CHAPTER 3
MATERIALS AND METHODS

3.1 Collection of Blood Samples

Blood samples were collected from Chiang Mai City traffic policemen on December 24 and 26, 1996, in collaboration with a research team from the Research Institute for Health Sciences (RIHES), Chiang Mai University. Stored blood specimens collected in April 1995 by the same staff were likewise used in the study. For both sets of samples, ethylenediaminetetraacetic acid dipotassium salt (K_2-EDTA) was used as anticoagulant.

3.2 Questionnaires

At the blood drawing venue, each participant was asked to fill out a questionnaire concerning personal information and potential determinants for blood lead levels. Topics covered by the questionnaire included demographic characteristics (such as education, age); occupational background and present nature of work like the the number of hours spent on the road per day and the kind of masks they use; as well as smoking and drinking habits.

3.3 Analytical Procedure

3.3.1 Apparatus

Voltammetric measurements were made with a microprocessor-controlled 693 VA Processor with built-in thermal printer equipped with a 694 VA Stand (Metrohm Ltd., Switzerland). The instrument was fitted with the following electrodes:

1. Working Electrode

Rotating disk electrode made up of two parts namely, a drive shaft (Metrohm, 6.1246.000) and electrode tip (Metrohm, 6.1204.1204) of glassy carbon (disk diameter 2.0 ± 0.1 mm) which were screwed together;

2. Reference Electrode

Reference system (Metrohm, 6.0728.020) of Ag/AgCl/c(KCl)=3 mole/l with ceramic diaphragm (3 mm diameter);

3. Auxiliary Electrode

Platinum (Pt) auxiliary electrode (Metrohm, 6.0343.000).

Measuring vessels made of lead-free borosilicate glass (Metrohm, 6.1415.150) with working volume of 5-70 ml were used for the analyses.

3.3.2 Solutions

Deionized, doubly-distilled water was used to prepare all solutions. Lead standard solutions were prepared by dilution of a lead (Pb) Titrisol Standard (Merck) containing 1.000 g ± 0.002 g Pb/litre.

Stock solutions of 1000 µg/ml Mn(II) and Al(III) were prepared from $MnSO_4.H2O$ (AR, Carlo Erba) and Al_2O_3 (AR, BDH), respectively. 1M stock solution of calcium chloride was prepared from $CaCl_2$ crystals (AR, Merck) and 0.06M Ethylenediaminetetraacetic acid (EDTA) stock solution from disodium EDTA salt (AR, Merck). A stock solution of 2000 µg/ml Hg(II) was prepared from $HgCl_2$ salt (AR, Carlo Erba).

Concentrated hydrochloric acid used was of Suprapur (Merck) grade. Methanol (AR, J.T. Baker) was used for rinsing the GCRDE.

The matrix modifying solution consisted of 1M HCl containing 200 mg/l each of Al(III) and Mn(II), 0.05 M $CaCl_2$, 0.001M EDTA, and 0.01% (v/v) Triton X-100 in reagent water.

The preplating solution consisted of 1 mM Hg(II) and 0.1M HCl.

3.3.3 Standards and QC Samples

Whole blood standards (Blood Lead Laboratory Reference System, BLLRS, Center for Disease Control, Atlanta, GA, USA) with three different Pb levels (34.3, 15.2, 10.3 µg/dl, respectively) were used to check accuracy of the method. Additionally, three other BLLRS materials (26.39, 26.38, 10.30 µg/dl, respectively) were used as standards to prepare calibration curves in sample matrix material. These BLLRS standards come from animal blood derived from livestock fed with diet containing specific $Pb(NO3)_2$ contents then haemolyzed with 1.5 mg/ml tripotassium EDTA then kept at –20°C.

Human blood quality control (QC) materials (University of Surrey, Guilford, UK) were likewise analyzed to validate the analytical method used in this study.

3.3.4 Preconditioning of GCRDE

Before using the glassy carbon rotating disk electrode (GCRDE), it was rinsed with distilled water to flush away any encrusted material on its surface. This was followed with a methanol rinse, and wiped dry. It was then polished on a microcloth disk wet with distilled water with a few drops of 0.05 µm Alumina suspension (Bioanalytical Systems, Indiana, USA). After rubbing the GC surface for 1-2 minutes on the disk using a smooth, circular motion, the electrode was again rinsed with distilled water, followed by methanol then wiped dry.

