected from the residential area age over 35 years old and stay in Mae-Sot district more than 10 years. Waist circumference is measure at the midpoint between the rib cage and the top of the lateral border of the iliac during minimal respiration. The blood pressure is measure in the seated position after 5 min of rest. Two measurements will make on all participants at 5 min intervals. The average of two measurements is used data analysis.

Blood and urine samples are collected from participants after an overnight fasting. The fasting time will verify before the blood specimen was taken. Participants who had not fast for at least 8 hrs did not have their blood draw. All biochemical examination [fasting glucose (Glu), blood urea nitrogen (BUN), creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride (TG)] were performed by using enzymatic methods on the Hitachi auto-analyzer (Roche, Switzerland). And the oxidative stress [lipid hydroperoxide [the method base on ferrous oxidation xylenol orange assay (FOX assay) principal], malondialdehyde [the method base on thiobabituric acid reactive substance (TBARS) principal] and total antioxidant

[the method base on the antioxidant in sample to inhibit the oxidation of ABTS· (2, 2'-Azino-di-[3-ethyl benzthiazoline sulphonate) to ABTS·+ by metmyoglobin)] are measured in these blood samples. Cadmium level will analyze in urine by Graphite furnace atomic absorption spectrophotometer (AA-Zeeman model). Urine creatinine, and NAG (NAG activity assay base on enzymatic hydrolysis of p-nitrophenyl-N-acetyl- β- D-glucosaminide) were measured in urine.

Materials and Instrument

1. Polypropylene test tubes (13 ml) with screw caps
2. Polypropylene microtube (2ml) with screw caps that contain O-ring seal were used for containing blood samples.
3. Spectro 22 spectrophotometer, being available for visible, near ultraviolet, and near infrared spectral wavelength, was employed for qualitative and quantitative analysis.
4. The Sunrise Touchscreen (Sunrise RC/TS-TC/TW/BC/6 Filter, Tecam Austria), the fully automatic 96-well micro plate reader, is utilized for the measurement of absorbance (optical density) of samples from the biological or non-biological by reading twelve well simultaneously.
5. Roche Hitachi 912 biochemistry (Japan) automatic analyzer is the instrument for performing chemical clinical analysis. It was used to analyze blood glucose, triglyceride, total cholesterol and HDL cholesterol
6. Continuously heat hotplate with heat control room temperature to 100°C or over.

Chemical reagent

1. Ammonium ferrous sulfate (Sigma-Aldrich, USA)
2. Xylenolorange (Sigma-Aldrich, USA)
3. Triphenylphosphine (Sigma-Aldrich, USA)
4. D-sorbitol (Sigma-Aldrich, USA)
5. Cumene hydroperoxide (Sigma-Aldrich, USA)
6. Phosphoric acid (Sigma-Aldrich, USA)
7. Thiobarbituric acid (Sigma-Aldrich, USA)
8. Tricholoacetic acid (MERCK, USA)

9. Phosphoric acid (Sigma-Aldrich, USA)
10. ABTS (Sigma-Aldrich, USA)
11. Trolox (Sigma-Aldrich, USA)
12. Myoglobin (Sigma-Aldrich, USA)
13. Sodium chloride solution
14. Citric acid solution
15. Sodium citrate solution
16. Citrate buffer
17. NAG- substrate solution
18. AMP buffer
19. *p*-Nitrophenol working standard solution

