Elemental Partitioning in *Hyalophera cecropia*

by

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Elemental Partitioning in *Hyalophera cecropia.*

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Abstract

Elemental Partitioning in *Hyalophera cecropia*

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Master of Science

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The concentration of 18 elements found in fifth instar *Hyalophera cecropia*, its feces, and sole food source, *Prunus serotina*, were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES). Test solutions were prepared using a clean chemistry protocol employing closed vessel microwave acid digestion of the specimens. The elemental partitioning data will be used to determine foliar consumption in future larval specimens.

Dedication

I dedicate this to my Husband, Larry, and my Mother, Marijane.

Acknowledgements

I have been in college many years, it seems. I have met many people, learning from each of them. I would like to acknowledge every person that has made me who I am today, but I don't know all their names. All human interaction shapes the being we become the day before we die. The weight of this inevitability, the consciousness of the future existence of that being, the self, is the whole of all human interaction. Every piece helped me along the way. Events that required strength and healing, they were life's torments and aged us every day, I am wiser. I am thankful for the wisdom I will have some day, and for the persons that I have known: every piece.

For this accomplishment, Master of Science Degree, I would like to thank my husband Larry. He has tolerated my mild mood swings, and never quelling, but cajoling my fleeting ideas of what I would like to be when I graduate. Thanks to my mom, Marijane, she was always an unwavering supporter. Thanks to my advisor, Daryl Mincey, for not answering all of my questions, it was more fun experimenting. Thanks to Dorothy Untch for listening to endless hours of whining and for the fabulous static reduction idea. Thanks to Ray Hoff who has a vast knowledge about absolutely everything and fixed all of it when it was broken. Thanks to Chris Romer, my first chemistry professor. She delivered engaging lectures about chemistry as well as the importance of SPAM, (the canned meat product), in Gulf War political commentary. Thanks to Steve Ray who took great pity on me in the dark days leading up to Pittcon '98. Thanks to Katie and Chuck Van Kirk who always made things seem a little brighter and a lot more humorous. Thanks to Pete Rook for sharing his space and taking me

under his wing. Thanks to Dr. Wagner and Dr. Mike for reading this thesis, which was delivered by a frantic, slightly unhinged author, quite close to the deadline. Thanks to YSU for making the necessary facilities, instrumentation and reagents available, as well as travel money for Pittcon '98. Thanks to John Poole, I was very glad to have loved such a scientist. Thanks to Doug Fowler, who has knowledge about many things and interest for everything. Thanks to my Aunt Caroline, it was always for something or other, all the time. Thanks everyone.

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Chapter One

Literature Review

Insect Budgets

The present literature does little to quantify elements other than nitrogen in insects.[1] The fact that mineral elements are important aspects of insect nutrition has been known for more than two decades,[1] but data regarding levels of these elements is scarce and has not resulted in an elemental mass balance for insect consumption of foliage.

A study of soil invertebrates and their surroundings reported concentrations of sodium, calcium, magnesium, and potassium in samples of lepidoptera and their food source using flame atomic absorption spectroscopy (FAA). The concentrations of the elements available for assimilation from their food source was inadequate to produce the concentrations found in the larvae. Therefore, the animal stored these elements from previous consumption. Even with the large standard deviations reported, it can be seen that there is a nominal difference between the food sources and the larval concentration (see Table 1.1).[2] Bioaccumulation of sodium and potassium were shown in all of the Canadian Woodland consumers studied, including lepidoptera (see Figure 1.1).[2] Analysis of the lepidoptera, *Pieris rapae,* was performed to determine whether sodium and potassium budgets differed with gender.[3] Field and lab insects were collected, killed in a jar containing sodium cyanide, then frozen. Each specimen was put into a separate 4-dram (1 dram = 3.551633 mL) polyvial, with concentrated nitric acid. After 21 hours, the samples were diluted with deionized water and filtered. Soil samples were collected near the puddles where the butterflies drank. The soil was extracted with ammonium

Table 1.1

Results of Ca, Mg, and K Analysis for Soil Invertebrates and their Surroundings.

	mg/g dry weight (x ± 95% confidence limits)			
	Calcium	Magnesium	Potassium	Sodium
Lepidopteran larvae (H)	1.95 ± 0.56	3.86 ± 0.50	33.52 ± 6.54	1.94 ± 1.76
Leaves	25.31 ± 6.91	3.53 ± 0.78	5.76 ± 1.91	0.04 ± 0.023

(H) - herbivore

This is based on the author's assumption that the leaves analyzed are the sole food source for the lepidoptera

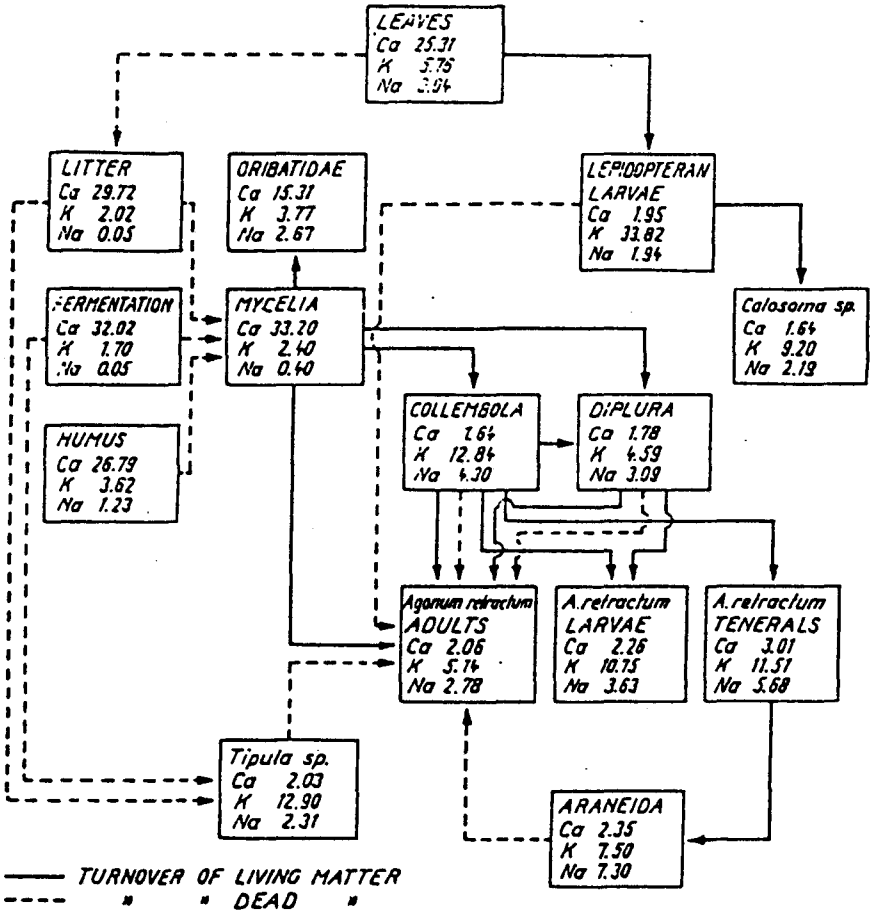

Figure 1.1. Food Web and Cycling of Ca, K, and Na , by Soil Invertebrates

acetate. The filtrates from the insects and the soil were analyzed for sodium and potassium, using flame atomic absorption (see Table 1.2).[3]

The toxin used to kill the insects does so through respiration. The analyte of interest, sodium, is the counter ion in the cyanide salt. Contamination of the sample is plausible, but unlikely if the procedure is done carefully. The salt is separated from the insects with a layer of Kim-wipes®. The sodium cyanide combines with water in the ambient air to produce hydrogen cyanide. Hydrogen cyanide has a vapor pressure of 760 mmHg at 25.9°C, whereas the sodium cyanide salt would have to be at 1497°C to have a comparable pressure: it is the hydrogen cyanide the animals are breathing. If the procedure was done carefully, traces of the sodium salt would not contaminate the specimens. If sodium did contaminate the sample that could account for every insect having elevated sodium levels. This would suggest bioaccumulation of sodium in the insect bodies.

There was no data published in regards to the levels of sodium and potassium in soil: citing only that "... it was complicated in many ways by the inextricable association of water with soil...". A known salt solution was prepared. Trays containing sand saturated with 0.1 M salt solutions were placed in the open with a male decoy pinned to each.[3] Activity was monitored for a total of 4.5 hours over a 3 day span; however, the butterflies that visited the control trays were not analyzed.

There was no published evidence that a budget had been determined; only a comparison of the lab reared males and females to the field collected males and females. The excrement was not considered.

Table 1.2

Results for the Determination of Na and K in Canadian Woodland Consumers

	Na and K expressed as percentage of dry weight (x ± S.D.)	
	Freshly emerged females	Field collected females
Dry weight	17.3 ± 4.8	20.2 ± 3.4
Body water	73.1 ± 2.6	65.1 ± 1.7
Na	0.136 ± 0.038	0.145 ± 0.044
K	1.728 ± 0.109	1.362 ± 0.109
	Freshly emerged males	Freshly collected males
Dry weight	20.0 ± 2.3	19.5 ± 2.3
Body water	76.0 ± 3.2	66.0 ± 2.3
Na	0.337 ± 0.055	0.151 ± 0.064
K	1.939 ± 0.225	1.994 ± 0.354

Copper and cadmium budgets were determined for fifth instar *Locusta migratoria*.[4] Larvae were fed with copper contaminated, cadmium contaminated, or uncontaminated maize foliage. It was determined that the excess copper was excreted in the feces, as the levels between control group and copper contaminated feeders were statistically the same. The cadmium levels in the larvae were elevated comparable to the elevated levels in the caterpillar's diet. The cadmium levels increased with the amount of time that locusts were fed the cadmium treated maize. The cadmium levels in the feces were also elevated, as there was excess cadmium contained in the undigested food.

Both copper and cadmium did bioaccumulate. In the results (see Figure 1.3), it is evident that the cadmium levels of the cadmium contaminated leaf feeders are much higher than control larvae. Inefficient assimilation of cadmium, was also found in the larvae of *Lymantria dispar* and *Panolis flammea.*[5,6]

Copper did bioaccumulate, meaning the food intake did not equal the concentrations in the feces and larva, but it is not drastically different from the control larvae.[4] This occurrence lends itself to the idea that a plateau effect may occur as does in the *Drosophila repleta.* Once the insect has stored enough copper, the bioaccumulation ceases and the excess copper is excreted in the feces.[7,8] In studies of different lepidoptera, some bioaccumulate copper, such as *Bupalus piniaris*, others like *Aglais urticae* do not.[6,9]

A study involving canopy arthropods, investigates the active role arthropods play in rejuvenating a clear-cut forest community.[10] Consumption by the chewing herbivores was estimated by measuring the area of leaf removed by chewing. Comparable leaves

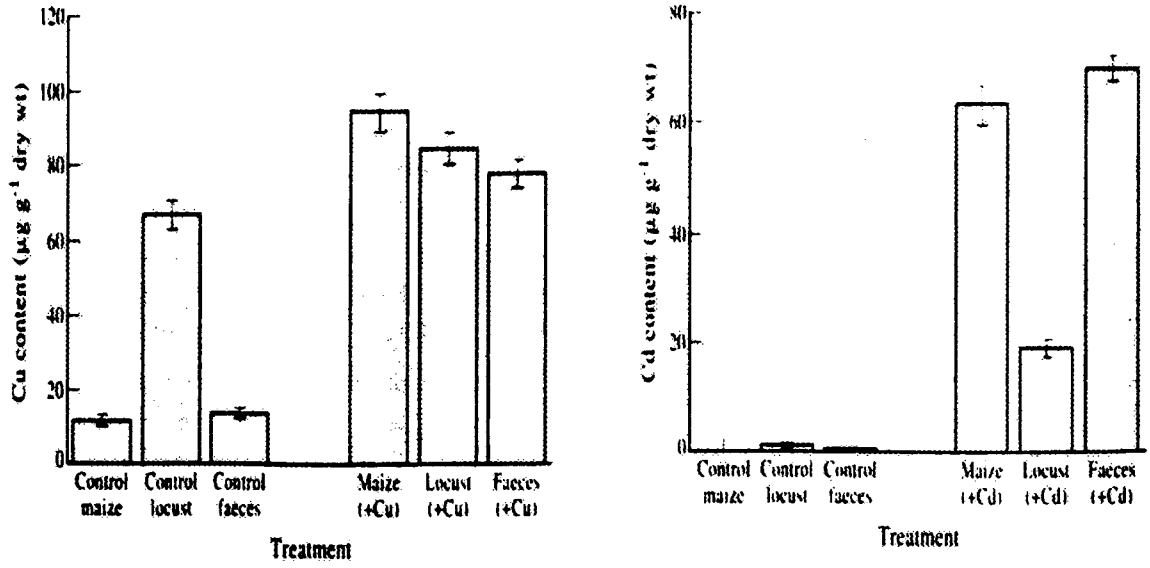

Figure 1.2. Comparison of Control Larvae and Larvae Fed Foliage Spiked with either Cd or Cr

collected in large nets, fumigated with chloroform and refrigerated. The insects were separated before digestion.