3.3.5 Thin Mercury Film (TMF) Preplating Procedure

TMF electrodes were prepared by plating mercury film on the GCRDE from a deaerated solution containing 0.1M HCl and 1 mM Hg(II) at –1.0 V for 300 s, while the electrode was rotated at 2000 rpm. The film thickness was 0.73 μm as shown in the calculation (Appendix I). This was based on the steady-state current measured (ca. 1×10^{-4} A) during the plating process, as well as the geometric surface area of the glassy carbon disk, and assuming a 100% current efficiency of the mercury deposition and a uniform distribution of the film.

3.3.6 Preparation of Calibration Curve

Calibration curves for quantitative analysis of samples were prepared each time Pb determinations were done. For this, 100 μl aliquot of a diluted BLLRS standard was used as the first calibration standard solution. Subsequently, the same solution was spiked with one 10-μl and four 20-μl of 1 μg/l aqueous Pb standard. Volume corrections were made with regard to the amounts of Pb standard added to get the exact Pb concentrations in the solution; these were then inputted in the VA Processor so that Pb concentrations are automatically calculated based on the calibration curve.

3.3.7 Voltammetric Procedure for Lead in Whole Blood

A 100-μl whole blood sample or standard was pipetted into a measuring vessel, 4.9 ml matrix modifying solution was added then the vessel was placed in the sample holder of the instrument.

The sample solution was mixed by rotating the electrode and bubbling with N_2 gas, stirred further for 5 s, and preelectrolyzed at –1.1 V for 120 s. The electrode was rotated at a speed of 2000 rpm. Then stirring was stopped, and after 5 s of quiescent time, the current was recorded in a square-wave mode (SQWMODE) from –600 mV

to –200 mV. A conditioning potential of -200 mV was applied for 30 s to the electrode to assure a complete oxidation of the analyte metal deposited during the previous experiment. Figure 1 shows the operation sequence and instrument settings used in the method. Stripping peak for Pb was identified to appear at potential -450 ± 40 mV.

Method: SQWASV2 .mth OPERATION SEQUENCE
Title : Pb detm'n. with calibration curve

		Instructions	t/s	Main parameters		Auxiliary parameters	
1		RDE		Rot.speed	2000 /min		
2	0	SMPL>M		V.fraction	0.100 mL	V.total	5.0 mL
3	C	<CAL					
4	C	CSOLN>M		addition into MEAS			
5		DOS>m		Soln.name	modifier	V.add	4.900 mL
6	!	CONDC		Cycles	10		
7		PURGE					
8		STIR	30.0	Rot.speed	2000 /min		
9		0PURGE					
0		<REP					
1		STIR	5.0	Rot.speed	2000 /min		
2		SEGMENT		Segm.name	SWASV		
3	!	CONDC		Cycles	10		
4		REP>1					
5	!	CONDC		Cycles	10		
6		RINSE		Cycles	1	V.rinse	mL
7	C	CAL>5					
8		END					

Method: SQWASV2 .mth OPERATION SEQUENCE
Method: SQWASV2 SEGMENT
SWASV

	Instructions	t/s	Main parameters		Auxiliary parameters	
1	RDE		Rot.speed	1000 /min		
2	SQWMODE		U.ampl	24 mV	Modul.freq.	100 Hz
			t.step	0.30 s	Prep.cycles	0
			t.meas	2.0 ms	Meas.cycles	4
3	MEAS	120.0	U.meas	-1100 mV		
4	0STIR	5.0				
5	SWEEP	21.0	U.start	-600 mV	U.step	6 mV
			U.end	-200 mV	Sweep rate	20 mV/s
6	MEAS	30.0	U.meas	-200 mV		
7	END					

Figure 1 Operation Sequence for Pb Analysis

Parameters for identification of Pb stripping peak and a sample voltammogram are shown in the following figure.

Method: SQWASV2 SUBSTANCES
Pb - SWASV

Recognition		Display / Plot	
U.verify	-450 mV	I.scale	auto
U.tol (+/-)	40 mV	U.div	50.00 mV/cm
U.width min	25 mV	U.begin	-300 mV
U.width max	120 mV	U.end	-600 mV
I.threshold	250 pA		

Baseline		Evaluation (for peaks only)	
Type	linear	Quantity	I.peak
Scope	whole		
dU.front	auto		
S.front	auto		
dU.rear	auto		
S.rear	auto		

========== METROHM 693 VA PROCESSOR (5.693.0021) ==========

Method	: SQWASV2	User	:	Date	: 97-01-20
Mode	: SQWMODE	Determ.	: 01201157	Time	: 11:57:05
Run	: 16				
Curve	: smoothed	Segment	: SWASV	V R	: * 0