Method and measurement of clinical characteristic

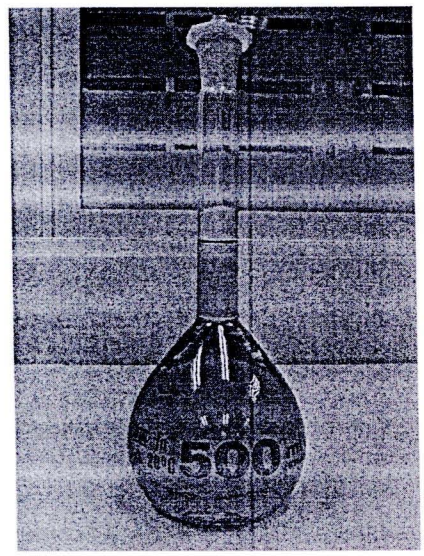
Body weight of volunteer was measured by a digital scale balance with an accuracy of 0.1 kg and height was measured to the nearest centimeter. Body mass index (BMI) was calculated as the ratio of body weight (kg) to height squared (m^2). Fasting glucose (Glu), blood urea nitrogen (BUN), creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride (TG)] were measured by Hitachi 912 analyzer. Low-density lipoprotein cholesterol (LDL-C) was measured by Friedelwald formular. Lipid hydroperoxide assay is base on FOX assay principal. Malondialdehyde (MDA) assay is base on the TBARS principal. Total antioxidant capacity measurement is base on the antioxidant in sample to inhibit the oxidation of ABTS˙ (2,2'-Azino-di-[3-ethyl benzthiazoline sulphonate]) to $ABTS^{\cdot+}$ by metmyoglobin. Cadmium level will analyze in urine by Graphite furnace atomic absorption spectrophotometer (atomic absorption-zeeman model). Urine creatinine and N-acetyl-β-D-glucosaminidase activity assay (NAG assay) (NAG activity assay base on enzymatic hydrolysis of p-nitrophenyl-N-acetyl- β- D-glucosaminide) were measured in urine.

Reagents preparation and assay procedure

Ferrous oxidation xylenol orange assay test for lipid hydroperoxide

Total hydroperoxides may also be determined using the ferrous oxidation in xylenol orange (FOX) assay, which can be used for hydroperoxides present in the aqueous (FOX1) and in the lipid (FOX2) phases (Jaffar Nourooz-Zadeh and Lester, 1999; Nouroozzadeh, et al., 1994). The ferrous oxidation of xylenol (FOX) assay was developed by Wolff and co-workers to analyze the hydroperoxides formed. The FOX method is based on the oxidation of ferrous (II) to ferric (III) ions by hydroperoxides under acidic conditions (Z.-Y. Jiang, et al., 1992; Z. Y. Jiang, et al., 1991; Sodergren, et al., 1998; Yin and Porter, 2005). Ferric ions are detected by UV absorbance at 560 nm after reaction with the ferric ion indicator, xylenol orange, generating a blue-purple complex with an absorbance maximum at 560 nm.

1. Fe^{2+} + hydroperoxides → Fe^{3}+ alkoxyl radical + OH^{-}
2. Fe^{3+} + XO → blue-purple complex (550-600 nm)



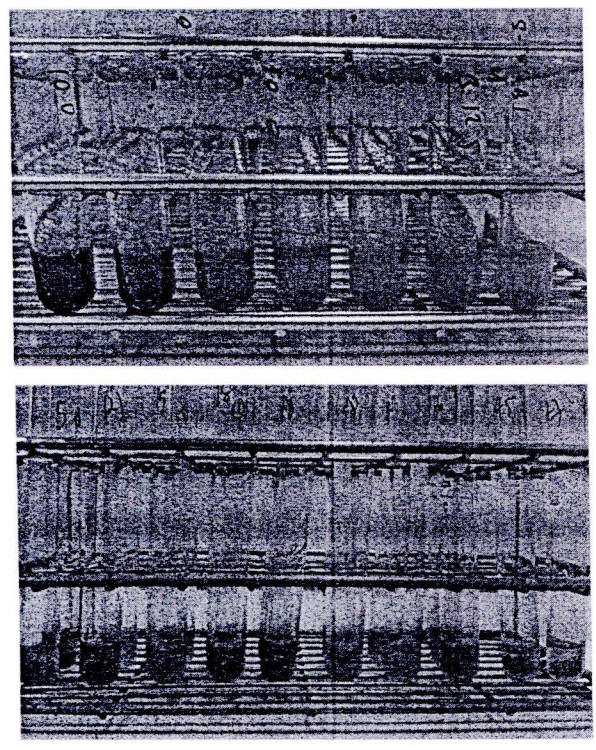


Figure 2 FOX reagent and FOX assay

FOX reagent 1000 ml

Dissolve 0.07g Xylenol orange, 0.1 g Ammonium ferrous sulfate and 18.22 g in Sulfuric acid 1000 ml