The specimens were ashed in a furnace by ramping from 250°C to 475°C and holding for 4 hours. They were then treated with 5 mL of concentrated nitric acid and heated to dryness. The entire process was repeated. 30% hydrogen peroxide solution was added, and the solution was diluted to volume with deionized water. The analysis for sodium, potassium, and calcium was performed on the diluted samples using FAA. The data collected from the analysis was extensive (see Table 1.3).[10]

The study concluded that when an area is disturbed, clear-cutting in this case, the arthropod community reacts. The population of a specific group and the eating habits of that population change. The results gave the relationship between biomass and nutrients. The nutrient consumption of the canopy arthropods and the nutrients the food source contained were estimated. The data was subject to high variances but a trend was evident (see Table 1.4).[10]

A study of heavy metal levels in soil animals included an analysis of feces, food, and animals with empty guts.[11] Sample preparation differed depending on the sample type and size. Oven dried plant and animal specimens of 10-120 mg dry weight and fecal samples from 10-75 mg dry weight, were placed in small glass vials with 1 mL of concentrated nitric acid. The solution was evaporated to dryness. 1 mL concentrated nitric acid and 1 mL 30% hydrogen peroxide was added to the residue and heated to dryness, this was repeated once. The final residue was treated with 0.16 N nitric acid. The feces did not completely dissolve. The larger samples were digested with 10 mL

Table1.3

Concentrations of Na, K, Mg, and Ca in Insects in an Undisturbed Watershed and a Clear-cut Watershed

CH = chewing herbivores, SH = sucking herbivores, O = omnivores (ants), P = predators.

watershed (WS 2) and a clearcut watershed (WS 7)

Nutrient Concentration (μg/g)

Nutrient	WS	Year	CH	SH	O	P	Others
Na	2	77	2268 (1275)	2608 (1275)	4399	4513 (1705)	3587 (47)
		78	3651 (950)	3913 (838)	7108	8654 (3318)	6368 (2565)
	7	77	3504 (2284)	3185 (765)	4307 (562)	5087 (1594)	2105 (231)
		78	3781 (813)	4552 (2085)	5850 (1343)	7298 (2735)	4301 (1618)
K	2	77	37829 (24419)	14633 (3952)	5123	15077 (6758)	7948 (3439)
		78	39921 (29381)	12598 (5264)	13585	17157 (8748)	18325 (2500)
	7	77	23525 (16326)	8639 (6234)	8377 (3217)	7034 (2926)	6912 (533)
		78	27573 (16947)	17193 (10323)	10166 (5490)	8294 (3100)	9394 (2683)
Mg	2	77	1807 (442)	1573 (113)	985	1364 (257)	1066 (8)
		78	3316 (1722)	2058 (437)	1853	2484 (561)	2409 (177)
	7	77	2339 (1143)	1772 (219)	1502 (82)	1776 (367)	1476 (31)
		78	2399 (925)	2599 (684)	1489 (118)	1558 (416)	2198 (703)
Ca	2	77	1819 (749)	864 (220)	2659	1026 (252)	1095 (240)
		78	8401 (10261)	875 (364)	2278	1818 (566)	1216 (562)
	7	77	2194 (1382)	2093 (2497)	2935 (865)	2112 (785)	641 (73)
		78	2773 (2362)	1101 (578)	1660 (522)	1496 (167)	904 (298)

Table 1.4

Biomass and Nutrients of Arthropod and Foliage in Undisturbed and Clear-cut Watersheds

	Biomass and nutrients (kg/ha)		
	WS 2	WS 7	
	Average	1977	1978
Amount of foliage			
Biomass	3000	1000	4000
Na	0.08	0.08	0.3
K	30	20	60
Mg	10	4	20
Ca	20	20	40
Amount Consumed			
Biomass	200	200	1000
Na	0.2	0.3	1
K	3	3	20
Mg	0.4	0.4	1
Ca	0.6	0.4	0.7
Amount in arthropods			
Biomass	2	1	3
Na	0.01	0.003	0.01
K	0.04	0.01	0.05
Mg	0.003	0.001	0.01
Ca	0.01	0.002	0.01

of concentrated nitric acid in a glass beaker. The beaker was placed on a hot plate, covered with a watch glass and heated for 30 min. A solution of nitric acid and hydrochloric acid was added. The test solutions/mixtures were diluted with 5 mL of deionized water and filtered when cooled. The test solutions were analyzed for zinc and copper using FAA, and for cadmium using GFAA. (see Table 1.5).[11] Feces were included with the data given and compared to other animals in the ecosystem (see Figure 1.3).[11]

The study compared the amount of a particular metal in an animal's food to the animal and its feces. Some of the consumers were shown to bioaccumulate certain elements. There is no mathematical relationship to equate the sum to the value of the food nutrient intake. It is implied by the elevated concentrations found in the animal, concentrations greater than those available from a particular food source at any one feeding.

Sample Digestion

There are many obstacles in preparing clean and statistically acceptable test solutions. The specimen digestion techniques, in the previously cited literature, required a lot of time, a number of different reagents, extended exposure to ambient air, the use of glassware, and often procedures resulted in incomplete digestions requiring filtering.

Sample preparation is the rate limiting step in most complex, analytical procedures. According to American Chemical Society (ACS) documentation, approximately 61% of procedure time is spent preparing samples for analysis.[12] In the

Table 1.5

Levels of Zn, Cd, and Cu in a Red Clover System

Mean ppm Dry Weight (SD)

	No. of Determinations	Total No. of Individuals Analyzed	Zinc	Cadmium	Copper
Living Clover					
Roots	5	-----	38 (9.2)	0.48 (0.11)	39 (8.6)
Stems	9	-----	36 (13.2)	0.14 (0.04)	16 (4.8)
Foliage	11	-----	104 (114.0)	0.16 (0.07)	44 (33.6)
Clover Litter					
Stem	6	-----	37 (14.2)	0.29 (0.07)	14 (4.3)
Mixed	7	-----	74 (24.4)	0.7 (0.21)	31 (17)
Mineral Soil	16	-----	83 (8.1)	0.4 (0.08)	26 (6.0)
Earthworms					
Sexually Mature Adults					
Lumbricus rubellus					
Tissue (S)	18-22	18-22	320 (129)	6 (1.9)	10 (3.0)
Faeces	15-23	-----	86 (9.7)	0.5 (0.13)	25 (4.1)
Allolobophora chloronica					
Tissue (S)	13	13	210 (45)	8 (2.4)	8 (2.4)
Aporrectodea spp.					
Tissue (D)	20	20	380 (114)	8 (2.5)	11 (3.0)
Sexually Immature Adults					
Lumbricus rubellus					
Tissue (S)	33-37	33-37	260 (84)	5.1 (1.36)	10 (2.1)
Faeces	22-24	-----	79 (22)	0.5 (0.25)	25 (4.8)
Slugs	9	9	214 (82.9)	2.2 (1.16)	100 (54)
Millipedes					
Tissue	7	97	321 (29.5)	0.2 (0.04)	221 (95.5)
Faeces	5	-----	340 (150.2)	1.1 (1.28)	28 (14.5)
Collembola	2	75	92		65
Coleoptera Carabidae					
Pterostichus melenarius					
Adults	6	6	116 (27.9)	0.3 (0.16)	13 (3.5)
Larvae	6	11	218 (88.9)	3.5 (1.55)	21 (7.2)
Staphylinid Adults	2	34	278	0.6	32

Figure 1.3. Food Web Relationship of Zn, Cd, and Cu in a Red Clover System

literature previously cited, various analyte solubilization techniques were employed. Adler and Pearson's specimens were treated with concentrated nitric acid at room temperature, requiring 12-21 hours, depending on the sample size.[3] Carter's procedure involved wet ashing with concentrated nitric acid, drying, and re-dissolving the residue.[2] Scholwalter et al., used a combination of dry ashing followed by wet ashing, a process requiring approximately 14 hours from specimen to test solution.[10]

Microwave Acid Digestion

Using closed vessel microwave acid digestion, test solution preparation time was greatly decreased: from specimen to test solution in 20 minutes. Microwave heating of acids for digestion of samples was demonstrated in 1975, using a home microwave modified for safe use with corrosive materials.[13] Microwave energy does not alter the structure of the molecules or ions: it causes the migration of ions and the rotation of dipoles.[14] Heat is produced due to the solution's resistance to ion migration and the alignment response of polar molecules to the electromagnetic field, followed by their return to disorder. In conventional conductive heating, only the portion of the solution in the vessel that is directly above the heating source has a higher temperature than the boiling point of the solution. The digestion vessel does not absorb microwave energy. The solution is heated more evenly because localized superheating is taking place throughout the solution. Wherever superheating is taking place, by definition, the temperature of the solution is above the boiling point of the solution at a given pressure (see Figures 1.4 & 1.5).[14] Substances that would not normally decompose at the normal boiling point

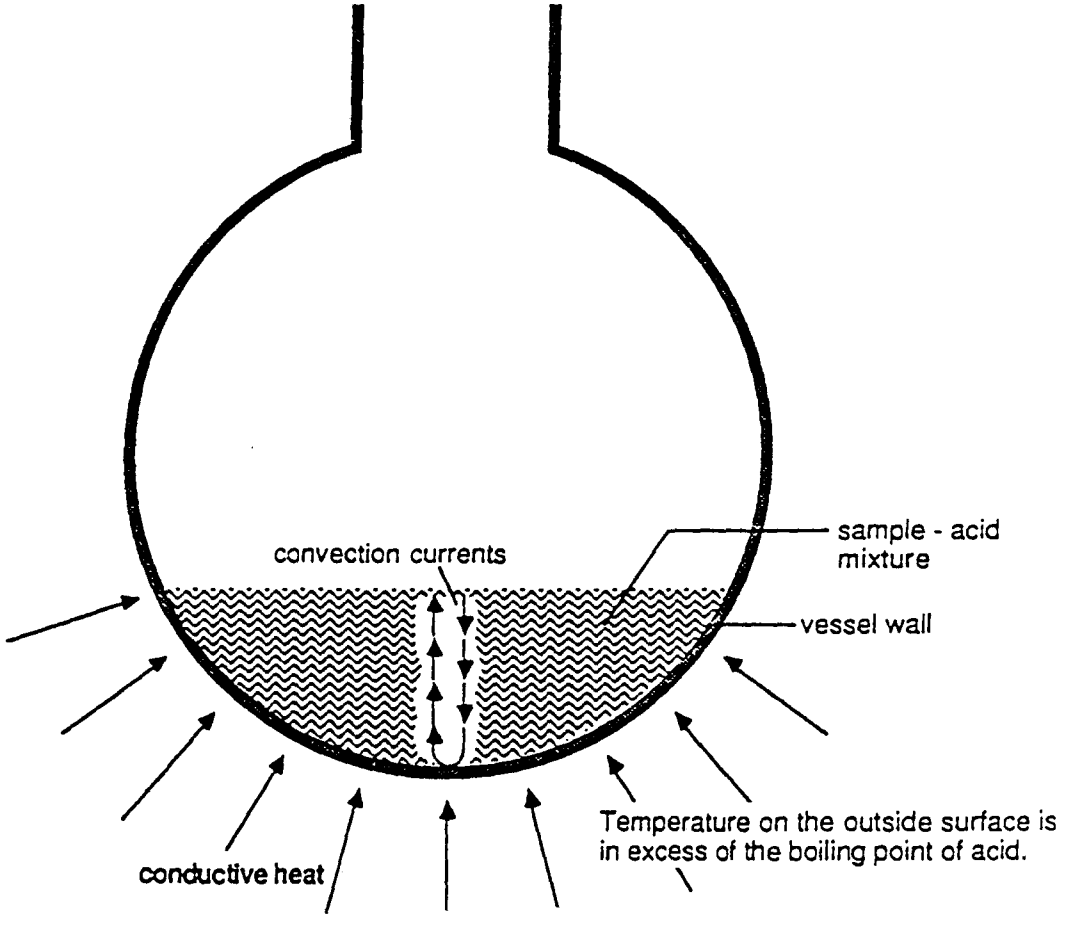

Figure 1.4. Conductive Heating of a Solution

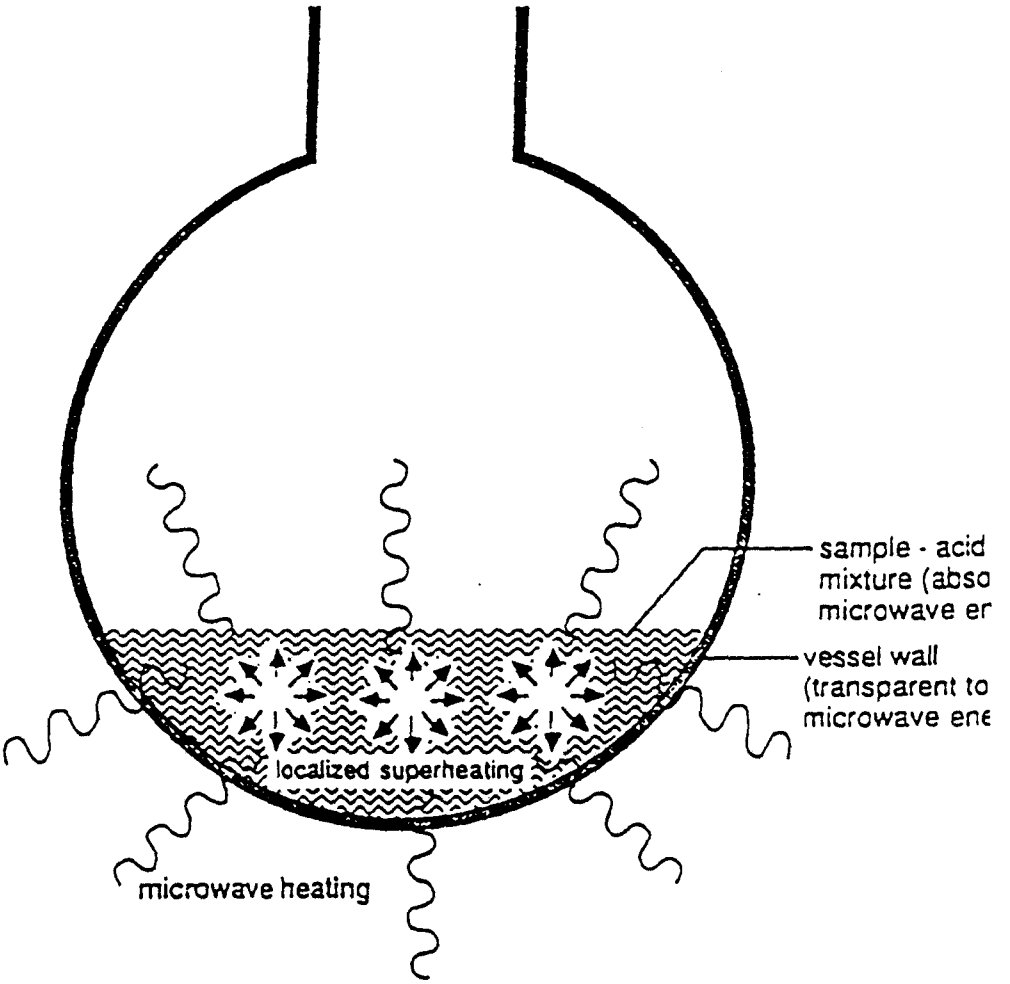

Figure 1.5. Microwave Heating of a Solution

of nitric acid (83°C), are added to the spectrum of analytes.[15] Substances that would normally enter into solution at normal boiling point temperatures, did so more quickly at elevated temperatures.[15] When the digestion vessel is sealed, (a digestion bomb), the pressure increases, and the temperatures achieved are well above the normal boiling point of the solution. In March 1990, the United States Environmental Protection Agency (USEPA), Contract Laboratory Program (CLP), approved the use of closed vessel microwave digestion and leaching techniques for soils and waters.[16] CEM Corporation applied to the USEPA for nationwide approval of their "closed vessel microwave digestion of waste water samples for metals determination". In 1992, the procedure for waters was accepted by the USEPA for water digestion as preparation for analysis with FAA, ICP-AES or DCP-AES, (direct current plasma).[17] Standard and revised methods for microwave assisted sample preparations for sediments, sludges, soils, oils, aqueous samples, and extracts were added to SW-846 by the USEPA in 1995.[18]

Many of the microwave methods for soils and sludges were extraction techniques rather than complete dissolution of the sample (see Appendix A). When digestion is incomplete, an additional step must be employed such as filtration or centrifugation, to separate the solid portion of the specimen remaining from the test solution. CEM Corporation had a wide array of dissolution procedures available for many different sample types including: agricultural, biological, environmental, and food. The digestion methods reviewed were those most closely related to the specimen type of interest, that resulted in the most complete destruction of the solid (see Appendix B). The organic

matter must be completely oxidized, so the viscosity of the solution is not increased by polysaccharides and residual hydrolysates of protein rendering the calibration with aqueous solutions invalid.[19] Complete digestion of the specimens was considered necessary and possible.

