CHAPTER III
METHODOLOGY

3.1 Introduction

In this research, soil and sugarcane samples were collected for the purpose of the determination of the bioavailability of Cd in sugarcane. Chemical analyses of the samples were carried out by means of total digestion and BCR sequential extraction. The following soil properties including: pH, organic matter content (OM) and oxidation-reduction potential (ORP) were determined to clarify their effects on the bioavailability of heavy metals in this study. The experimental design in this study included sample collection, preparation, and procedure in soil and sugarcane analysis, and the process of data analysis as shown in Figure 3.1. All the apparatus used is given in Appendix A.

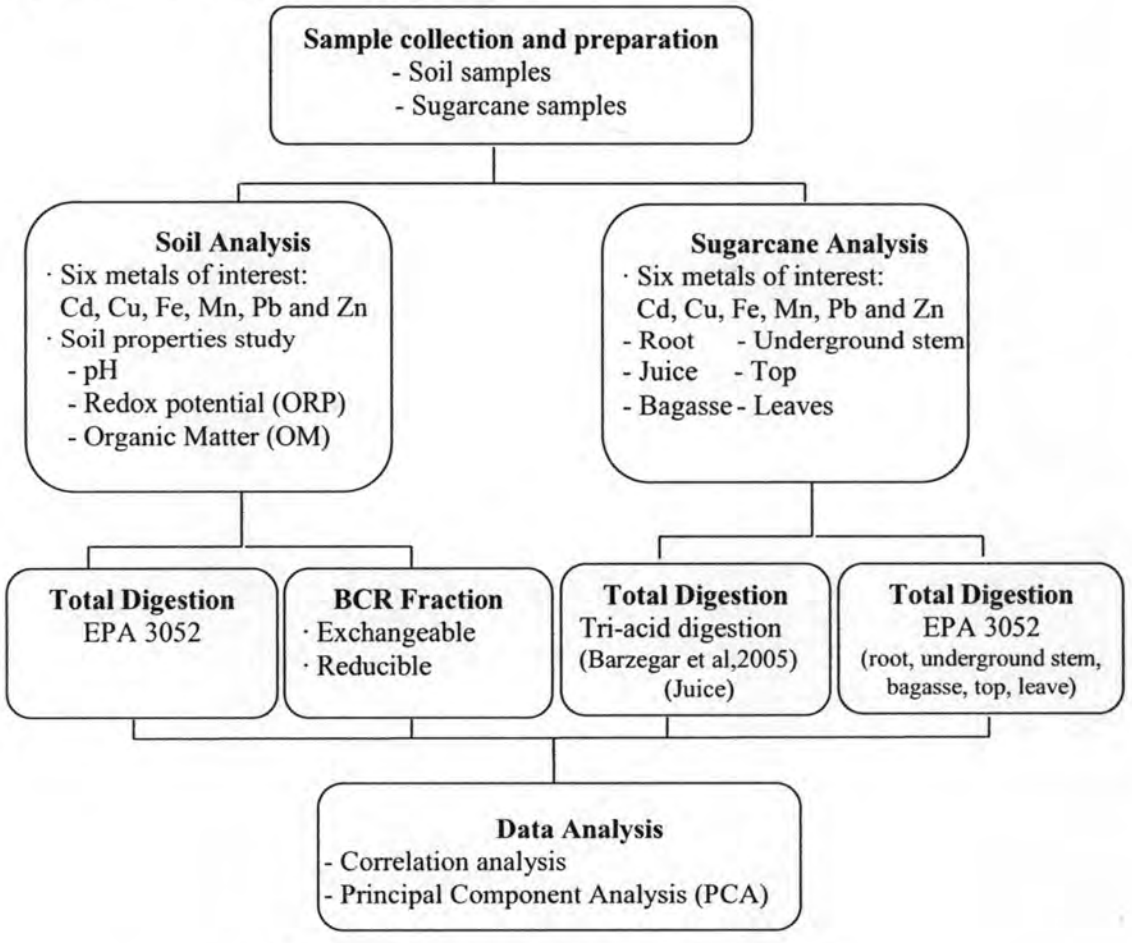

Figure 3.1 Experimental design in this study.