BHT and TPP reagent 250 ml

Dissolve 22.04 g BHT and 0.66 g TPP in methanol 250 ml

Assay procedure

1. Add 90 µl of sample/standard was taken in test tubes
2. Added 10 µl TPP and 400 mmol/l BHT and mix well
3. Incubate 15 min
4. Add 900 µl of FOX reagent and mix well
5. Incubate 30 min at room temperature
6. Measurement absorbance at 560 nm by spectrophotometer

The Thiobabituric acid test (TBA test) for Thiobabituric acid reactive substance (TBARS)

The TBA test is performed to measure the amount of MDA present in the sample. MDA is generated as a degradation product from peroxidized lipids (Janero, 1990). It has been shown to be formed from PUFAs containing at least two double bonds. The basis of the TBA methods is the reaction of MDA with TBA at low pH and high temperature to form a colored complex, the MDA-TBA complex, with an absorption maximum at 532 nm that can be measured by visible absorption spectrophotometer (Tangvarasittichai, 2009; Wong, et al., 1987).

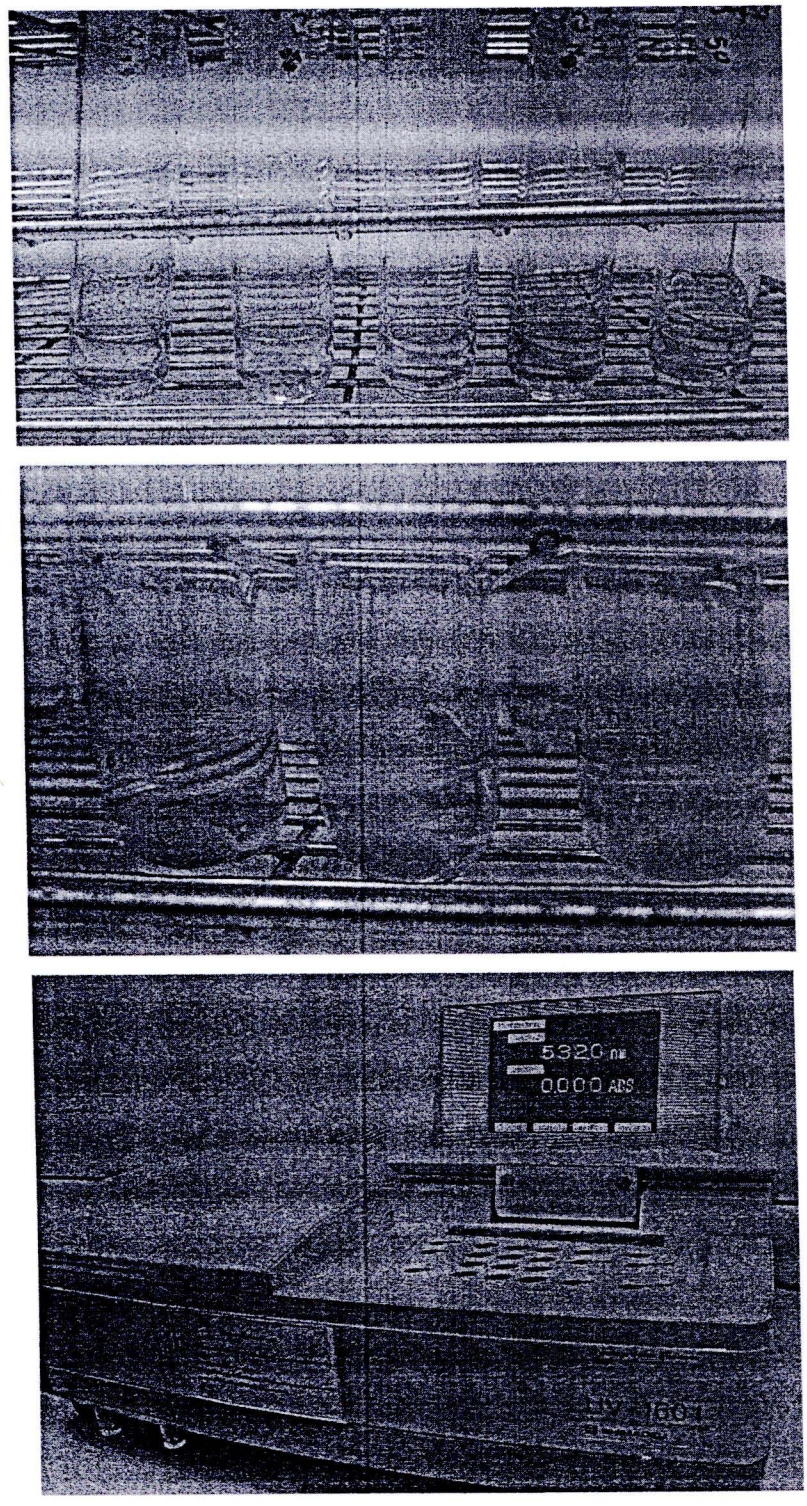


Figure 3 Thiobabituric acid test

Thiobarbituric acid

Thiobarbituric acid was dissolved in HPLC-grade water to 250 μM.

Phosphoric acid

Phosphoric acid was dissolve in HPLC-grade water to 100 μM.

Tricholoacetic acid

Tricholoacetic cid was dissolve in HPLC-grade water to 100 mg/dl

Assay procedures

1. Add 50 μl of sample 750 μl of phosphoric acid and 250 μl of thiobabitouric acid and tightly close scroll cap tube