The samples and reagents inside of the digestion bomb do not come in contact with the air in the laboratory environment. Since the vessels are sealed, volatile reagents cannot escape, decreasing the volume of reagent necessary, thereby decreasing possible contamination from the reagent to the sample. The more volatile elements do not escape during digestion unless the vessel was improperly vented, in which volatile elements may escape due to rapid decrease in pressure resulting in uncontrolled boiling, allowing volatile elements to escape in aerosol form.[20]

Clean Chemistry Techniques

Preventing contamination of a test solution during preparation is an obstacle in all trace metal analysis. Once a sample is contaminated, it is difficult to pinpoint the source, especially when the analysis takes place in an unclean environment. The levels of trace metals in dust in ambient air, were determined to be unacceptable when compared with the detection limits achievable in trace metal analysis (see Table 1.6).[21]

In the area of biological collection and preparation, some major sources of contamination are identified[22]: dust, dirt, cosmetics, disinfectant, talc, metallic corrosion and residue. Elevated zinc levels were attributed to the powder inside of latex gloves. The yellow plastic stoppers for volumetric flasks were the cause of elevated cadmium levels in trace metal analysis.[23] Elevated levels of trace metals have been attributed to

Table 1.6

Trace Element Levels in Dust from the Laboratory Atmosphere

Element	mg/kg Dust	Element	mg/kg Dust
Al	3,000	Mg	2,390
As	55	Mn	116
Br	23	Na	2,950
Ca	2,690	Ni	70
Cd	3	P	1,150
Cl	2	Pb	2,150
Co	9	S	20,000
Cr	39	Sb	15
Cu	213	Sn	10
F	1	Sr	14
Fe	3,230	Ti	258
I	3	V	259
K	7,920	Zn	1,640

metal scalpels and needles used to extract specimens. Nickel and chromium levels were doubled in a liver sample taken by contact with a scalpel blade. Positive errors from 30% to 50% were found for copper, manganese, and zinc for samples taken by contact with a needle.[24]

A study was done, implementing cleanroom protocol, for determination of trace metals in specimens, without major remodeling or renovation to the laboratory. The problem encountered by Prevatt's group was lack money or available space to build a cleanroom facility. An analytical plan was constructed addressing each step of the procedure. All glassware was avoided, only high density polyethylene (HDPE), low density polyethylene (LDPE), and Teflon® that was leached in dilute nitric acid, were used in contact with the sample. Ziploc®-type bags were used for storage of polymer volumetric ware and sampling hoses when they were not in use. Modifications to the filtering procedure included the reduction of static charge on the filtering apparatus. After being leached in dilute nitric acid for 24 hours, the polymer ware was rinsed and allowed to sweat in a Ziploc®-type bag for 24 hours. The droplets that formed on the surface absorbed the static charge. The apparatus was rinsed with deionized water before use.

The ideas for procedure modifications given above were incorporated into the trace metal analysis of the specimens of interest as well as many new ideas that were sparked by Prevatt's study. Filtering was not necessary for the test solutions prepared in the manner described in the procedure section. However, the method for reducing static charge was used for the UDV-10 bomb liners.

Chapter Two

Project information

The *Hyalophera cecropia*, or Robin moth has a range from the Eastern North America to the Rocky Mountains, and from Canada to the deep south.[25] The larvae of the *Hyalophera cecropia* must be in its fifth instar before they form a cocoon and begins their metamorphic process. An instar is a stage in the life of an arthropod (as an insect) between two successive molts. In the fifth and final instar, the larvae measure 9-11 cm and are green with yellow dorsal tubercles, blue lateral tubercles, and four red thoracic tubercles, with a circle of gray around each spiracle (Figure 2.1).[25] The excrement from this caterpillar is rather large, resembling a raisin.

Hyalophera cecropia larvae can exist on many different food plants, including the cyanide containing leaves of *Prunus serotina.* The leaves of the black cherry tree are dark green and glossy, measuring 6-12 cm in length and 2 to 5.5 cm in width (Figure 2.2).[26] The twigs, leaves, pit, and bark all contain hydrocyanic acid.[26] This medium sized tree, measuring 12-19 m, has a nearly black bark when mature; resembling "burnt corn flakes" (Figure 2.3).[27] The flowers are small and white appearing in 4-10 cm racemes from May to July: depending on climate. The black cherry tree produces black, shiny, juicy, edible fruit, measuring 0.7-1 cm.[26]

Hyalophera cecropia eggs were taken to Lawrence County, Western Pennsylvania, from the Biology Department, at Youngstown State University, placed on the leaves of black cherry trees, surrounded with netting, and allowed to

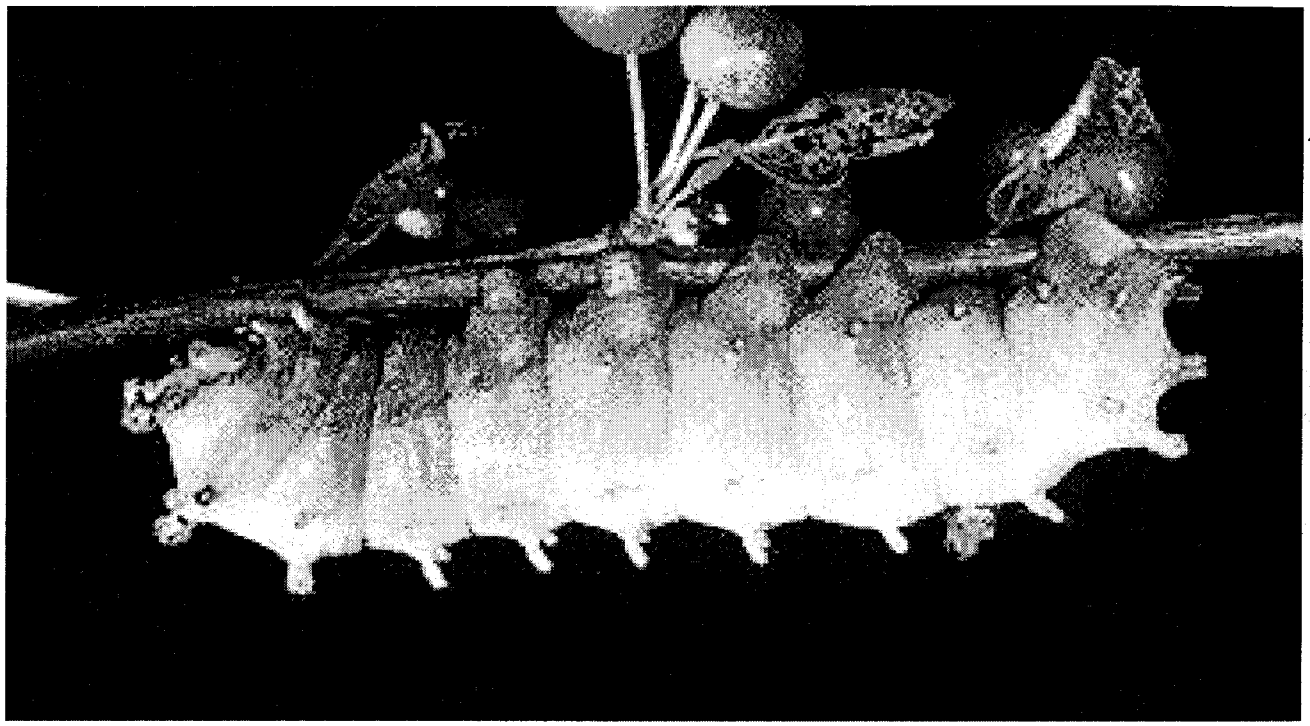

Figure 2.1. Fifth Instar *Hyalophera cecropia*

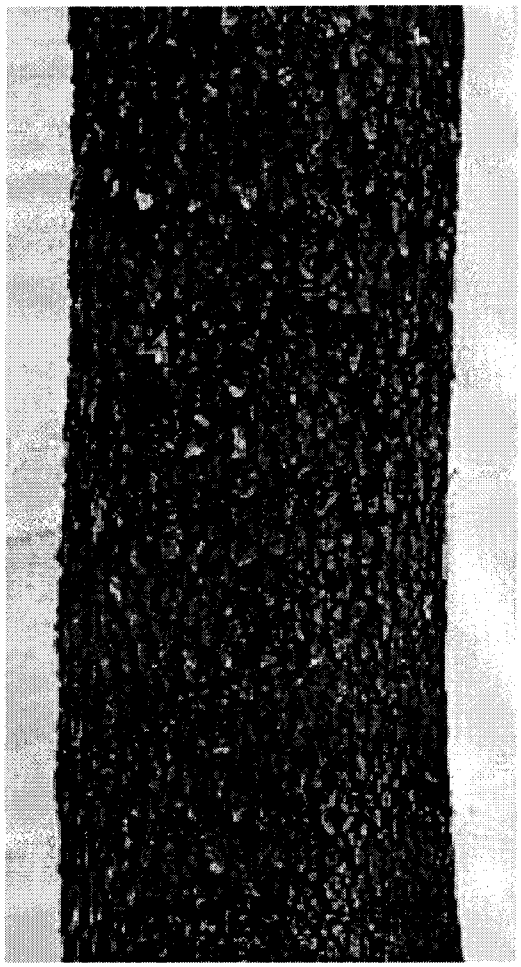

Figure 2.2. Bark of the *Prunus serotina*

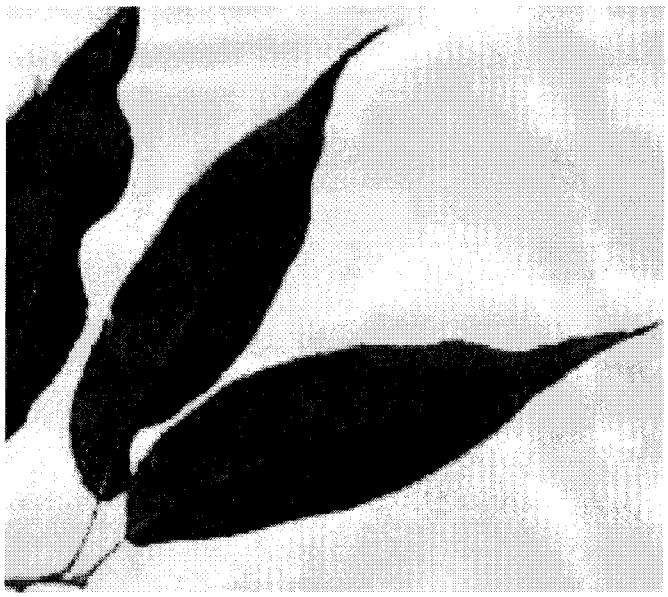

Figure 2.3 Leaves of *Prunus serotina*

develop normally. The larvae were collected in their fifth instar, along with there feces, and the leaves of the black cherry tree. Knowing the elemental ratios determined in this study, when these field larvae are collected, predictions can be made about their food source.

The lab larvae used as specimens were reared from eggs, by Dr. Lauren Schroeder, in the Biology Department at Youngstown State University. They were exposed to 14 hours of light and 10 hours of dark at 27°C and 80% relative humidity. The larvae fed daily on black cherry leaves: the uneaten portions, the leaf residuals, were removed each day. The individual caterpillars were housed in cardboard cones fashioned from a 5 x 7 inch note card covered with cheesecloth. Feces were collected in glass vials that were taped to the bottom of each of the cones.

The specimens of feces, leaves, and leaf residuals, were dried at 60°C in a vacuum over $CaCl_2$, ground into a fine powder, then stored in a dessicator. The fifth instar larvae were collected after they stopped feeding and prepared to pupate. They were killed by freezing at -20°C, dried, and ground into a fine powder. The petroleum ether soluble fraction, the lipids, was removed from the larvae prior to being stored in a dessicator.

With the specimens described above, an elemental mass balance was determined. Most of the data relative to food use by an organism is based on mass units.[28] In this study, the equation I = G + M + F was evaluated for the larval development of *Hyalophera cecropia,* resulting in an input/output elemental mass balance.

$$I = G + M + F$$

I ≡ ingestion, mass or caloric equivalent of food eaten

G ≡ growth, mass or caloric equivalent of larval tissue

M ≡ maintenance, mass or caloric equivalent expended in maintaining life processes; primary respiration

F ≡ excrement, mass or caloric equivalent of material eliminated from the gut

Excrement includes unassimilated food, digestive enzymes, enteric bacteria, nitrogenous waste products, secretions, and sloughed off intestinal lining cells.[28] The purpose of this study was to relate mass and energy budgets of the cecropia larvae. The equation above was modified as follows:

$$V = G + M$$

V ≡ larvae, includes growth and maintenance

$$I = V + F$$

$$I = 1.07$$

$$V = 0.16$$

$$F = 0.82$$

The values of I, L, and F were determined by Dr. Lauren A. Schroeder at the Biology Department at Youngstown State University.