3.2 Study Sites

Soil and sugarcane samples were collected in March, July and October 2007 from a cadmium and zinc contaminated area in Mae Sot District, Tak Province. A total of eighteen sampling sites were chosen from the heavy metals concentration zoning map of previous work (National Research Center for Environmental and hazardous Waste Management, 2005) in order that the chosen sample sites would distribute throughout evenly accordingly to the high, medium and low concentration of cadmium content. Sugarcane growing fields are denoted as M, J, and O according to the time collecting the samples which are March, July, October 2007, respectively; and all details for sampling sites is given in Appendix B. The criteria for selecting the sampling sites are as follows:

3.2.1 High concentration zone (> 20 mg/kg): M(1-3), J(25-30), J(31-33), J(34-39), O(51-57), O(58-60) and O(61-66)

3.2.2 Medium concentration zone (3-20 mg/kg): M(4-8), M(9-12), M(13-15), M(16-18), M(22-24), J(40-45), and O(67-72)

3.2.3 Low concentration zone (< 3 mg/kg): M(19-21), J(46-51), O(74-78) and O(79-81)

3.3 Sample collection and preparation

3.3.1 Soil sample collection and preparation

Eighty-one soil samples were collected from the surface layer (0-30 cm depth). The soil locations were determined by using a global positioning system (GPS) receptor. Soil samples were immediately stored and sealed in polypropylene bags and kept at 4 °C before taken to the laboratory for further processing and analysis. At the laboratory, the samples were oven dried at 105 ± 5 °C in an electric oven until a constant weight is achieved; then the dried samples were crushed to fine powders using an agate mortar before passing through a 200-mesh sieve. They were carefully screened and then put in polypropylene containers until the analysis. The particles less than 200 mesh (< 74μm) in the soil samples, which are representative of true soil, were used for analysis in this study and stored in polyethylene bottles in a

desiccators at room temperature prior analysis (Tokalioğlu, 2003). Precautions were taken to avoid contamination during sampling, drying, grinding, sieving and storage.

3.3.2 Sugarcane sample collection and preparation

Eighty-one sugarcane samples were harvested and collected concurrently with the soil collection from the mentioned thirteen sugarcane growing fields where the soils have been sampled. After removing, the whole sugarcane plants were carefully cleaned by a tap water jet spray to detach soil particles, followed by thoroughly rinsed with deionized water (excess water drops attached to the outer surface of the plants were then removed). Then the sugarcanes were separated into six parts which are root, underground stems, shoot, juice, top, and leaves. Shoot were extracted to get juice and wasted mass of bagasse. Roots, underground stems, bagasse, top and leaves were then dried at 105 °C; ground and dry weights were recorded.

Analytical reagent grade chemical or better and distilled deionized water (18 MΩ, Milli-Q or equivalent) from a ELGA water purification unit (ELGA, England) were used for preparing all solutions throughout this study. All standard reagent solutions were stored in polyethylene bottles. Blank determinations were performed by using the same reagents in equal quantities throughout the experiments to ensure that the samples are free of contamination.

3.4 Soil Analysis

3.4.1 Soil properties determination

Eighty-one soil samples were analyzed for the selected soil properties as followings:

3.4.1.1 Soil pH (1:2 soil/water suspensions): by pH meter.

3.4.1.2 Soil organic matter content (OM): by wet digestion according to the Walkley-Black procedure (Benton Jones, 2001).

3.4.1.3 Oxidation-reduction potential (ORP): by Oxidation-reduction potential meter.

3.4.2 Metal determination in soil samples

Soil samples were extracted for heavy metal fractions by a BCR sequential extraction procedure and were analyzed for total concentration by a total acid digestion.

3.4.2.1 Total digestion (EPA 3052)

For the determination of total metal content in the soil, the samples were directly digested using the microwave-assisted acid digestion procedure according to the Standard Method of US-EPA, 3052 (1996). 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 completion of specific reaction. After cooling, the digested solution was then centrifuged, if necessary, at 3000 rpm for 10 minutes to clear supernatant or filtered through ashless Whatman No.41 filter paper and stored in polyethylene bottles at 4°C until subsequent procedure of trace element analysis. The filtrate was used for the determination of metals using inductively coupled plasma-optical emission spectrometry (ICP-OES). All of the analyses were carried out in duplicate number.

3.4.2.2 BCR sequential extraction

The sequential extraction procedure applied in this study was composed of step one and step two of procedure proposed by the Standards, Measurements and Testing programme of the European Union, SM&T (Figure 3.2). Soils sampled in March, July, and October 2007 were carried in duplicate number for each sample. Each reagent used in this scheme represents an increase in strength of chemical extractant. In this study, it is designed to separate heavy metals into two operationally defined fractions: exchangeable (BCR1) and reducible (BCR2) fractions. Therefore, step 1 and 2 of the BCR extraction procedure were performed. All the sample analyzes were run in duplicate, by using two portions of 0.5 g sub-samples of each dry soil sample and then placed into 50 mL of polypropylene centrifuge tubes. The preparation of reagent solution is given in Appendix C. The summary of extraction procedure is described below:

Step 1: Exchangeable /Acid soluble fraction (BCR1)

Acetic acid (20 mL of 0.11 mol/L solutions) was added and shaken for 16 hrs at room temperature of 22 ± 5 °C. No delay should occur between the addition

of the extractant solution and the beginning of the shaking. The extract was separated from the solid residue by centrifugation for 10 minutes at 3000 rpm, decanted the supernatant liquid into a polyethylene container, and stored in a refrigerator at about 4 °C prior to analysis. The residue then was washed with 10 mL distilled water, shaking for 15 minutes on the end-over-end shaker and centrifuging for 10 minutes at 3000 rpm. Discard the supernatant, but not any of the solid residues.

