CHAPTER III

METHODOLOGY

3.1 Sample Collection

Soil and sugarcane sampling were gathered from the Cd contaminated area in Mae Sot district, Tak Province in March, July and October 2007. The soil and sugarcane samples were collected according to the level of their contamination that had been studied and assigned by the National Research Center for Environmental and Hazardous Waste Management in 2005. Geographic Information System (GIS) instrument was used to locate the position of the sampling stations.

3.1.1 Soil sampling

Soil samples were collected from the ground surface to a depth of 30 cm. About 1-2 kg samples were collected from each site and put in the plastic bags and then sealed in order to prevent oxidation reactions of heavy metals in the soil with atmospheric oxygen. Each sample was labeled in order to identify the station of sampling. Soil properties were determined as shown in Table 3.1.

Table 3.1 Parameters and analytical method for physical and chemical properties of soil samples

Soil properties	Method
pH	pH Meter (w/v, soil/water suspension)
Oxidation-Reduction Potential (ORP)	ORP meter
Organic matter content (OM)	Walkley and Black Method
Moisture content	Oven dried at 105° C

3.1.2 Sugarcane Sampling

Sugarcane samples were taken from the same stations of soil sampling. The whole part of sugarcane including above ground and underground were collected. These sugarcane samples were wash and air dried. Then they were separated into 6 parts; root, underground stem, bagasse, juice, top (shoot) and leave to analyzed for Cd accumulation.

3.2 Sample Preparation

The collected soil samples were air dried for 2-3 days and the impurities were removed from the samples and then the samples were dried at 105 °C for 24 hr in an oven. The dried soil samples were crushed into fine particle sizes and ground to pass through a 2 mm sieve and thoroughly mixed to homogenize. These fine samples were kept in plastic bag containments before extraction procedures for determination of metals.

Sugarcane shoots and roots were first washed thoroughly with high pressurized tap water and then with de-ionized water. These the sugarcane samples were separated into six parts, roots, underground stems (setts), bagasse, sugarcane juice, leaves and top. Sugarcane samples were dried in an oven at 105 °C for 2 days to constant weight and dry matter yields were determined. After that, sugarcane samples were ground with electric mill and thoroughly mixed to homogenize. These fine samples were kept in plastic bag containments before extraction procedures for determination of metals. For the juice, it was kept in refrigerator until digestion.

3.3 Reagents and glassware

All reagents used were of analytical reagent grade or better. All glassware and plastic containers were previously soaked into 1:2 (v/v) HNO_3 overnight and rinsed thoroughly with de-ionized water before used in experiment.

3.4 Total digestion

The prepared soil and ground sugarcane (underground stems (setts), roots, bagasse, leave and top) samples were directly digested using the microwave assisted acid procedure to the EPA-Method 3052. (1996), in order to quantify the total metal contents. For the total Cd and Zn determination, two replicates of 0.5 g of representative samples were accurately weighed and digested with a mixture of acid (10 mL HNO_3, 3 mL HF and 15 mL HBO_3) in inert Teflon vessels. Temperature of the vessel in the Microwave Digestion System was raised to 180 ± 5 °C and remaining for 9.5 minutes to ensure the leaching process by microwave digestion system. After cooling, the solution was filtered through a Whatman filter paper No. 41. The filtered solutions were diluted with de-ionized water 50 ml in volumetric flask. These solutions were stored in polyethylene containers and finally stored at 4 °C for subsequent analysis. The presence of the metals was determined by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

The sugarcane (juice) samples were digested according to Tri-acid mixture digestion, in a mixture of 10:1:4 (v/v/v) of 14 M nitric acids (HNO_3), 36.8 M sulphuric acids (H_2SO_4) and 15.3 M perchloric acids ($HClO_4$) (Jackson, 1973). The digested solution were centrifuged, if necessary, at 3000 rpm for 10 minutes to clear supernatant and filtered through Whatman No.41 filter paper. The filtrate solutions were diluted with de-ionized water to 50 ml in volumetric flask. These solutions were stored in polyethylene containers and finally stored at 4 °C for further analysis. The presence of the metals was determined by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