The concentrations of the elements determined by ICP-AES and GFAA are included as follows:

$$i\,(1.07) = v\,(0.16) + f\,(0.82)$$

x ≡ specific element

{x} ≡ the concentration of the specific element in ppm

$i \equiv \{x\}$ in the the difference of the mass of (leaves - leaf residuals)normalized to 0.500 g

$v \equiv \{x\}$ in the normalized mass, 0.500 g, of a larvae specimen

$f \equiv \{x\}$ in the normalized mass, 0.500 g, of a feces specimen

$\therefore [v\,(0.16) + f\,(0.82)]/[i\,(1.07)] = 1$

The elements, whose partition ratio is equal to 1, can be applied to elemental levels found in field larvae. The data from the larval analysis would be indicative of their food source and their eating habits. Deviation from 1 was expected in certain elements. In cases where bioaccumulation takes place, the ratio was greater than 1. If an element was lost through respiration, (excretions other than feces), the value would be less than 1.

Chapter Three

Materials

Polymer ware

Materials used in the preparation and analysis of the test solutions were chosen specifically to minimize the possibility of contamination. The pre-treatment of glassware takes weeks to complete and requires large volumes of acid. Glassware was replaced with comparable polymer ware made by Nalgene®: beakers were made of Halar fluoropolymer resin and volumetric flasks were made of polypropylene. Metal spatulas and scoopulas were replaced with stainless steel spatulas coated with Teflon® TFE (tetrafluoroethylene) and plastic coffee stirrers from McDonald's©. The reagents used were the purest attainable. It would be preferable, if this were on a larger scale, to purchase a distilling apparatus and purify the acids as needed.

The only portion of the digestion bomb apparatus that came into direct contact with the sample were the liners and covers of the Ultimate Digestion Vessel (UDV-10), which are made of TFM® PTFE.

Glove-bags

Since the laboratory air contains many contaminants, the materials were housed in three separate glove bags, (JV manufacturing Co. Spilfyter "HANDS-IN-BAG") which were flushed, then filled with argon.

The first glove bag contained a Mettler AE 100 analytical balance, vials of the specimens, Kim-Wipes®, TFM® UDV-10 liners and covers, and spatulas. The second glove-bag contained a Labsystems Digital Finnpipette, volume 2.0-10.0 mL, disposable pipet tips, two 250 mL Nalgene® beakers, one empty for waste and one filled with trace metal grade nitric acid from Fisher A509SK-212 Safe-Cote®, and covered with Parafilm®, one 50 mL Nalgene® beaker, and an "HF Master" thermowell with threaded connector.

The third glove bag contained a Nalgene® funnel, 100.0 mL Nalgene® volumetric flasks, two 250 mL Nalgene® beakers for rinse waste, Parafilm®, 50 mL graduated, previously labeled, freestanding, polypropylene centrifuge tubes, a 250 mL rinse bottle filled with 18 MΩ deionized water, and an 18 MΩ deionized water line that came directly from the deionized water faucet through a plastic hose joiner, into the glove bag through previously leached Tygon® tubing.

Chapter Four

Methods

Specimen Treatment

Since there were no available clean room facilities for test solution preparation, an alternate method was devised to achieve clean room conditions. The three hands-in-bag stations were connected with rubber tubing, which carried argon to specific bags through releasing and tightening C-type clamps placed on the tubing. The surfaces of all objects that would occupy the bags, including sample vials, were wiped with a Kim-wipe® moistened with deionized water. The materials were placed in the respective bags as the argon was flowing to keep dust from entering the artificial atmosphere. After all of the items needed were placed in the bag, the opening was folded and clamped, and the bag was allowed to fill to a workable volume with argon gas. The argon gas tank was shut off, and the clamp on the hose leading to the bag was closed.

The position of each bagged station in the series was important, as well as what was kept in each bag. The first station, the balance bag, contained no liquids. A polystyrene weighing boat containing dessicant was covered with a Kim-wipe® and placed in the bag. The balance contained metal and corrosion had to be eliminated to prevent the glove bag from becoming contaminated. Even though the weighing station's atmosphere was indirectly connected to the acid station, the balance was never exposed to corrosive acid vapors. The inlet hoses for the second and third station were flushed and

clamped closed before the argon was used to flush and fill the first station. To eliminate any corrosive vapors in the lines, the hose was removed from the straight connector, which was positioned approximately 20 cm from the entrance of the hose into the bag, and flushed with argon. When the straight connector was pulled apart outside of the bag, pressing lightly on the inflated bag would flush out the hose from the inside of the bag outward to the surroundings. The tubing was reconnected and clean argon, free of corrosive vapors, was available to keep the glove bag pressurized constantly. The glove bag was only opened to place materials as needed, at which time the argon flow would be turned on to restore pressure. The bag had another outlet hose directly in the front of the bag used to reduce pressure in the bag when necessary. The argon flows directly from the argon gas tank, passing through the Tygon® tubing, through a polypropylene T-connector, through a polypropylene straight connector, then into the glove bag.

The purpose for the second bag was to pipet acid into the liners containing accurately weighed portions of specimens. Before the clamp was opened to allow argon to flush and fill the acid bag, the tubing leading to the balance bag was securely clamped. The second station had an argon inlet hose that traveled from the T-connector, through a Y-connector, and into the top of the bag. There was a piece of Tygon® tubing, cut and rejoined with a straight connector between the second station, which was the acid station, and the third station which was the dilution station. The connecting tube allows acid fumes to be pushed by the flowing argon, from the acid bag, through the dilution bag, the tubing, out of the other side of the dilution bag, and into a hood.

The third station was the glove bag containing the materials to quantitatively transfer the contents of the UDV-10 liners into the volumetric flasks for dilution. A deionized water line was secured, with tape, through a hole in the glove bag, to refill the rinse bottle as needed for dilution. This minimized the number of times the bag had to be opened. A hose from the side of the bag into a hood, ventilated the bag while the argon was flowing, to continually flush the heavy orange-brown cloud found above the solution in the UDV-10 liner after digestion. The clamp to the balance bag was closed before the argon was delivered to the dilution bag. The argon for this bag traveled through a T-connector, through a Y-connector and into the top of the glove bag to flush and fill the dilution station. The tubing entering into the side of the glove bag that was connected to the acid bag is clamped closed during dilutions.

Microwave Digestion

The CEM Corporation, (Matthews, N.C.), Microwave Sample Preparation System (MSP-1000), was used for the microwave acid digestion. The MSP-1000 operates at 950 ± 50 watts. The microwave power was programmable in 1% increments from 1-100% power.[29] It was equipped with a 25-pin printer port and a 9-pin, RS 232, IBM compatible computer port. The microwave was programmed from a CPU outside of the microwave. Data from microwave digestions, including pressure and temperature, were recorded.

The MSP-1000 was greatly improved from the CEM Microwave Digestion System (MDS-80) available in the laboratory. The MSP-1000 was equipped with a Temperature Control System (TCS), as well as a Pressure Control System (PCS).[29] The

PCS involved pushing water through a small Teflon® tube with a syringe while the two-way valve was in the open position. The valve was closed and the pressure line containing the water was attached to the reference vessel before the closed vessel was heated. The pressure exerted by the contents of the digestion bomb on the water inside of the tubing was conveyed to the pressure transducer. The pressure information was displayed on the MSP-1000 display panel, printed directly from the microwave unit, displayed on the computer monitor, or stored and printed from the computer. The pressure system was very similar to the modifications made to the MDS-80, with much more advanced data reporting methods. The TCS involved a fiber-optic temperature probe that was encased in the "HF Master" thermowell, made of industrial diamond, to protect the probe from the destructive acid. The probe itself was invisible to microwave radiation and would not heat independently of the solution. The temperature detection was the result of a phosphor at the end of the fiber-optic probe.[29] Fluorescent light is emitted by the phosphor after it has been excited by a pulse of optical energy, and the decay of the fluorescent signal was dependent on temperature. By measuring the duration of the decay, an accurate determination of the temperature could be determined when the rate of decay is compared to calibration information stored in the computer's memory.

The vessels used for the digestion of the specimens were the UDV-10 from the CEM Corporation (see Table 3.1).

Table 3.1

UDV-10 Specifications

Vessel Volume:	100 ml (liner)
Liner Weight:	100 g
Maximum Operating Temperature:	260°C
Maximum Control Temperature:	200°C
Max Material Design Pressure:	1500 psig (100 bar)
Maximum Operating Pressure:	1000 psig (70 bar)
Maximum Control Pressure:	600 psig (41 bar)
Maximum Organic Sample Size:	0.5 grams
Materials of Construction:	
Liner:	TFM® PTFE
Insulator & Sleeve Assembly:	Teflon® PFA and Advanced Composite
Cover:	Teflon® PFA
Support Module:	Polypropylene w. TFM® heat shield
Vent Fitting:	Teflon® PFA (Color Coded - Gold)
Ferrule Nuts:	Teflon® PFA

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TFM is a registered trademark of Hoechst.

Analytical Instrumentation

Inductively Coupled Plasma Atomic Emission Spectroscopy

A plasma is a gas whose atoms are ionized: argon plasma is made up of Ar^+ and free electrons. Plasma is a non-combustion excitation source, and can reach temperatures much greater than combustion excitation. Inductively coupled plasma, (ICP), technology was first employed in the 1960's as a technique to improve crystal growing and deposition.

Most of the analysis was performed using an Applied Research Laboratories Inc., (ARL), Model 3410 ICP Spectrometer, with Argon Plasma Minitorch®, (ARL is now Thermo-Jarrel-Ash). An inductively coupled plasma is produced by a 3-turn radio frequency, (RF), coil positioned around a quartz torch, fashioned from three concentric tubes: the inner tube, the intermediate tube, and the outer tube (see Figure 4.1).[30] The coil is attached to a water cooled RF generator, which delivers an AC current of approximately 30MHz, at a power level of 2kW. This RF induces a high frequency (HF) oscillating magnetic field. Argon gas escapes through the top of the intermediate tube of the quartz torch, a Tesla discharge spark enters into the gas in the magnetic field, and the gas is ionized. The magnetic field acts on the ionized gas causing an eddy current of cations and electrons. The charged particles attempt to move along with the magnetic field and collide with argon atoms, producing more charged particles.[31] The alternating current produces a magnetic field which switches polarity, causing the direction of the cation and electron flow to change, resulting in more collisions, producing more

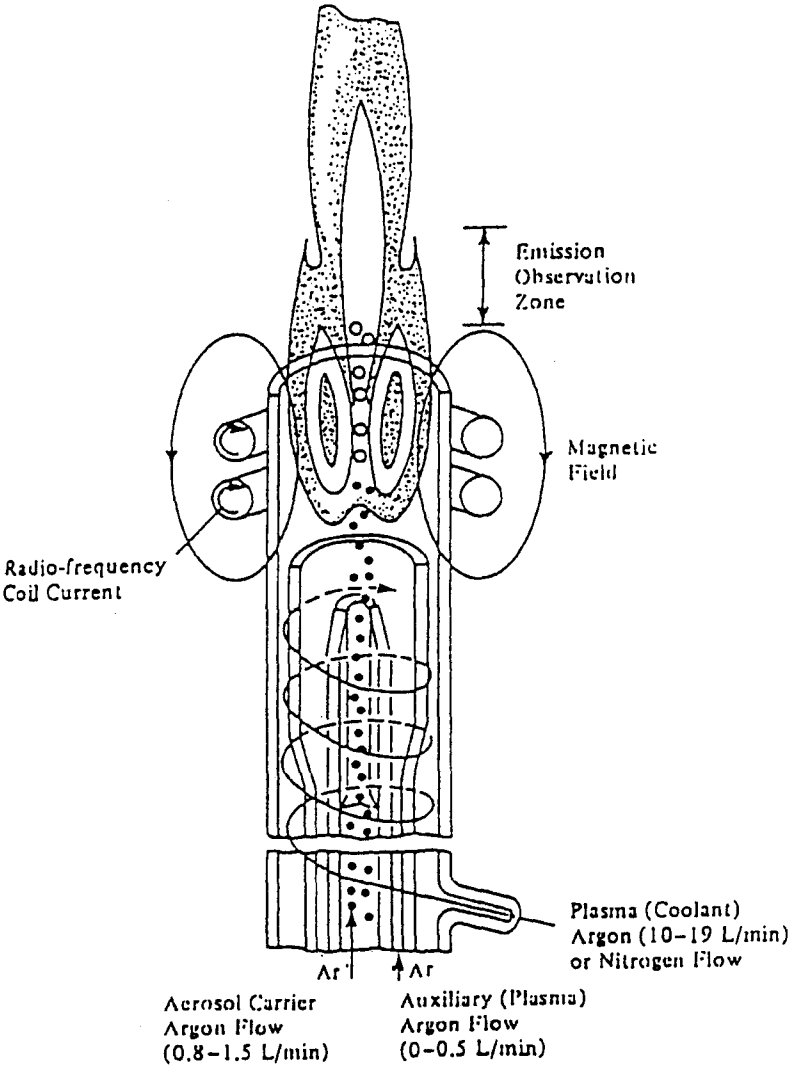

Figure 4.1. Argon Plasma Torch

electron flow in the argon gas causes the intense heating of about 10,000K. The inner quartz tube conveys sample received from the nebulizer, to the argon plasma torch. The argon flowing tangentially through the outer tube, insulates the plasma from the surroundings and helps to stabilize the plasma cone.

The test solution, delivered as an aerosol through the inner cone to the argon plasma, experiences temperatures of 6,000-8,000K: atomization and excitation of the atoms and ions occurs. The tail above the plasma cone contains all of the analyte atoms and ions that have been excited by the heat of the plasma. The optical window views the area above the cone. The radiation emitted by the excited electrons returning to the ground state emit light at a specific wavelength particular to an energy level transition of a specific element. The narrow emission profile of the transition reduces self-absorption, which would decrease the intensity of the radiation received by the detector. For a sequential ICP-AES, the radiation strikes a diffraction grating, driven by a stepper motor. The diffraction grating is set at a particular wavelength, and is allowed to scan a particular range around that wavelength. The grating reflects a particular wavelength of light onto a mirror, which directs the radiation through an exit slit and into a photomultiplier tube (PMT) (see Figure 4.2). The signal received by the PMT is compared to signals received during calibration with defined concentrations. The concentration of the analyte responsible for the emission is then computed.