2. Boil sample tube in boiling water for 1 hour

3. After that, immediately bring all sample tube in ice for 10 minutes after that add 50 μl of trichloroacetic acid mix and incubate at room temperature for 5 minute

4. Add 400 μl of HPLC-grade water and centrifuge at 3500 rpm for 15 minutes

5. Gently draw supernatant and read absorbance at 532 nm

Total Antioxidant capacity measurment

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids (Halliwell and Chirico, 1993), food extracts (C Rice-Evans and Burdon, 1993). Two types of approach have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen- or electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical. From the ABTS [2, 29-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical generation as the cation form that the basis of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS [2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] to $ABTS^{\cdot}+$ by metmyoglobin. The amount of $ABTS^{\cdot}+$ produced can be monitored by reading the absorbance at 405 nm Color is reverse correlated to total antioxidant.

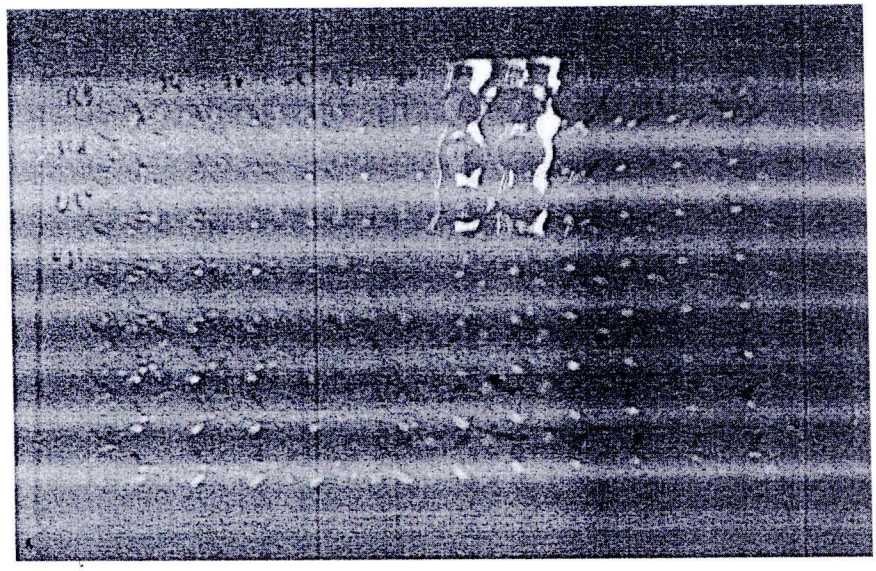


Figure 4 Total Antioxidant capacity measurement

Reagent preparation

Antioxidant assay buffer (10x) (phosphate buffer saline)

Dilute 10x assay buffer with HPLC-grade water (for example 3 ml of assay buffer and 27 ml of HPLC-grade water).