3.5. Extraction Procedures

In this study, four different extraction procedures were employed to compare the extraction efficiency. The single extraction with the use of DTPA, EDTA and $CaCl_2$ as extractants and the first step of the Community Bureau of Reference (BCR) method are showed in Table 3.2. These method were chosen for the evaluation of bioavailability of heavy metals in soil into sugarcane. Extraction was performed in a batch-wise manner (e.g. shaking), followed by centrifuge or filtration according to the following procedure:

3.5.1 EDTA procedure

Two-gram prepared soil samples were transferred to an extraction bottle in which 20 ml of 0.05 mol/l EDTA was added. The solution pH value was adjusted to 7. The obtained mixture was then shaken by an end-over-end shaker operating at 30 rpm for 1 h at 20+2 °C (Wear and Evans, 1968). The extractants were then separated from the residue by 10 min centrifuge at 3000 g, decanted into polyethylene containers and finally stored at 4 °C for analysis. (See Appendix A)

3.5.2 DTPA procedure

Ten-gram prepared soil samples were transferred to an extraction bottle in which 20 mL of 0.005 mol/l DTPA solution was added. The mixture was mixed by shaking by an end-over-end shaker as described above for 2 h in a room at 20+2 °C (Lindsay and Norvell, 1978). The solution pH value was adjusted to 7.3. The extractants were then separated from the residue by 10 min centrifugation at 3000 g, decanted into polyethylene containers and finally stored at 4 °C for analysis. (See Appendix A)

3.5.3 $CaCl_2$ procedure

Two-gram soil was transferred to an extraction bottle in which 20 mL of 0.01 mol/l $CaCl_2$ was added. The obtained mixture was shaken by an end over-end shaker operating at 30 rpm for 3 h in a room at 20+2 °C (Novozamsky et al., 1993). The extractants were then separated from the residue by 10 min centrifugation at 3000 rpm1, decanted into polyethylene containers and finally stored at 4 °C for analysis. (See Appendix A)

3.5.4 First-step BCR procedure

The SM&T method was proposed by the Standards, Measurements and Test program (SM&T-formerly BCR) of the European Union. This method is the sequential extraction method but the only first step was used in this experiment. In the first step, acetic acid (20 ml of 0.11 M solution) was added to 0.5 g of sample accurately weighed in 50 ml polyethylene centrifuge tubes. The tubes were shaken for 16 h by an end-over-end mechanical tumbler at 30 rpm. The extractants were then separated from the residue by 10 min centrifugation at 3000 g, decanted into

polyethylene containers and finally stored at 4 °C for analysis. The residues were washed with 10 ml de-ionized water by shaking for 15 min, centrifuged and discarded. (See Appendix A)

Table 3.2 Extraction procedures used in the experiment

Extraction method	Procedure
EDTA	2.0 g soil in 20 ml 0.05 M EDTA adjusted by ammonia solution to pH 7.0, shaking for 1 hr.
DTPA	10.0 g soil in 20 ml 0.005M DTPA + 0.01M TEA + 0.01M $CaCl_2$ adjusted to pH 7.3, shaking for 2 hr.
$CaCl_2$	2.0 g soil in 20 ml 0.01 M $CaCl_2$, shaking for 3 hr.
BCR1	1.0 g soil in 20 ml 0.11 M acetic acid, shaking for 16 hr.

3.6. Quality Assurance and Quality control

For each batch samples, prepare blank samples by using each reagent extraction procedures without samples and follow the same condition as common samples. This blank is useful in determining if samples are being contaminated. For Quality control of samples analysis, all samples are done in duplicates both of total concentration and bioavailability fraction of Cd and Zn by four extraction procedures.

3.7. Statistical Analysis

The correlation coefficient analysis was performed by bivariate using SPSS version 11.5. Spearman correlation coefficient was determined by correlating total metal contents in soil and sugarcane with extractable metals by four different extraction procedures, to examine the most suitable extraction procedures to be used in this area of Mae Sot, Tak province.