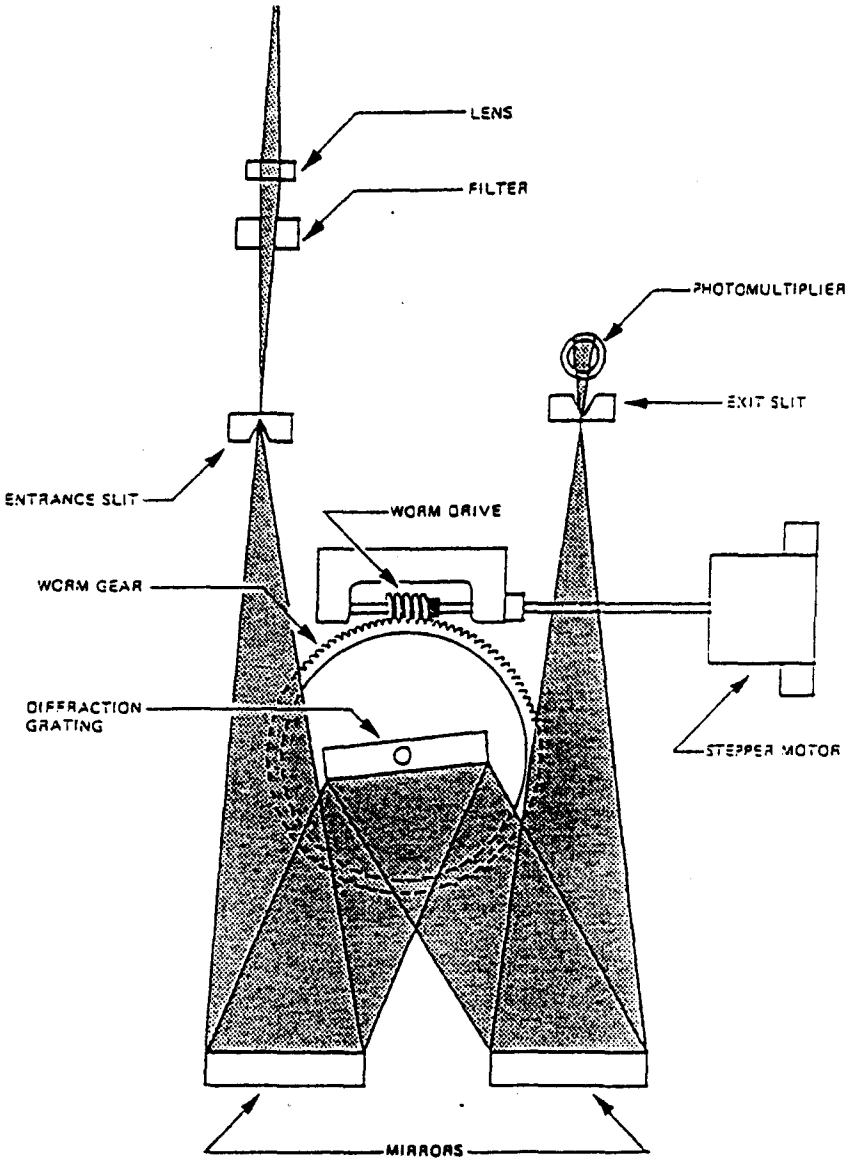

Figure 4.2. Schematic Representation of the ARL Monochrometer

Chapter Five

Procedure

Treatment of Containers

The use of glass was avoided throughout the procedure. All volumetric flasks, beakers, UDV-10 liners, covers, disposable pipet tips, and funnels were treated as follows:

1) Placed into a large Nalgene® tub
2) Filled tub with 10% (v/v) nitric acid, let soak 48 ± 2 hours
3) Emptied tub
4) Rinsed each piece and tub with deionized water
5) Filled tub with deionized water, let soak 48 ± 2 hours
6) Repeated steps 2 - 5 once
7) Stored pieces in a garbage bag, flushed with argon, until needed

The UDV-10 liners were treated as above with the following additional treatment:

8) Filled with 10.0 mL of concentrated nitric acid
9) Heated according to digestion program, after proper assembly
10) Allowed to cool
11) Discarded contents
12) Final rinse with deionized water
13) Repeated steps 8-12 once
14) Sealed in Ziploc®-type bags to sweat for 24 hours

15) Rinsed with deionized water

16) Excess water was removed from surface

17) Stored in argon flushed Ziploc®-type bags in an argon flushed garbage bag

After the initial leaching, volumetric ware, etc., that was not disposable, was rinsed with deionized water, 10% (v/v) nitric acid, then rinsed with deionized water once more. They were placed inside of the argon filled trash bag until needed. The moisture inside of the bag was not of concern since the volumetric ware was rinsed again prior to use.

The UDV-10 vessel covers had to fit tightly to prevent leakage while heating. The cover was made so the seal becomes tighter as the portion fitting inside of the vessel liner is pushed against the liner with increased pressure. To be sure of a snug fit, the vessel covers were reformed after each successive heating, prior to being cleaned, using the Seal Energizing Device supplied by CEM Corporation. The portion of the simple lever device that came in contact with the liner covers was made of Teflon®, and posed no concern as a source of contamination.

Specimen

Capped vials containing larvae, feces, leaves, and leaf residuals were received by the Chemistry Department of YSU from the Biology Department of YSU. When received, the specimens were numbered and recorded. The vials containing the larvae

noted that the lipids had been removed from the larvae specimens. The specimen vials were stored in a dessicator until digestion.

Glovebags

While preparing the stations, it was imperative that the list of supplies was available as a checklist. It is time consuming to have to remove clamps and open up the bag to place objects that were necessary but forgotten. Each object entering the bag was wiped with a damp Kim-wipe® before being placed in the bag, even the Kim-wipe® box.

The corners of the bag were occupied by heavy items or taped on the outside to the bench top surface. This prevented the bag from "rolling" when arms were in the sleeves. All hoses entering the bag were securely taped to seal the opening. Cotton gloves were worn on the hands, inside the gloves to absorb sweat that collected inside the plastic. Elastic bands were tied in loops and placed around the sleeve, inside of the glove bag, to help secure the loose plastic that could be a hazard while moving in the bag. Items in the bag were placed so the gloved hands could move freely.

Balance Station

The glove bag was flushed and filled with argon and the clamp was closed directly below the T-connector. Inside the station, the mass of the specimens and Standard Reference Material (SRM) (Citrus Leaves) were determined by difference. The vial was uncapped, placed on the balance, the balance was tared, a portion of the sample was transferred to the UDV-10 liner and the vial was returned to the balance. When the

negative mass appearing on the digital display was between 0.3 - 0.5 g, the mass was recorded ±0.0001 g. The vial was capped and set aside. The cover was placed on top of the liner. When all of the specimen portions were transferred to the UDV-10 liners, and all of the caps were secure, the argon flow was turned on and the bag was opened. The sample vials, any used Kim-wipes®, and the covered digestion vessel liners were removed. The bag was resealed, and the argon flow was turned off, and the tubing was clamped after the T-connector.

Acid Station

The glove bag was flushed and the large bag opening in the front was folded and clamped. Tubing leading from the bag to the hood was opened, and a slow stream of argon continued to flow from the tank through the bag. 10.0 mL aliquots of concentrated HNO_3 were pipetted from a 250 mL beaker to the vessel liners containing specimen. After acid was transferred, vessel liners were capped. The thermowell was placed into the cover of the reference vessel and secured with the threaded connector, prior to capping the reference vessel (see Figure 5.1). The covered vessel liners were removed the bag was thoroughly flushed and vented into a hood to remove any remaining acid vapors.

Assembly of the Ultimate Digestion Vessels (UDV-10)

After the capped liners containing the acid and the specimen were removed from the acid station, the outer surface was wiped to remove any corrosive residue from the acid glove bag. The liners were slid securely into the insulator inside of the advanced

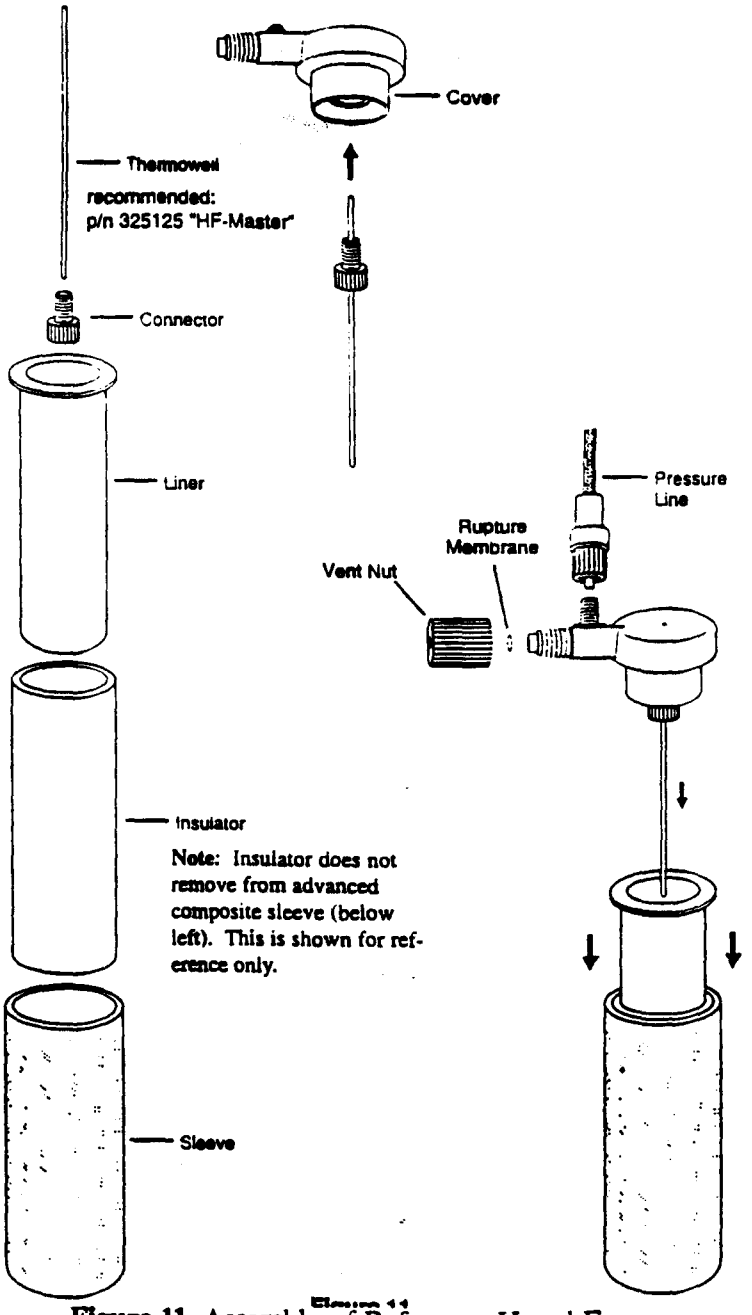

Figure 11. Assembly of Reference Vessel For Pressure/Temperature Control of UDV-10

Figure 5.1. Assembly of Reference Vessel for Pressure/Temperature Control

composite sleeve. The digestion bombs were placed into the support modules and secured with Teflon® slide lock. The gold colored Teflon® PFA vent nut was fitted with a rupture membrane and screwed onto the vent hole.

The reference vessel had a particular cover that had been fitted with the HF Master thermowell, while in the acid bag. The vent nut was affixed in the same manner as the other covers, but there was an additional opening for the pressure sensing line that was attached before the microwave digestion procedure. There was a hole in the top of the support module that allows the fiber-optic temperature probe to enter into the protective thermowell to determine the temperature of the digesting mixture.

Microwave Carousel

The fully assembled UDV-10 digestion bombs were slid into the slots notched in the microwave carousel. The carousel was placed squarely onto the drive lug positioned in the microwave oven. The fiber-optic probe was gently slid into the hole in the top of the support module and secured by pressing the protective sheath around the probe, slightly into the top of the hole. The pressure sensing tubing was flushed into a beaker by opening the two-way valve and depressing a syringe. The two-way valve was closed, and the pressure tubing was screwed on to the opening adjacent to the vent nut. The door was closed, and the carousel rotated to be sure the fiber-optic line and the pressure tubing cleared the fan on the interior of the microwave. The protective shield was lowered in front of the microwave door. Depending on the run, the numbers of vessels varied.

Microwave Digestion Program Variables

The microwave could be programmed either by the computer, or the keypad next to the display. The IBM compatible software from CEM, Preplink, was used. The software was not upgraded for the UDV-10 vessels. The program was done with the Heavy Duty Vessel (HDV) selection. The digestion program variables remained identical for each digestion (see Table 5.1).

After the program variables were entered, they were downloaded to the microwave. The command to proceed was clicked in, and the digestion program began. The current pressure and temperature readings were displayed on the monitor, as well as time elapsed. Another available screen showed the Time vs. Temperature and Time vs. Pressure plots. The data was printed in graphical and raw 'temperature at time' data, recorded every second.

When the digestion program was complete, the vessels were allowed to sit in the microwave until the pressure displayed on the monitor was 8 psi or less. The swivel ferrule nut coupling the pressure tubing to the reference vessel was unscrewed, a small amount, approximately 5 mL, of thick, dark orange gas escaped through the vent hole. All of the vessels were placed in a hood. The pressure had already been released from the reference vessel, so the rest of the vessels were vented by loosening then removing the gold vent nut. Vessels were removed from the support module and set aside to cool for approximately 10 min. The liners were slid from the insulating liner of the advanced composite sleeve.