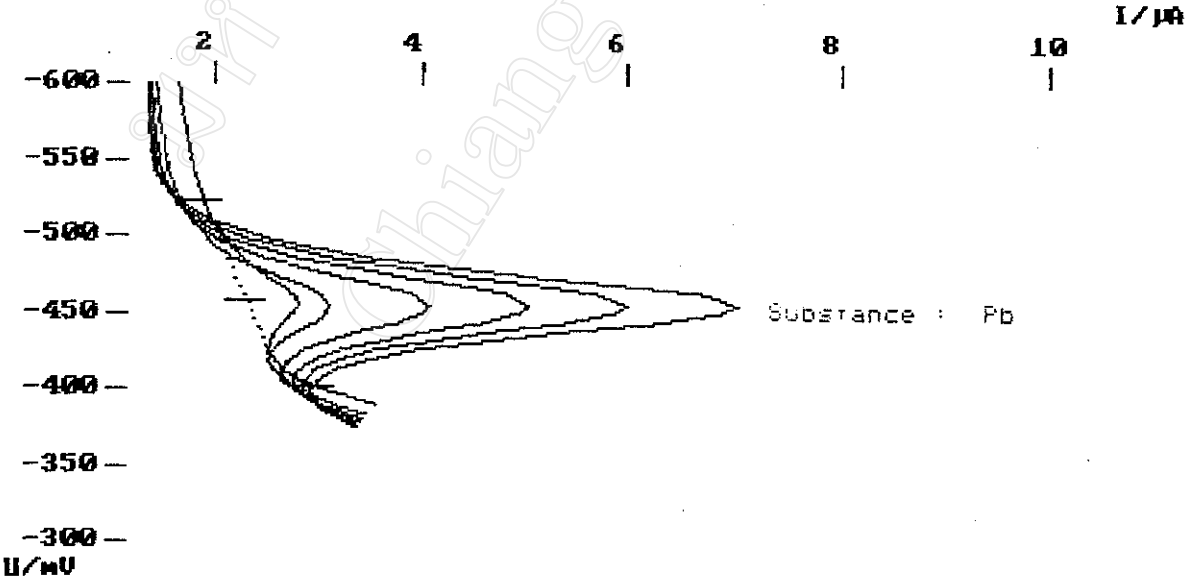

Figure 2 Parameters for Identification of Pb Stripping Peak and Sample Voltammogram

3.3.8 Operation of Metrohm 693 VA Processor

The following steps describe the operation of the instrument used for analysis in the present study:

1. Switch on the 693 VA Processor with main switch at the rear of the instrument. The instrument is initialised. If all is in order, the dialogue page "MONITORING" appears on the screen.

2. Switch to dialogue page "METHODS" by pressing the yellow <Methods> key. The dialogue page "METHODS" appears showing the list of stored methods in the instrument.

3. Use the cursor keys to select the method "SQWASV2", which then becomes black-backed.

4. Press the softkey <Copy to>. The softkey bar changes and presents the destinations for copying the selected method. Press the softkey <Working storage>.

Once the method is copied to the working storage, one can check the contents of the method using the dialogue pages; i.e., "OPERATION SEQUENCE" shows the main program for the determination of lead; "SEGMENT" indicates the program for measured value acquisition; "SUBSTANCES" shows the parameters for calibration of the substance lead; "CALCULATION" shows the calculation for the final results; and "DOCUMENTATION" indicates the automatic output of reports and curves.

5. To perform the determination, switch to dialogue page "MONITORING" by pressing the yellow <Monitoring> key.

6. Switch the program run mode to "determination". Check if the right parameters are entered on the dialogue page (e.g. "Sample size/SO").

7. Press the red <Start> key to begin the determination. The status display in the header of the dialogue page "MONITORING" switches to "*BUSY*". On completion of the program, the reports and curves entered on the "DOCUMENTATION" page are outputted on the built-in thermal printer.

3.4 Data Analysis

Statistical analyses of determined PbB levels and questionnaire data were made using SPSS and Epi Info 6.0 systems. Variations among PbB levels were examined by different factors as assessed with questionnaire data. Analysis of variance (ANOVA) was used to test the significance of association of factors such as age, drinking and smoking habits, the number of hours spent on the road, and the types of mask used with PbB levels. P-values <0.05 were considered significant (Appendices VII-VIII). The prevalence odds ratio for PbB levels ≥10 μg/dl with respect to certain variables was calculated using the Epi Info 6.0 system. Paired t-test was used to compare the PbB levels of the same individuals across the two years that PbB determinations were made.