CHAPTER III
METHODOLOGY

3.1 Apparatus, Instruments and Chemicals

3.1.1 Apparatus

1) Cut pieces of mature sugarcane stem (setts), LK 92-11 ecotype, obtained from Kampaengpetch province
2) Soil used in pot experiment was collected from Mae Ku sub-district, Tak province
3) Granular commercial fertilizer 16-16-8 NPK formulation
4) Plastic bag (diameter of 45 cm.)
5) Whatman filter paper No. 40 (Ø 110 mm.)
6) GF/C (Glass Micro Filters) filter paper (Ø 70 mm.)
7) Deionized water
8) Parafilm
9) Laboratory glassware; beaker, cylinder, volumetric flask, pipette, watch glass, funnel, glass rod etc.
10) 2 mm- sieve
11) Planting equipment; hoe, shovel, watering can etc.
12) 60 ml. plastic bottles
13) Zip lock plastic bags

3.1.2 Instruments

Instruments	Model/ Brand
GPS (Global Positioning System) receiver	Map76, Garmin
Atomic Absorption Spectrometer (AAS)	AAnalyst 800, Perkin Elmer
Microwave Digester	ETHOS SEL, MILESTONE
Hot Air Oven	ULE 500, MEMMERT
Hood	Wiwatsan
Hot Plate	Cimarec 2, Thermolyne
pH Meter	Sension 2, HACH
Analytical balance 4 digits	BP 221S, Sartorius
Vacuum pump	N035AN.18-IP20,
Shaker	OS-2, Green SSeriker2
Blender	RT04A

3.1.3 Chemicals

Chemicals	Company/Country
Nitric acid (65% HNO_3)	MERCK, Germany
Hydrochoric acid (37% HCl)	MERCK, Germany
Sulphuric acid (95-97% H_2SO_4)	MERCK, Germany
Perchloric acid (70% $HClO_4$)	Panreac, European Union
Hydrogenperoxide (30% H_2O_2)	MERCK, Germany
Dethylenetriamine pentaacetic acid; DTPA ($C_{14}H_{23}N_3O_{10}$)	Fluka, Switzerland
Triethanolamine ($N(CH_2CH_2OH)$)	Unilab, Australia
Calcium chloride ($CaCl_2.2H_2O$)	Univar, Australia
Ammonium metavanadate	Carlo Erba, Italy
Ammonium molybdate	J.T. Baker, USA.
Ammonium fluoride	J.T. Baker, USA.
Ascobic acid	Fluka, Switzerland

3.2 Field experiment

3.2.1 Study area

According to the report of National Research Center for Environmental and Hazardous Waste Management; NRC-EHWM (2005) in the distribution and bioavailability in cultivated soil and crops in the vicinity of zinc mine in Mae Sot, the cadmium contaminated areas in Mae Sot district, Tak province were zoned in accordance with the cadmium concentration in soil into three ranges including the areas with cadmium concentration less than 3, 3-20 and more than 20 mg Cd/kg soil on a dry weight basis, respectively.

Sugarcane cultivation has been promoted to replace rice farming in these areas. Somkid Jamjumrus (personal communication, October 12, 2006) mentioned that in planting sugarcane, soil was plowed to ensure that it was broken into fine even particles to promote roots of new plants to reach nutrients and moisture. After that, mature sugarcane stems are cut into sections and laid horizontally. All of these sugarcane fields received an initial application of a granular commercial fertilizer 16-16-8 NPK formulation at the rate of 50 kg fertilizer/rai. Repeat application of fertilizer was conducted at the same rate between four and five months later.

3.2.2 Site selection and Sampling points

Three different sugarcane cultivated fields in the area of Mae Sot district were selected for the field experiment. Each field had different levels of cadmium concentrations. The Geographic Information System (GIS) was used for site selection and sampling point in those three sugarcane cultivated fields. The co-ordinates of sampling points in the field experiment are illustrated in Table 3.1. The three sugarcane cultivation fields which were selected for the field experiment including:

1) Mae Ku (< 3 mg Cd/kg of soil on dry weight basis)
2) Mae Tao 1 (3-20 mg Cd/kg of soil on dry weight basis)
3) Mae Tao 2 (> 20 mg Cd/kg of soil on dry weight basis)

The map of site selection and sampling points is present in Appendix A.

Table 3.1 Co-ordinates of sampling points in field experiment

Area	Cd contaminated level	Plot	X-axis	Y-axis
Mae Ku	<3	1	458625	1839156
		2	458642	1839156
		3	458655	1839156
Mae Tao 1	3-20	1	456460	1842030
		2	456444	1842035
		3	456423	1842038
Mae Tao 2	>20	1	456550	1843022
		2	456558	1843019
		3	456571	1843018

Soil samples were collected (0-30 cm from surface horizon) from those three areas and then they were air-dried and passed through a 2-mm sieve to before determining the properties of soil. The analysis methods used for the determination of basic physical and chemical properties of soils are presented in Table 3.2.