Table 5.1

Microwave Digestion Variables

Stage	(1)	(2)	(3)	(4)	(5)
Power	100 %	100 %	0 %	0 %	0 %
Pressure	0200	0600	0020	0020	0020
Run Time	5:00	15:00	00:00	00.00	00.00
Time @ P	00.00	15:00	00.00	00.00	00.00
Temperature	180 °C	180 °C	0 °C	0 °C	0 °C
Fan Speed	100 %	100 %	100 %	100 %	100 %

Number of Vessels:	8
Volume per Vessel:	10 mL
Sample Weight	0.4 g
Acid:	Nitric

Dilution Station

Capped vessel liners were placed in the glove bag that was being continually flushed with argon. The tubing connecting the acid bag and the dilution bag was clamped closed. The front opening was folded and clamped closed. The tubing leading from the bag into the hood was left open. The flow of gas into the hood evacuated the corrosive vapors released into the bag during the transfer and dilution of the test solutions.

The solution was quantitatively transferred by funnel and rinse bottle into a volumetric flask. The solution was at room temperature to avoid bubbles forming on the inside of the volumetric flask. The top of the flask neck was kept dry to insure that no drops would enter the solution after it was properly diluted. The volumetric flask was sealed with Parafilm® and mixed. The contents of the volumetric flasks were divided between two, previously labeled, freestanding, 50 mL centrifuge tubes. A deionized water line was fed directly into the dilution bag allowing for easy refilling of the rinse bottle.

Chapter Six

Results and Discussion

The data and subsequent values determined through calculation are tabulated on pages 68-84. The data listed on pages 68-71, was a result of three replicate measurements performed sequentially and averaged by the ICP-AES software. Each specimen was sampled three times, giving the three determinations for each specimen. The concentration values given on pages 72-75, were normalized to 0.5000g. The adjusted concentrations were multiplied by a value given by L. A. Schroeder, Biology Department at Youngstown State University. The products of the calculations were used in the equation (G+F)/I (see Page 25). The variable, I, defined previously was not adjusted using residual concentrations. The mass of residual compared to mass of whole leaf is presently unknown. The adjustment, when made will be negligible; expected to fall within the standard deviation relevant to each of the values.

Some values were removed in the following tabulations. The Q-test was applied and values were dismissed. Test solution, 21,1, was subjected to a gross error and was not analyzed. The remaining vacancies are a result of unavailable data.

A number of ratios determined in this project have a mean value of 1, within the standard deviation: phosphorus, sulfur, manganese, zinc, iron, potassium, and calcium. These ratios will be useful to biologists in determining the foliar consumption of the field caterpillars.

The results of the analysis of budget A, seemed extremely high in a number of instances. The Q-test was performed, and values were removed before determining the

average ratio (G+F)/I. The larval concentrations exhibited in budget A suggest possible contamination of the specimen.

Chapter Seven

Conclusion

The ratio (G+F)/I, when equal to 1, is useful to biologists in determining natural foliage consumption of field caterpillars. This would otherwise be impossible, given the enormous amount of variables present in any natural process. Values that are greater than 1, could be a result of bioaccumulation of the element if the reason for the high value is a result of an increased concentration found in the larva. Values that are less than 1 could be a result of elemental loss through respiration or volatilization.

A number of ratios determined in this project have a mean value of 1, within the standard deviation: phosphorus, sulfur, manganese, zinc, iron, potassium, and calcium. These ratios will be useful to biologists in determining the foliar consumption of the field caterpillars.

The continuing analysis of specimens to statistically improve the ratio is well underway. The digestion technique has been modified, now employing the ACV vessel, achieving complete digestion in five minutes. All of the specimens were digested using the new technique. Resulting test solutions will be analyzed by ICP-AES, ICP-MS, and GFAA. Comparison of the methods will help define detection limits and matrix effects in a large array of elements in a very simple, well digested 10% nitric acid matrix. The combination of methods and the number of determinations will greatly improve the validity of the ratios useful in biological and environmental applications.

Sodium budgets were popular in the literature. The solution was tested for sodium, but the levels present were too low for detection. The sodium budget for these lepidoptera will be determined by analysis with ICP-MS.

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APPENDIX A

USEPA METHOD 3051

MICROWAVE ASSISTED ACID DIGESTION OF SEDIMENTS, SLUDGES, SOILS, AND OILS

1.0 SCOPE AND APPLICATION

1.1 This method is applicable to the microwave assisted acid digestion of sludges, sediments, soils, and oils for the following elements:

Aluminum	Cadmium	Iron	Molybdenum	Sodium
Antimony	Calcium	Lead	Nickel	Strontium
Arsenic	Chromium	Magnesium	Potassium	Thallium
Boron	Cobalt	Manganese	Selenium	Vanadium
Barium	Copper	Mercury	Silver	Zinc
Beryllium				

1.2 This method is provided as an alternative to Method 3050. It is intended to provide a rapid mulitelement acid leach digestion prior to analysis so that decisions can be made about site cleanup levels, the need for TCLP testing of a waste and whether a BDAT process is providing acceptable performance. If a decomposition including hydrochloric acid is required for certain elements, it is recommended that Method 3050A be used. Digests produced by the method are suitable for analysis by flame atomic absorption (FLAA), graphite furnace atomic absorption (GFAA), inductively coupled plasma emission spectroscopy (ICP-ES) and inductively coupled plasma mass spectrometry (ICP-MS). Due to the rapid advances in microwave technology, consult your manufacturer's recommended instructions for guidance on their microwave digestion system and refer to the SW-846 "DISCLAIMER" when conducting analyses using Method 3051.

2.0 SUMMARY OF METHOD

2.1 A representative sample of up to 0.5 g is digested in 10 mL of concentrated nitric acid for 10 minutes using microwave heating with a suitable laboratory microwave unit. The sample and acid are placed in a fluorocarbon (PFA or TFM) microwave vessel. The vessel is capped and heated in the microwave unit. After cooling, the vessel contents are filtered, centrifuged, or allowed to settle and then diluted to volume and analyzed by the appropriate SW-846 method (Ref. 1).

3.0 INTERFERENCES

3.1 Very reactive or volatile materials that may create high pressures when heated may cause venting of the vessels with potential loss of sample and analytes. The complete decomposition of either carbonates, or carbon based samples, may cause enough pressure to vent the vessel if the sample size is greater than 0.25 g when used in the 120 mL vessels with a pressure relief device that has an upper limit of 7.5 ± 0.7 atm (110 ± 10 psi).

4.0 APPARATUS AND MATERIALS

4.1 Microwave apparatus requirements.

4.1.1 The microwave unit provides programmable power with a minimum of 574 W, which can be programmed to within ± 10 W of the required power. Typical units provide a nominal 600 W to 1200 W of power. Pressure, or especially temperature, monitoring and control of the microwave unit are desirable.

4.1.2 The microwave unit cavity is corrosion resistant and well ventilated.

4.1.3 All electronics are protected against corrosion for safe operation.

4.1.4 The system requires fluorocarbon (PFA or TFM) digestion vessels (120 mL capacity) capable of withstanding pressures up to 7.5 ± 0.7 atm (110 ± 10 psi) and capable of controlled pressure relief at pressures exceeding 7.5 ± 0.7 atm (110 ± 10 psi).

4.1.5 A rotating turntable is employed to insure homogeneous distribution of microwave radiation within the unit. The speed of the turntable should be a minimum of 3 rpm.

> CAUTION: Those laboratories now using or contemplating the use of kitchen type microwave ovens for this method should be aware of several significant safety issues. First, when an acid such as nitric acid is used to assist sample digestion in microwave units in open vessels, or sealed vessels equipped, there is the potential for the acid gases released to corrode the safety devices that prevent the microwave magnetron from shutting off when the door is opened. This can result in operator exposure to microwave energy. Use of a unit with corrosion resistant safety devices prevents this from occurring.
>
> CAUTION: The second safety concern relates to the use of sealed containers without pressure relief valves in the unit. Temperature is the important variable controlling the reaction. Pressure is

needed to obtain elevated temperatures but must be safely contained. However, many digestion vessels constructed from certain fluorocarbons may crack, burst, or explode in the unit under certain pressures. Only unlined fluorocarbon (PFA or TFM) containers with pressure relief mechanisms or containers with PFA-fluorocarbon liners and pressure relief mechanisms are considered acceptable at present.

Users are therefore advised not to use kitchen type microwave ovens or to use sealed containers without pressure relief valves for microwave acid digestions by this method. Use of laboratory-grade microwave equipment is required to prevent safety hazards. For further details consult reference 2.

CAUTION: There are many safety and operational recommendations specific to the model and manufacturer of the microwave equipment used in individual laboratories. These require the analyst to consult the specific equipment manual, manufacturer and literature for proper and safe operation of the microwave equipment and vessels.

4.2 Volumetric graduated cylinder, 50 or 100 mL capacity or equivalent.

4.3 Filter paper, qualitative or equivalent.

4.4 Filter funnel, glass or disposable polypropylene.

4.5 Analytical balance, 300 g capacity, and minimum ± 0.01 g.

5.0 REAGENTS

5.1 All acids should be sub-boiling distilled where possible to minimize the blank levels due to metallic contamination. Other grades may be used, provided it is first ascertained that the reagent is of sufficient purity to permit its use without lessening the accuracy of the determination. If the purity of a reagent is questionable, analyze the reagent to determine the level of impurities. The reagent blank must be less than MDL in order to be used.

5.1.1 Concentrated nitric acid, HNO_3. Acid should be analyzed to determine levles of I impurity. If the method blank is less than the MDL, the acid can be used.

5.2 Reagent Water. Reagent water shall be interference free. All references to water in the method refer to reagent water unless otherwise specified (Ref. 3).

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1 All samples must have been collected using a sampling plan that addresses the considerations discussed in Chapter Nine of this manual.

6.2 All sample containers must be prewashed with detergents, acids and water. Plastic and glass containers are both suitable. See Chapter Three, sec. 3.1.3 of this manual, for further information.

6.3 Samples must be refrigerated upon receipt and analyzed as soon as possible.

7.0 PROCEDURE

7.1 Calibration of Microwave Equipment

NOTE: If the microwave unit uses temperature feedback control capable of replicating the performance specifications of the method, then the calibration procedure may be omitted.

7.1.1 Measurement of the available power for heating is evaluated so that the absolute power in watts may be transferred from one microwave unit to another. For cavity type microwave equipment, this is accomplished by measuring the temperature rise in 1 kg of water exposed to microwave radiation for a fixed period of time. The analyst can relate power in watts to the partial power setting of the unit. The calibration format required for laboratory microwave units depend on the type of electronic system used by the manufacturer to provide partial microwave power. Few units have an accurate and precise linear relationship between percent power settings and absorbed power. Where linear circuits have been utilized, the calibration curve can be determined by a three-point calibration method (7.1.3), otherwise, the analyst must use the multiple point calibration method (7.1.2).

7.1.2 The multiple point calibration involves the measurement of absorbed power over a large range of power settings. Typically, for a 600 W unit, the following power settings are measured: 100, 99, 98 97, 95, 90, 80, 70, 60, 50, and 40 % using the procedure described in section 7.1.4. This data is clustered about the customary working power ranges. Nonlinearity has been commonly encountered at the upper end of the calibration. If the unit's electronics are known to have nonlinear deviations in any region f proportional power control, it will be necessary to make a set of measurements that bracket the power to be used. The final calibration point should be at the partial power setting that will be used in the test. This setting should be checked periodically to evaluate the integrity of the

calibration. If a significant change is detected (±10 W), then the entire calibration should be reevaluated.

7.1.3 The three-point calibration involves the measurement of absorbed power at three different power settings. Measure the power at 100% and 50% using the procedure described in section 7.1.4. From the 2-point line calculate the power setting corresponding to the required power in watts specified in the procedure. Measure the absorbed power at the partial power setting. If the measured absorbed power does not correspond to the specified power within ±10 W, use the multiple point calibration in 7.1.2. This point should also be used to periodically verify the integrity of the calibration.

7.1.4 Equilibrate a large volume of water to room temperature (23 ± 2 °C). One kg of reagent water is weighed (1,000.0 g ± 0.1 g) into a fluorocarbon beaker or a beaker made of some other material that does not significantly absorb microwave energy (glass absorbs microwave energy and is not recommended). The initial temperature of the water should be 23 ± 2 °C measured to ± 0.05 °C. The covered beaker is circulated continuously (in the normal sample path) through the microwave field for 2 minutes at the desired partial power setting with the unit's exhaust fan on maximum (as it will be during normal operation). The beaker is removed and the water vigorously stirred. Use a magnetic stirring bar inserted immediately after microwave irradiation and record the maximum temperature within the first 30 seconds to ± 0.05 °C. The covered beaker is circulated continuously (in the normal sample path) through the microwave field for 2 minutes at the desired partial power setting with the units exhaust fan on maximum (as it will be during normal operation). The beaker is removed and the water vigorously stirred. Use a magnetic stirring bar inserted immediately after microwave irradiation and record the maximum temperature within the first 30 seconds to ± 0.05 °C. Use a new sample for each additional measurement. If the water is reused both the water and the beaker must have returned to 23 ± 2 °C. Three measurements at each power setting should be made.

The absorbed power is determined by the following relationship:

Equation 1 $$P = [(K)(C_p)(m)(\Delta T)]/t$$

Where:

P = the apparent power absorbed by the sample in watts (W) (W = J/s)

K = the conversion factor for thermochemical calories/sec to watts (=4.184).

C_p = the heat capacity, thermal capacity, or specific heat (cal/g °C) of water.

m = the mass of the water sample in grams (g).

ΔT = the final temperature minus the initial temperature (°C).

t = the time in seconds (s)

Using the experimental conditions of 2 minutes and 1 kg of distilled water (heat capacity at 25 °C is 0.9997 cal/g °C) the calibration equation simplifies to:

Equation 2: $P = (\Delta T)(34.86)$

> NOTE: Stable line voltage is necessary for accurate and reproducible calibration and operation. The line voltage should be within manufacturer's specification, and during measurement and operation should not vary by more than ±2 V. A constant power supply may be necessary for microwave use if the source of the line voltage is unstable.
>
> Electronic components in most microwave units are matched to the units' function and output. When any part of the high voltage circuit, power source, or control components in the unit have been serviced or replaced, it will be necessary to recheck the units' calibration. If the power output has changed significantly (±10 W), then the entire calibration should be reevaluated.