Antioxidant assay ABTS stock solution

Dissolved ABTS (Sigma-Aldrish USA) with 1x buffer to 300 mM.

Antioxidant assay ABTS substate working solution

Add 5 % hydrogenperoxide 25 µl in 10 ml of ABTS substrate working solution.

Antioxidant assay metmyoglobin

Dissolved myoglobin 10 mg with 2.85 ml of 1x assay buffer.

Procedure

1. Prepare myoglobin working solution by dilute myoglobin stock solution 100 folds
2. Add 10 µl of sample, 50 µl of working myoglobin and 150 µl of ABTS substate working solution to each well in ELISA plate
3. Incubrate for five minutes at room temperature
4. Read absorbance at 405 nm using plate reader

_N_-acetyl- β- D-glucosaminidase activity assay

NAG activity assay

_N_-acetyl-β-glucosaminidase (NAG), a proximal tubule lysosomal enzyme, has been extensively studied and has proven to be a sensitive, persistent, and robust indicator of tubular injury. Increased NAG levels have been reported with nephrotoxicant exposure, delayed renal allograft function, chronic glomerular disease, diabetic nephropathy, as well as following cardiopulmonary bypass procedures. The two advantages of using NAG are (a) sensitivity, subtle alterations in the epithelial cells in the brush border of the proximal tubules result in shedding of NAG into the urine and the amount of shed enzyme can be directly correlated to tubular injury; and (b) quantitation, simple and reproducible enzymatic assays are well established to measure the analyte colorimetrically using a spectrophotometer (Edelstein, 2008).

NAG activity assay base on enzymatic hydrolysis of _p_-nitrophenyl-_N_-acetyl-β- D-glucosaminide were measured in urine.

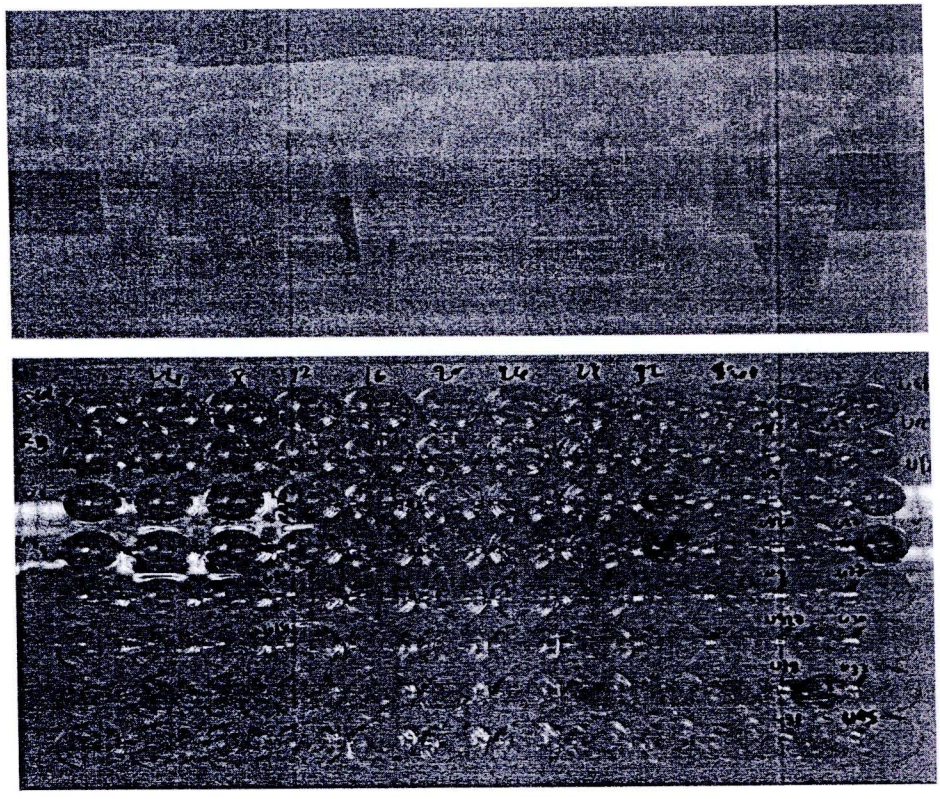


Figure 5 _N_-acetyl- β- D-glucosaminidase activity assay

Reagent preparation

Sodium chloride solution 0.15 mol/L

Citric acid solution 0.2 mol/L

In a 200 ml volumetric flask, dissolve 8.4 g of citric acid monohydrate in distilled water, dilute to the calibration mark and store at 4 °C