Step 2: Reducible fraction (BCR2)

To the residue from Step 1 (BCR1), 20 mL of freshly prepared hydroxylamine hydrochloride (0.5 mol/L) were added and then shaken for 16 hrs at room temperature of 22 ± 5 °C. No delay should occur between the addition of the extractant solution and the beginning of the shaking. The extract was separated from the solid phase by centrifugation and decantation as described in Step 1 (BCR1). The residue then was washed with 10 mL distilled water, shaking for 15 minutes on the end-over-end shaker and centrifuging for 10 minutes at 3000 rpm. Decant the supernatant and stored at 4 °C.

All laboratory-ware is of borosilicate glass, polypropylene or PTFE. Vessels in contact with samples or reagents were cleaned by soaking with 4 mol/L HNO_3 (overnight) and rinsed repeatedly with distilled water before use. For each fraction of the sequential extraction procedure, particulates in the digested solution were removed by filtration before analysis through Whatman No. 41 filter paper. The determinations of metals in the extract were performed by a mechanism end-over-end-shaker, at a speed of 30 ± 10 rpm. Extracts were analyzed by ICP-OES for the six metals (Cd, Cu, Fe, Mn, Pb and Zn) of interest.

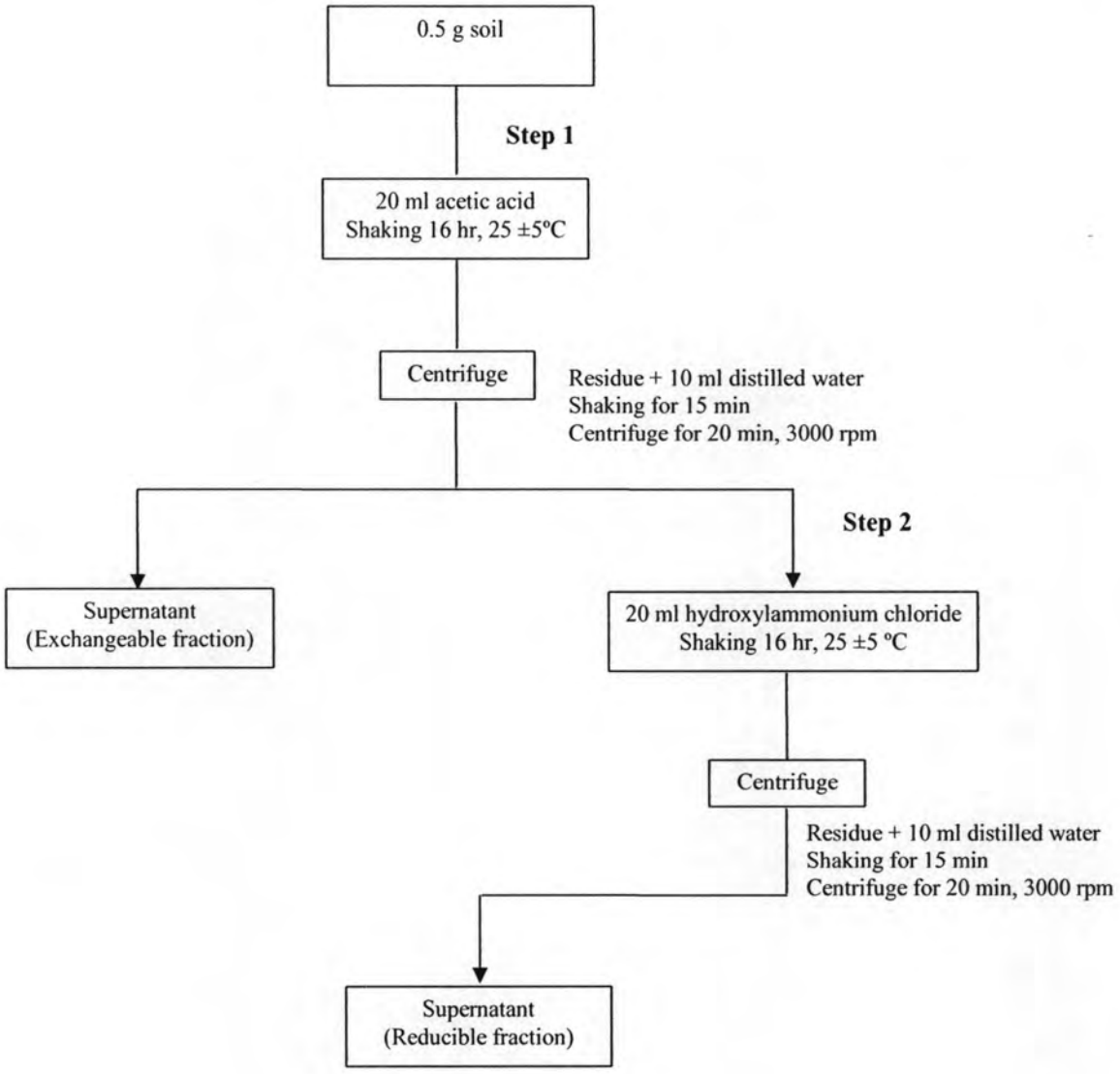

Figure 3.2 First-two steps BCR Sequential Extraction Scheme

3.5 Sugarcane analysis

For plant analysis, only 30 samples (O52-81) collected in October 2007 were analyzed for the whole part of sugarcane namely: root, underground stem, bagasse, juice, top, and leaves. While for other samples, only three parts; bagasse, root and juice were analyzed. Root, underground stem, bagasse, top, and leaves were digested with microwave-assisted acid digestion procedure according to the standard method of US EPA 3052 with slightly modification on digestion procedure. Metals in juice were determined by using the tri–acid digestion (Jackson, 1973).

3.5.1 Total digestion (EPA 3052)

Root was digested according to the standard method EPA 3052 as described for soil analysis. Underground stem, bagasse, top, and leaves were digested according to the acid digestion in a closed vessel microwave (12 mL HNO_3 and 3 mL H_2O_2). The descriptive information for microwave digestion is given in Appendix D.

3.5.2 Total digestion (Tri-acid digestion)

For the digestion of juice, a 25 mL sample was digested with repeated additions (5 mL) of a mixture of acid 10:1:4 (v/v/v) (HNO_3, H_2SO_4 and $HClO_4$) on the hot plate at 95 ± 5 °C for 30 minutes. Let the samples cool down and repeat this step over and over until get clear solution. Particulates were then removed by filtration, by centrifugation, or by allowing the sample to settle. The sample is now ready for analysis by ICP.