Three replicates of soil and sugarcane samples were collected from sugarcane cultivated fields in Mae Ku, Mae Tao1 and Mae Tao2 area at the end of the second and sixth month of the cultivation. Sugarcane samples were taken from the same area used in soil samples collection.

Table 3.2 Analytical methods for the physical and chemical properties of soils used in the experiment

Parameters	Analysis method
pH	pH Meter (soil:water = 1:1)
Organic matter (OM)	Walkley and Black Method
Moisture content	Oven-dried 105° C
Total nitrogen	Kjeldahl Method
Total phosphorus	Vanadomolybdate Method
Potassium	Ammonia Acetate 1N pH 7.0 Extraction
Total cadmium	Microwave Digestion (USEPA 3052)
Total zinc	Microwave Digestion (USEPA 3052)
Soil texture	Hydrometer Method

3.3 Pot experiment

3.3.1 Soil preparation

The soil collected from Mae Ku, Mae Sot district, Tak province was used in pot experiment. Soil was collected from the 0-30 cm surface horizon and allowed to air dry before being crushed to pass through a 2-mm sieve. Background cadmium and zinc in soil was determined by USEPA method 3052 (USEPA, 1996) (see Appendix B). Basic physical and chemical properties of soil were determined using the same method as for field experiment. The analysis methods are presented in Table 3.2.

3.3.2 Plant preparation

Cut pieces of mature sugarcane stem (setts) were obtained from Kampangpetch province. The USEPA method 3052 was used for the analysis of cadmium and zinc in sett samples (Appendix B). A Perkin Elmer Atomic Absorption Spectrometer Model AAnalyst 800 (Perkin Elmer Instruments LLC, Unberlingen, Germany) was used. The results showed that background cadmium and zinc concentrations in setts were non detectable.

3.3.3 Fertilizer analysis

A granular commercial fertilizer 16-16-8 NPK formulation was used in this study. pH value of the fertilizer was determined in soil suspensions (1:1 soil to water ratio) using a pH meter. Background cadmium and zinc contained in the fertilizer were determined by USEPA 3052 method.

3.3.4 Experimental design and procedure

The experiment was conducted in a randomized block design with three replications with 20 kg of soil per pot. Each pot was planted with a sett. A month later, the first application of fertilizers was conducted. A granular commercial NPK fertilizer 16-16-8 formulations (16% N, 16% P_2O_5 and 8% K_2O) was applied to each pot at the rate of 0 (control group), 50, 100, and 200 kg/rai. The calculation method for amount of fertilizer applied to soil is shown in Appendix C. The control group did not receive any fertilizer addition, but was subjected to all other manipulation steps. Repeat application of fertilizers was conducted at the same rate about four months (the fifth month after cultivation) after the first application.

3.4 Samples collection and preparation

3.4.1 Soil samples

Soil samples were collected at the end of the second and the sixth months after the sugarcane cultivation. Soil pH was determined in soil suspensions (1:1 soil to water ratio) using a pH meter. Samples were oven-dried at 105°C for 24 hr to constant weight and to determine dry matter yields then they were crushed to pass through a 2-mm sieve and thoroughly mixed to homogenize. For determination of available cadmium and zinc in soil, soil samples were allowed to air-dry for 72 hr and then crushed to pass a 2-mm sieve and mixed to homogenize before analysis.

3.4.2 Sugarcane samples

Sugarcane samples were collected at the end of the second and the sixth month of the cultivation. Samples were cleaned and washed with tap water twice and rinsed with deionized water. After sampling, the sugarcane samples were separated into five parts, underground stems (setts), roots, bagasses, sugarcane juice and leaves Samples collected at the end of the second month of the cultivation were cut into only four parts as sugarcane juice was not obtainable because of incomplete maturity of sugarcane. Samples were oven-dried at 105°C for 24-48 hr until reaching a constant the weight and dry matter yields of sugarcane were determined. After that, samples were ground with an electric mill and thoroughly mixed to homogenize.

3.5 Samples analysis

3.5.1 Soil samples

Total soil cadmium and zinc were determined by the USEPA method 3052 (microwave assisted acid digestion of siliceous and organically based matrices) (USEPA, 1996). All samples were made up to 25 ml by deionized water and preserved at 4° C until analyzed. Available cadmium and zinc in soil were estimated by DTPA extraction method (Lindsay and Norvell, 1978). Total cadmium and available cadmium concentrations in the solutions were determined by graphite furnace atomic absorption spectrophotometer (GFAAS) whereas total zinc and available zinc in solutions were analyzed by flame atomic absorption spectroscopy (FAAS). Total phosphorus was determined by vanadomolybdate method (AOAC, 1975) and available phosphorus in soil was analyzed by Bray II method (Bray and Kurtz, 1945). All sample analysis methods are presented in Appendix B.