Sodium citrate solution 0.2 mol/L

In a 200 ml volumetric flask, dissolve 10.3 g of anhydrous trisodium citrate in distilled water, dilute to the calibration mark and store at 4 °C

Citrate buffer, pH 4.4, 0.1 mol/L

Put 56 ml of citric acid solution and 44 ml of sodium citrate solution in a beaker and allow the solution to equilibrate at 37 °C in a water bath. Check the pH with a pH meter and adjust to pH 4.4 by adding citric acid solution or sodium citrate solution.

NAG- substrate solution 10 mmol/L

In a 100 ml volumetric flask, dissolve 342 mg of p-nitrophenyl-N-acetyl- β-D-glucosaminide in citrate buffer and dilute to the mark. Dispense this solution in 10 ml aliquots into screw-capped polystyrene test tubes and store the tubes at -20 °C until the day of use. Under the condition, the NAG-substrate solution is stable for months. Before use thaw the NAG-substrate solution and equilibrate it in a water bath at 37 °C.

AMP buffer, 0.75 mol/L, pH 10.25

In a 200 ml volumetric flask, dissolve 18.84 g of 2-amino-2methyl-1-propanol hydrochloride in 50 ml of distilled water. With a pH meter adjust the pH to 10.25 at 25 °C by adding NaOH 6 mol/L solution. Dilute to the calibration mark with distilled water and store at 4 °C

***p*-Nitrophenol working standard solution 100 µmol/L**

With a calibrated volumetric pipette, transfer 1 ml of 10 mmol/L p-nitrophenol stock standard solution into 100 ml volumetric flask and dilute to the calibration mark with 0.15 mol/L NaCl solution. Prepare this solution immediately before use.

Procedure

1. Label four spectrophotometer cuvets "RB" (reagent blank), "S" (standard), "U" (Urine), "UB" (urine blank)
2. Into these respective cuvets pipette the following solution:
   2.1 Cuvet RB 0.1 ml of NaCl solution
   2.2 Cuvet S 0.1 ml of p-nitrophenol working standard solution
   2.3 Cuvet U and UB 0.1 ml of urine
3. Equilibrate the cuvet in water bath at 37 °C
4. Pipette 0.1 ml of NAG-substrate solution into cuvet RB, S and U
5. Incubate the samples for precisely 15 min at 37 °C
6. Add 0.1 ml of AMP buffer all the cuvet and add 0.1 ml of NAG substrate to cuvet UB
7. Measure the absorbance of the cuvet at 405 nm.

Graphite Furnace Atomic Absorption Spectrophotometry

Graphite furnace atomic absorption spectrometry (GFAAS) (also known as Electrothermal Atomic Absorption Spectrometry (ETAAS)) is a type of spectrometry that uses a graphite-coated furnace to vaporize the sample. Briefly, the technique is based on the fact that free atoms will absorb light at frequencies or wavelengths characteristic of the element of interest (hence the name atomic absorption spectrometry). Within certain limits, the amount of light absorbed can be linearly correlated to the concentration of analyte present. Free atoms of most elements can be produced from samples by the application of high temperatures. In GFAAS, samples are deposited in the small graphite or pyrolytic carbon coated graphite tube, which can then be heated to vaporize and atomize the analyte. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. Applying the Beer-Lambert law directly in AA spectroscopy is difficult due to variations in the atomization efficiency from the sample matrix, and non-uniformity of concentration and path length of analyte atoms (in graphite furnace AA). Concentration measurements are usually determined from a working curve after calibrating the instrument with standards of known concentration.