7.2 All digestion vessels and volumetric ware must be carefully acid washed and rinsed with reagent water. When switching between high concentration samples and low concentration samples, all digestion vessels should be cleaned by leaching with hot (1:1) hydrochloric acid (greater than 80 °C, but less than boiling) for a minimum of two hours followed with hot (1:1) nitric acid (greater than 80 °C, but less than boiling) for a minimum of two hours and rinsed with reagent water and dried in a clean environment. This cleaning procedure should also be used whenever the prior use of the digestion vessels is unknown or cross contamination from vessels is suspected. Polymeric or glass volumetric ware and storage containers should be cleaned by leaching with more dilute acids (approximately 10% v/v) appropriate for the specific plastics used and then rinsed with reagent water and dried in a clean environment. To avoid precipitation of silver, ensure that all HCl has been rinsed from the vessels.

7.3 Sample Digestion

7.3.1 Weigh the fluorocarbon (PFA or TFM) digestion vessel, valve and cap assembly to 0.001 g prior to use.

7.3.2 Weigh a well-mixed sample to the nearest 0.001 g into the fluorocarbon sample vessel equipped with a single-ported cap and a pressure relief

valve. For soils, sediments, and sludges use no more than 0.500 g. For oils use no more than 0.250 g.

7.3.3 Add 10 ± 0.01 mL concentrated nitric acid in a fume hod. If a vigorous reaction occurs, allow the reaction to stop before capping the vessel. Cap the vessel and torque the cap to 12 ft-lbs (16 N-m) or according to the unit manufacturer's directions. Weigh the vessels to the nearest 0.001 g. Place the vessels in the microwave carousel.

CAUTION: Toxic nitrogen oxide fumes may be evolved, therefore all work must be performed in a properly operating ventilation system. The analyst should also be aware of the potential for a vigorous reaction. If a vigorous reaction occurs, allow to cool. Before capping the vessel.

CAUTION: When digesting samples containing volatile or easily oxidized organic compounds, initially weigh no more than 0.10 g and observe the reaction before capping the vessel. If a vigorous reaction occurs, allow the reaction to cease before capping the vessel. If no appreciable reaction occurs, a sample weight up to 0.25 g can be used.

CAUTION: All samples known or suspected of containing more than 5-10 % organic mater4ial should be predigested in a hood for at least 15 minutes.

7.3.4 Properly place the carousel in the microwave unit according to the manufacturer's recommended specifications and, if used, connect the tubes. Any vessels containing 10 mL of nitric acid for analytical blank purposes are counted as sample vessels. When fewer than the recommended number of samples are to be digested, the remaining vessels should be filled with 10 mL of nitric acid to achieve the full complement of vessels. This provides an energy balance since the microwave power absorbed is proportional to the total mass in the cavity (Ref. 4). Irradiate each group of sample vessels for 10 minutes. The temperature of each sample should rise to 175 °C in less than 5.5 minutes and remain between 170-180 °C for the balance of the 10 minute irradiation period. The pressure should peak at less than 6 atm. For most soil, sludge, and sediment samples (Ref. 5). The pressure will exceed these limits in the case of high concentrations of carbonate or organic compounds. In these cases the pressure will be limited by the relief pressure of the vessel to 7.5 ± 0.7 atm (110 ± 10 psi). All vessels should be sealed according to the manufacturers recommended specifications.

7.3.4.1 Newer microwave units are capable of higher power (W) that permits digestion of a larger number

of samples per batch. If the analyst wishes to digest more samples at a time, the analyst may use different values of power as long as they result in the same time and temperature conditions defined in 7.3.4. That is, any sequence of power that brings the samples to 175 °C in 5.5 minutes and permits a slow rise to 175 - 180 °C during the remaining 4.5 minutes (Ref. 5).

Issues of safety, structural integrity (both temperature and pressure limitations), heat loss, chemical compatibility, microwave absorption of vessel material, and energy transport will be considerations made in choosing alternative vessels. If all of the considerations are me and the appropriate power settings provided to reproduce the reaction conditions defined in 7.3.4, then these alternative vessels may be used (Ref. 1,2).

7.3.5 At the end of the microwave program, allow the vessels to cool for a minimum of 5 minutes before removing them from the microwave unit. When the vessels have cooled to room temperature, weight and recorded the weight of each vessel assembly. If the weight of acid plus sample has decreased by more than 10 % from the original weight, discard the sample. Determine the reason for the weight loss. These are typically attributed to loss of vessel seal integrity, use of a digestion time longer than 10 minutes, too larege a sample, or improper heating conditions. Once the source of the loss has been corrected, prepare a new sample or set of samples for digestion beginning at 7.3.1.

7.3.6 Complete the preparation of the sample by carefully uncapping and venting each vessel in a fume hood. Transfer the sample to an acid-cleaned bottle. If the digested sample contains particulates which may clog nebulizers or interfere with injection of the sample into the instrument, the sample may be centrifuged, allowed to settle, or filtered.

7.3.6.1 Centrifugation: Centrifugation at 2,000-3,000 rpm for 10 minutes is usually sufficient to clear the supernatant.

7.6.3.2 Settling: Allow the sample to stand until the supernatant is clear. Allowing a sample to stand overnight will usually accomplish this. If it does not, centrifuge or filter the sample

7.6.3.3 Filtering: The filtering apparatus must be thoroughly cleaned and pre-rinsed with dilute (approximately 10 % v/v) nitric acid. Filter the sample through qualitative filter paper into a second acid-cleaned container.

7.3.7 Dilute the digest to a known volume ensuring that the samples and standards are matrix matched. The digest is now ready for analysis for elements of interest using the appropriate SW-846 method.

7.4 Calculations: The concentrations determined are to be reported on the basis of the actual weight of the original sample.

8.0 QUALITY CONTROL

8.1 All quality control data must be maintained and available for reference or inspection for a period of three years. This method is restricted to use by, or under supervision of, experienced analysts. Refer to the appropriate section of Chapter One for additional quality control guidance.

8.2 Duplicate samples should be processed on a routine basis. A duplicate sample is a sample brought through the whole sample preparation and analytical process. A duplicate sample should be processed with each analytical batch or every 20 samples, whichever is the greater number. A duplicate sample should be prepared for each matrix type (ie.. soil, sludge etc.).

8.3 Spiked samples or standard reference materials should be included with each group of samples processed or every 20 samples, whichever is the greater number. A spiked sample should also be included whenever a new sample matrix is being analyzed.

9.0 METHOD PERFORMANCE

9.1 Precision: Precision data for Method 3051, as determined by the statistical examination of inter-laboratory test results, is located in Tables 1 and 2.

9.2 Repeatability: If successive results are obtained by the same analyst with the same apparatus under constant operating conditions on identical test material, then the difference between the average result for each analyst will not, with 95 % probability, exceed the repeatability value. For example, in the case of lead, an average on only 1 case in 20 would exceed

$$0.206\ x$$

in the long run, where x is one result in μg/g (Ref. 6).

9.3 Reproducibility: If two successive measurements are made independently by each of two different analysts working in different laboratories on identical test material, then the difference between the average result for each analysts will not, with 95 %

probability exceed the reproducibility value. For example, in the case of lead, an average of only 1 case in 20 would exceed

$$0.303\ x$$

in the long run, where x is the average of two successive measurements in μg/g (Ref. 2).

As can be seen in Table 1, repeatability and reproducibility differ between elements, and usually depend on that element's concentration. Table 2 provides an example of how users of the method can determine expected values for repeatability and reproducibility: nominal values of lead have been used for this model (Ref. 6)

9.4 Bias: In the case of SRM 1085 - wear metals in oil, the bias of this test method is different for each element. An estimate of bias, as shown in Table 3, is:

$$\text{Bias} = \text{Amount Found} - \text{Amount Expected}$$

However, the bias estimate inherits both the uncertainty in the measurements made using Method 3051 and the uncertainty on the certificate. So whether the bias is real or only due to measurement error must also be considered. The concentrations found for Al, Cr. And Cu using Method 3051 fall within their certified ranges on SRM 1085, and 95 % percent confidence intervals for Fe and Ni overlap with their respective certified ranges: therefore, the observed biases for these elements are probably due to chance and should be considered insignificant. Biases should not be estimated at all for Ag and Pb because these elements were not certified. Therefore, the only two elements considered in this table for which the bias estimates are significant are Mg and Mo.

10.0 REFERENCES

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Appendix B

MICROWAVE APPLICATION NOTE FOR ACID DIGESTION

Sample Type: Citrus Leaves (NBS SRM 1572)
Wheat Flour (NBS SRM 1567)
Rice Flour (NBS SRM 1568)
Tomato Leaves (NBS SRM 1573)
Pine Needles (NBS SRM 1575)

Summary:

This method provides for the acid dissolution of citrus leaves, wheat flour, rice flour, tomato leaves and pine needles in a closed Teflon PFA vessel using microwave heating for analysis by spectroscopic or wet chemical methods.

Required Equipment:

MDS-81D Microwave Instrument, Teflon PFA Vessels (120 mL size) with pressure relief valve, Digestion Turntable, Capping Station.

Reagents:

Hydrochloric acid (37 %) and nitric acid (70 %).

Method:

1. Transfer 0.5 g of powdered sample into a vessel. Add 10 Ml of nitric acid and allow each vessel to stand for 30 minutes. Add 5 mL of hydrochloric acid. Place a safety valve and cap on the vessel and then tighten cap using the Capping Station. Place the vessel in the turntable and attach a venting tube.

2. Repeat step 1 until the turntable contains 12 vessels.

3. Turn the MDS-81D exhaust on to the maximum fan speed. Activate the turntable so that it is rotating.

4. Program the instrument for 4 minutes time and 100 % power in program 1, and 8 minutes time and 50 % power in program 2. Depress the START key and allow the sample mixtures to heat.

5. Allow the sample solutions to cool to room temperature and manually vent each vessel. Open the vessels, filter if necessary, and transfer to appropriate containers.

NOTE: This procedure is a reference starting point for sample digestion using the MDS-81D and may need to be modified or changed to obtain the required results on your sample.

CAUTION: Manual venting of CEM closed vessels should only be performed when the vessel contents are at or below room temperature to avoid the potential for chemical burns. When venting vessels, it is recommended that hand, eye and body protection be worn.

label	mass (g)	concentration (ppm)								
food		P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A-1,1	0.3740	6.956	5.217	0.091	0.016	36.718	0.378	1.787	11.082	0.428
A-1,2	0.3286	6.430	4.338	0.080	0.022	32.215	0.340	1.584	9.931	0.353
A-1,3	0.3502	7.182	5.153	0.099	0.104	33.949	0.543	1.568	9.496	0.375
B-2,1	0.3536	8.767	4.600	0.127	0.037	38.535	0.418	1.735	13.762	0.629
B-2,2	0.3675	12.208	5.501	0.182	0.263	39.737	0.369	1.822	13.968	0.665
B-2,3	0.3310	9.854	4.549	0.147	0.141	35.752	0.577	1.553	12.232	0.514
C-3,1	0.3383	7.916	5.221	0.175	0.207	34.239	0.507	2.508	9.862	0.984
C-3,2	0.3604	9.184	5.910	0.200	0.270	35.953	0.636	2.743	9.954	0.683
C-3,3	0.3536	8.424	5.433	0.186	0.241	35.819	0.595	2.692	10.036	0.831

label	mass (g)	concentration (ppm)								
residuals		P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A- 10,1	0.3689	6.301	4.866	0.115	0.022	37.637	0.399	1.813	11.996	0.532
A-10,2	0.3717	6.295	4.933	0.112	0.042	37.243	0.451	1.907	12.448	0.454
A-10,3	0.4058	6.561	5.358	0.123	0.013	40.964	0.730	2.271	13.538	0.586
B-11,1	0.4392				0.000	52.439	0.499	2.173	17.654	0.595
B-11,2	0.4463	11.763	5.242	0.192	0.006	53.623	0.494	2.221	17.739	0.796
B-11,3	0.4688	12.404	5.484	0.204	0.000	56.188	0.787	2.533	18.376	0.555
C-12,1	0.4197	7.841	5.300	0.209	0.032	46.716	0.718	2.536	12.535	0.908
C-12,2	0.4401	8.355	5.484	0.220	0.023	49.253	0.734	3.688	12.919	1.425
C-12,3	0.4452	8.533	5.512	0.022	0.022	49.281	0.875	3.877	13.259	1.602

label	mass (g)	concentration (ppm)								
feces		P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A-20,1	0.4510	7.443	6.198	0.120	0.075	53.172	0.873	2.833	20.043	1.227
A-20,2	0.4232	6.840	5.937	0.115	0.065	50.550	0.814	2.669	19.422	1.434
A-20,3	0.4236	6.699	5.792	0.110	0.060	50.381	0.793	2.683	18.803	1.418
B-21,1	0.4558									
B-21,2	0.4759	14.391	4.673	0.181	0.094	69.328	0.810	2.635	24.239	1.288
B-21,3	0.4824	14.967	4.914	0.184	0.044	69.674	0.842	2.762	24.720	0.926
C-22,1	0.4224	8.240	5.455	0.271	0.086	58.749	0.988	4.778	16.750	1.607
C-22,2	0.3617	7.051	4.402	0.191	0.078	47.708	0.791	3.948	14.285	0.731
C-22,3	0.5340	10.649	6.903	0.279	0.114	71.807	1.184	5.835	20.702	1.434

label	mass (g)	concentration (ppm)								
larva		P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A-30,1	0.3290	13.291	10.523	0.225	0.948	33.814	0.029	0.014	4.224	0.05
A-30,2	0.3485	14.275	10.948	0.231	0.878	35.918	0.158	0.021	5.372	0.05
A-30,3	0.3239	12.176	9.287	0.202	0.816	32.459	0.038	0.013	4.883	0.05
B-31,1	0.2728	9.982	8.225	0.248	0.473	50.100	0.108	0.017	5.204	0.05
B-31,2	0.3465	3.000	10.475	0.298	0.510	0.173	0.359	0.030	5.122	0.05
B-31,3	0.3228	12.729	9.712	0.286	0.597	0.107	0.104	0.018	4.889	0.05
C-32,1	0.3324	12.725	10.504	0.233	0.332	30.182	0.057	0.016	5.204	0.05
C-32,2	0.3327	12.773	10.928	0.241	0.322	29.692	0.089	0.037	5.122	0.05
C-32,3	0.3279	12.300	10.446	0.229	0.369	29.115	0.044	0.018	4.889	0.05