3.5.2 Sugarcane samples

Total cadmium and total zinc in underground stems (setts), roots, bagasses and leaves of sugarcane were determined by the USEPA method 3052 (microwave assisted acid digestion of siliceous and organically based matrices). Sugarcane juice was analyzed by Tri-acid digestion method (Jackson, 1973). Following digestion, all samples were made up to 25 ml using deionized water and store at 4° C until analyzed. The digested solutions were analyzed for cadmium by graphite furnace atomic absorption spectrophotometer (GFAAS) whereas zinc was analyzed by flame atomic absorption spectrophotometer (FAAS). All sample analysis methods are presented in Appendix B.

The schematic diagram of experimental metric is illustrated in Figure 3.1.

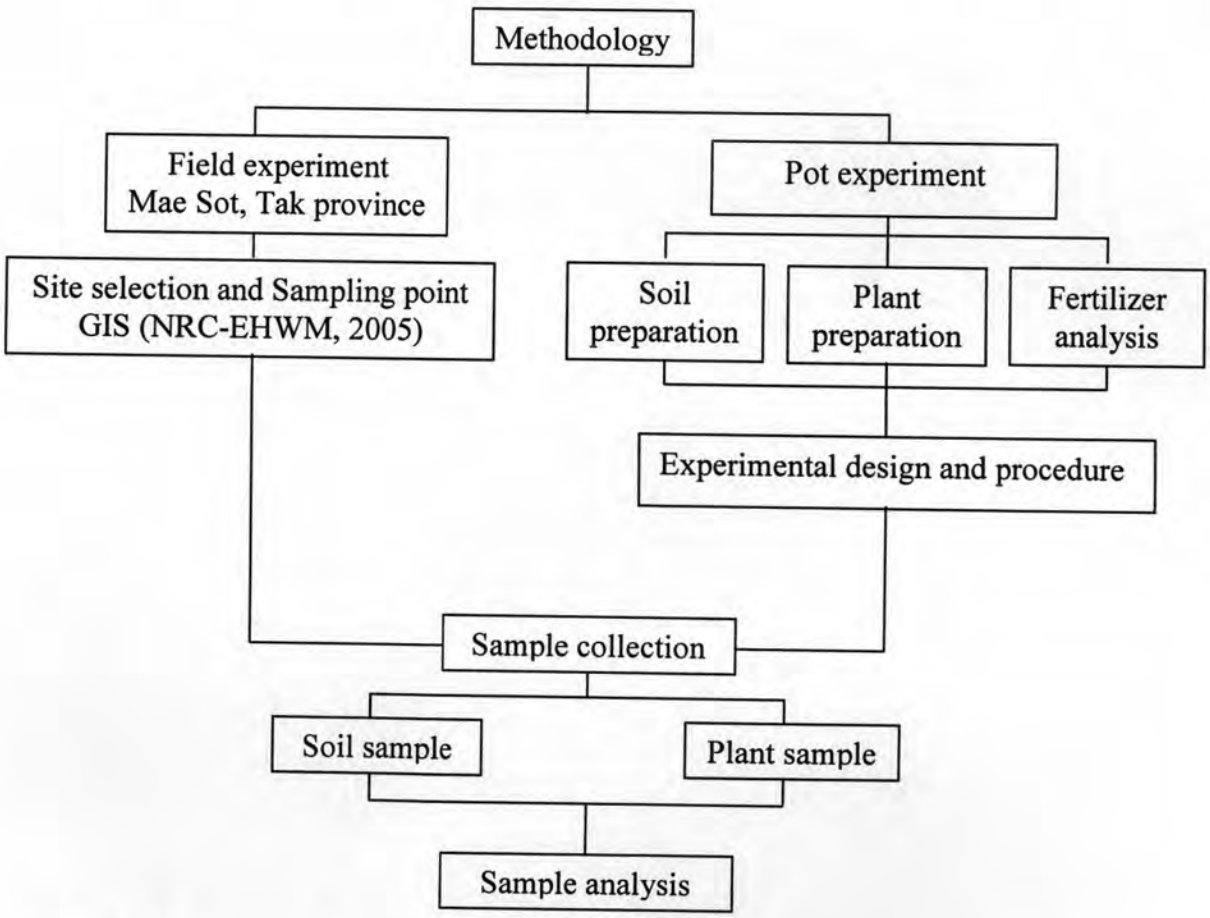

Figure 3.1 The schematic diagram of experimental metric

3.6 Statistical analysis

Analysis of variance was performed using Statistical Package for the Social Sciences (SPSS) program. Mean separations were compared using ANOVA with significant level at $P < 0.05$ and the Duncan's New Multiple Range Test (DMRT) with orthogonal contrast to test the difference of total concentration of cadmium and zinc in soils and sugarcane, available cadmium and zinc, total phosphorus and available phosphorus. The Duncan's New Multiple Range Tests was used to obtain the groupings of the mean values of metals uptake that are not significantly different among themselves.