GFAA spectrometry instruments have the following basic features: (1) a source of light (lamp) that emits resonance line radiation; (2) an atomization chamber (graphite tube) in which the sample is vaporized, (3) a monochromator for selecting only one of the characteristic wavelengths (visible or ultraviolet) of the element of interest; (4) a detector, generally a photomultiplier tube (light detectors that are useful in low-intensity applications), that measures the amount of absorption; (5) a signal processor-computer system. (strip chart recorder, digital display, meter, or printer)

Cadmium in urine by Graphite furnance atomic absorption

Urine samples are diluted with Tritron x-100 and diammonoum hydrogen phosphate, which acts as a matrix modifier, and 1% HNO3 to remove interference matrix during drying and ashing strep then be measured cadmium by Graphite furnance atomic absorption spectrophotometer

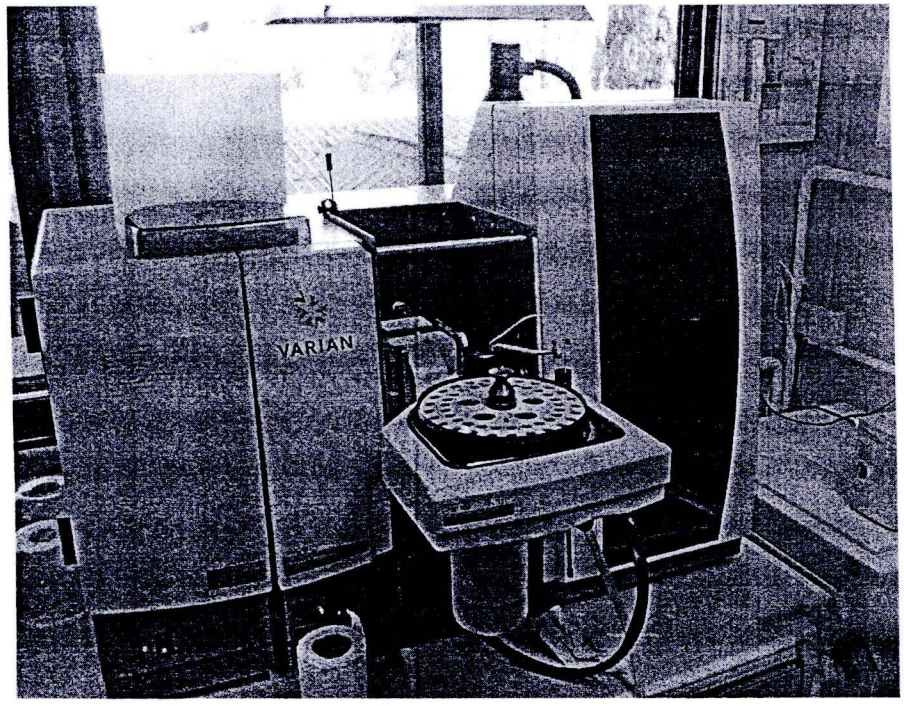


Figure 6 Graphite Furnace Atomic Absorption Spectrophotometry, AA-zeman, Germany

Reagent preparation

Modifier diluents solution

Substance in the unit of 0.1% (W / V) Triton X-100 and 0.2% (W / V) Diammonium hydrogen phosphate, and weighed Triton X-100 0.1 g in volumetric flask 100 ml, add water about 20 ml, to be heated in water bath at 40 °C, weighed Diammonium hydrogen phosphate 0.2 g, mixed in the volumetric flask, add 1% HNO3 100 µl, adjust the volume to reach 100 ml.

1% HNO3

Add water 50 ml in a volumetric flask 100 ml, pipette conc. HNO3 1 ml into the volumetric flask to adjust the volume to reach 100 ml.

Procedure

1. Pipette diluents 900 µl into micro-centrifuge tube
2. Add urine 100 µl and mixed by mixer, poured into sample cup
3. Measurement level of cadmium by Graphite furnace atomic absorption spectrophotometer

Statistical analyses

The results are analyzed by descriptive statistic and report as mean, range and percent, we compares by the ANOVA to determine the relationships between variables will assess by Pearson correlation.