3.6 Quality control

For quality control, analytical blanks and certified reference material (CRM) with known concentrations of elements were analyzed using the same procedures and reagents. The accuracy of the analytical procedures for total digestion was checked using a soil certified reference material (CRM025-050, RTC). This CRM soil is the moderately contaminated soil available from the Western United States (Peter Matúš et al., 2005). All samples and reference materials were run in duplicate for the total acid digestion.

3.7 Analytical methods

Metal contents of the first-two fractions from BCR sequential extraction and total metal contents in the filtered solution were analyzed by Inductively Coupled Plasma Spectrometry-Optical Emission Spectrometers (ICP-OES). Multi-element standard working solutions of the elements analyzed were used and prepared from the corresponding 1000 mg/L Merck titrisol solutions using 2% HNO_3 (from nitrate salts of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn in 2 % HNO_3).

3.8 Data analysis

Statistic studies were conducted by using the correlation analysis and principal component analysis (PCA). Statistical data processing of the principal component analysis (PCA) were carried out using the SPSS program package software (version 11.5). Direct Oblimin rotation was performed for PCA analysis. Correlations between cadmium available to sugarcane influenced by pH, organic matter, oxidation-reduction potential and other metals (Cu, Fe, Mn, Pb, and Zn) were performed and calculated based on the correlation matrix.

Principal Component Analysis (PCA) is a powerful technique that attempts to explain the variance of a larger set of inter-correlated variables and transforming it into a smaller set of interdependent variables (Principal Component, PCs) (Sinha et al., 2007). In this research, PCA was performed on data matrices consisted of information on the total concentration of six interested metals (Cd, Cu, Fe, Pb, Mn, and Zn) present in soils, available metals concentration (BCR1) in soil and soil characteristics (pH, organic matter content (OM), oxidation-reduction potential) in order to decrease the number of descriptor responsible for the highest percentage of a total variance of the experimental data (Ražić et al, 2006). Thus, total and available metals (Cd, Cu, Fe, Pb, Mn, and Zn) in soil samples were taken as variables. Soil characteristics included soil pH, organic matter content (OM) and oxidation-reduction potential (ORP) were also included in the input matrix as variables.