label	mass (g)	concentration (ppm)								
food		Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A-1,1	0.3740	0.048	35.356	0.153	0.297	0.049	0.001	0.032	0.000	0.093
A-1,2	0.3286	0.039	31.461	0.134	0.210	0.047	0.001	0.030	0.004	0.082
A-1,3	0.3502	0.012	33.156	0.144	0.267	0.116				0.085
B-2,1	0.3536	0.048	54.497	0.249	0.329	0.149	0.026	0.056	0.128	0.158
B-2,2	0.3675	0.054	55.463	0.260	0.375	0.292	0.067	0.106	0.234	0.142
B-2,3	0.3310	0.034	51.057	0.232	0.278					0.162
C-3,1	0.3383	0.052	30.431	0.182	0.442	0.212	0.045	0.089	0.209	0.085
C-3,2	0.3604	0.041	32.023	0.195	0.383	0.200	0.072	0.114	0.297	0.089
C-3,3	0.3536	0.048	31.822	0.192	0.411	0.220	0.048	0.085	0.228	0.088

label	mass (g)	concentration (ppm)								
residuals		Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A- 10,1	0.3689	0.045	42.265	0.274	0.244	0.062	0.008	0.039	0.033	0.127
A-10,2	0.3717	0.059	43.025	0.279	0.307	0.048	0.007	0.032	0.005	0.129
A-10,3	0.4058	0.080	47.330	0.306	0.343	0.072	0.035	0.046	0.026	0.141
B-11,1	0.4392	0.073	72.298	0.350	0.396	0.028	0.004	0.017	0.016	0.224
B-11,2	0.4463	0.073	72.509	0.345	0.423	0.087	0.007	0.029	0.040	0.225
B-11,3	0.4688	0.079	75.756	0.368	0.499	0.087	0.035	0.058	0.019	0.236
C-12,1	0.4197	0.075	38.180	0.236	0.649	0.107	0.010	0.040	0.051	0.111
C-12,2	0.4401	0.083	38.894	0.246	0.704	0.094	0.012	0.043	0.049	0.115
C-12,3	0.4452	0.078	40.036	0.249	0.698	0.109	0.022	0.053	0.044	0.117

label	mass (g)	concentration (ppm)								
feces		Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A-20,1	0.4510	0.093	68.566	0.346	0.643	0.106	0.017	0.030	0.065	0.206
A-20,2	0.4232	0.118	66.542	0.334	0.614	0.097	0.010	0.039	0.044	0.196
A-20,3	0.4236	0.099	65.215	0.330	0.606	0.102	0.013	0.032	0.075	0.194
B-21,1	0.4558									
B-21,2	0.4759	0.048	98.720	0.493	0.718	0.119	0.020	0.050	0.082	0.296
B-21,3	0.4824	0.048	99.224	0.507	0.726	0.099	0.011	0.034	0.042	0.301
C-22,1	0.4224	0.063	55.127	0.385	0.818	0.065	0.015	0.121	0.005	0.159
C-22,2	0.3617	0.035	46.154	0.321	0.588	0.076	0.018	0.039	0.012	0.133
C-22,3	0.5340	0.012	66.264	0.486	0.910	0.117	0.021	0.048	0.033	0.200

label	mass (g)	concentration (ppm)								
larva		Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A-30,1	0.3290	0.048	12.680	0.003	0.039	0.121	0.014	0.154	0.056	0.013
A-30,2	0.3485	0.046	13.742	0.006	0.053	0.196	0.018	0.158	0.068	0.016
A-30,3	0.3239	0.052	12.419	0.003	0.051	0.131	0.016	0.138	0.072	0.013
B-31,1	0.2728	0.051	10.983	0.003	0.057	0.039	0.000	0.278	0.000	0.008
B-31,2	0.3465	0.041	14.562	0.003	0.092	0.123	0.007	0.271	0.040	0.013
B-31,3	0.3228	0.048	13.574	0.003	0.063	0.064	0.008	0.276	0.007	0.011
C-32,1	0.3324	0.050	16.311	0.004	0.076	0.081	0.001	0.122	0.000	0.015
C-32,2	0.3327	0.050	14.788	0.003	0.060	0.161	0.009	0.133	0.009	0.013
C-32,3	0.3279	0.049	16.034	0.003	0.067	0.080	0.002	0.109	0.016	0.015

label	mass (g)	concentration (ppm)								
food	normalized	P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A-1,1	0.5000	9.299	6.975	0.122	0.021	49.088	0.505	2.389	14.816	0.572
A-1,2	0.5000	9.784	6.601	0.122	0.033	49.019	0.517	2.410	15.111	0.537
A-1,3	0.5000	10.254	7.357	0.141	0.148	48.471	0.775	2.239	13.558	0.535
B-2,1	0.5000	12.397	6.505	0.180	0.052	54.490	0.591	2.453	19.460	0.889
B-2,2	0.5000	16.610	7.484	0.248	0.358	54.064	0.502	2.479	19.004	0.905
B-2,3	0.5000	14.885	6.872	0.222	0.213	54.006	0.872	2.346	18.477	0.776
C-3,1	0.5000	11.700	7.717	0.259	0.306	50.604	0.749	3.707	14.576	1.454
C-3,2	0.5000	12.741	8.199	0.277	0.375	49.879	0.882	3.805	13.810	0.948
C-3,3	0.5000	11.912	7.682	0.263	0.341	50.649	0.841	3.807	14.191	1.175

label	mass (g)	concentration (ppm)								
residuals	normalized	P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A- 10,1	0.5000	8.540	6.595	0.156	0.030	51.012	0.541	2.457	16.259	0.721
A-10,2	0.5000	8.468	6.636	0.151	0.056	50.098	0.607	2.565	16.745	0.611
A-10,3	0.5000	8.084	6.602	0.152	0.016	50.473	0.899	2.798	16.681	0.722
B-11,1	0.5000				0.000	59.698	0.568	2.474	20.098	0.677
B-11,2	0.5000	13.178	5.873	0.215	0.007	60.075	0.553	2.488	19.873	0.892
B-11,3	0.5000	13.230	5.849	0.218	0.000	59.927		2.702	19.599	0.592
C-12,1	0.5000	9.341	6.314	0.249	0.038	55.654	0.855	3.021	14.933	1.082
C-12,2	0.5000	9.492	6.230	0.250	0.026	55.957	0.834	4.190	14.677	1.619
C-12,3	0.5000	9.583	6.190	0.025	0.025	55.347	0.983	4.354	14.891	1.799

label	mass (g)	concentration (ppm)								
feces	normalized	P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A-20,1	0.5000	8.252	6.871	0.133	0.083	58.949	0.968	3.141	22.221	1.360
A-20,2	0.5000	8.081	7.014	0.136	0.077	59.724	0.962	3.153	22.947	1.694
A-20,3	0.5000	7.907	6.837	0.130	0.071	59.468	0.936	3.167	22.194	1.674
B-21,1	0.5000									
B-21,2	0.5000	15.120	4.910	0.190	0.099	72.839	0.851	2.768	25.466	1.353
B-21,3	0.5000	15.513	5.093	0.191	0.046	72.216	0.873	2.863	25.622	0.960
C-22,1	0.5000	9.754	6.457	0.321	0.102	69.542	1.170	5.656	19.827	1.902
C-22,2	0.5000	9.747	6.085	0.264	0.108	65.950	1.093	5.458	19.747	1.011
C-22,3	0.5000	9.971	6.463	0.261	0.107	67.235	1.109	5.463	19.384	1.343

label	mass (g)	concentration (ppm)								
larva	normalized	P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A-30,1	0.5000	20.199	15.992	0.342	1.441	51.389	0.044	0.021	6.419	0.076
A-30,2	0.5000	20.481	15.707	0.331	1.260	51.532	0.227	0.030	7.707	0.072
A-30,3	0.5000	18.796	14.336	0.312	1.260	50.107	0.059	0.020	7.538	0.077
B-31,1	0.5000	18.295	15.075	0.455	0.867		0.198	0.031	9.538	0.092
B-31,2	0.5000		15.115	0.430	0.736		0.518	0.043	7.391	0.072
B-31,3	0.5000	19.717	15.043	0.443	0.925		0.161	0.028	7.573	0.077
C-32,1	0.5000	19.141	15.800	0.350	0.499	45.400	0.086	0.024	7.828	0.075
C-32,2	0.5000	19.196	16.423	0.362	0.484	44.623	0.134	0.056	7.698	0.075
C-32,3	0.5000	18.756	15.929	0.349	0.563	44.396	0.067	0.027	7.455	0.076

label	mass (g)	concentration (ppm)								
food	normalized	Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A-1,1	0.5000	0.064	47.267	0.205	0.397	0.066	0.001	0.043	0.000	0.124
A-1,2	0.5000	0.059	47.871	0.204	0.320	0.072	0.002	0.046	0.006	0.125
A-1,3	0.5000	0.017	47.339	0.206					0.000	0.121
B-2,1	0.5000	0.068	77.060	0.352	0.465	0.211	0.037	0.079	0.181	0.223
B-2,2	0.5000	0.073	75.460	0.354	0.510	0.397	0.091	0.144	0.318	0.193
B-2,3	0.5000	0.051	77.125	0.350	0.420					0.245
C-3,1	0.5000	0.077	44.976	0.269	0.653	0.313	0.067	0.132	0.309	0.126
C-3,2	0.5000	0.057	44.427	0.271	0.531	0.277		0.158	0.412	0.123
C-3,3	0.5000	0.068	44.997	0.271	0.581	0.311	0.068	0.120	0.322	0.124

label	mass (g)	concentration (ppm)								
residuals	normalized	Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A- 10,1	0.5000	0.061	57.285	0.371	0.331	0.084	0.011	0.053	0.045	0.172
A-10,2	0.5000	0.079	57.876	0.375	0.413	0.065	0.009	0.043	0.007	0.174
A-10,3	0.5000	0.099	58.317	0.377	0.423	0.089		0.057	0.032	0.174
B-11,1	0.5000	0.083	82.306	0.398	0.451	0.032	0.005	0.019	0.018	0.255
B-11,2	0.5000	0.082	81.233	0.387	0.474	0.097	0.008	0.032	0.045	0.252
B-11,3	0.5000	0.084	80.798	0.392	0.532	0.093	0.037	0.062	0.020	0.252
C-12,1	0.5000	0.089	45.485	0.281	0.773	0.127	0.012	0.048	0.061	0.132
C-12,2	0.5000	0.094	44.188	0.279	0.800	0.107	0.014	0.049	0.056	0.131
C-12,3	0.5000	0.088	44.964	0.280	0.784	0.122	0.025	0.060	0.049	0.131

label	mass (g)	concentration (ppm)								
feces	normalized	Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A-20,1	0.5000	0.103	76.016	0.384	0.713	0.118	0.019	0.033	0.072	0.228
A-20,2	0.5000	0.139	78.618	0.395	0.725	0.115	0.012	0.046	0.052	0.232
A-20,3	0.5000	0.117	76.977	0.390	0.715	0.120	0.015	0.038	0.089	0.229
B-21,1	0.5000									
B-21,2	0.5000	0.050	103.719	0.518	0.754	0.125	0.021	0.053	0.086	0.311
B-21,3	0.5000	0.050	102.844	0.525	0.752	0.103	0.011	0.035	0.044	0.312
C-22,1	0.5000	0.075	65.254	0.456	0.968	0.077	0.018	0.143	0.006	0.188
C-22,2	0.5000	0.048	63.801	0.444	0.813	0.105	0.025	0.054	0.017	0.184
C-22,3	0.5000	0.011	62.045	0.455	0.852	0.110	0.020	0.045	0.031	0.187

label	mass (g)	concentration (ppm)								
larva	normalized	Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A-30,1	0.5000	0.073	19.271	0.005	0.059	0.184	0.021	0.234	0.085	0.020
A-30,2	0.5000	0.066	19.716	0.009	0.076	0.281	0.026	0.227	0.098	0.023
A-30,3	0.5000	0.080	19.171	0.005	0.079	0.202	0.025	0.213	0.111	0.020
B-31,1	0.5000	0.093	20.130	0.005	0.104	0.071	0.000	0.510	0.000	0.015
B-31,2	0.5000	0.059	21.013	0.004	0.133	0.177	0.010	0.391	0.058	0.019
B-31,3	0.5000	0.074	21.025	0.005	0.098	0.099	0.012	0.428	0.011	0.017
C-32,1	0.5000	0.075	24.535	0.006	0.114	0.122	0.002	0.184	0.000	0.023
C-32,2	0.5000	0.075	22.224	0.005	0.090	0.242	0.014	0.200	0.014	0.020
C-32,3	0.5000	0.075	24.450	0.005	0.102	0.122	0.003	0.166	0.024	0.023

average normalized concentration (ppm) with standard deviation (S.D.)

sample no.	Ti	S.D.	Ca	S.D.	Ba	S.D.	Al	S.D.	As	S.D.
A1	0.047	0.026	47.493	0.330	0.205	0.001	0.358	0.055	0.069	0.004
B2	0.064	0.011	76.548	0.943	0.352	0.002	0.465	0.045	0.304	0.132
C3	0.067	0.010	44.800	0.323	0.270	0.001	0.589	0.061	0.301	0.020
A10	0.080	0.019	57.826	0.518	0.375	0.003	0.389	0.051	0.079	0.013
B11	0.083	0.002	81.016	0.308	0.390	0.004	0.486	0.042	0.074	0.037
C12	0.090	0.003	44.879	0.653	0.280	0.001	0.786	0.013	0.119	0.011
A20	0.120	0.018	77.203	1.316	0.389	0.006	0.718	0.007	0.118	0.003
B21	0.050	0.000	103.282	0.619	0.522	0.005	0.753	0.001	0.114	0.016
C22	0.045	0.032	63.700	1.607	0.452	0.007	0.878	0.081	0.097	0.018
A30	0.073	0.007	19.386	0.290	0.006	0.002	0.071	0.011	0.222	0.052
B31	0.084	0.014	20.723	0.513	0.005	0.001	0.112	0.019	0.116	0.055
C32	0.075	0.000	23.736	1.310	0.005	0.001	0.102	0.012	0.162	0.069

average normalized concentration (ppm) with standard deviation (S.D.)

sample no.	Cr	S.D.	Cu	S.D.	Se	S.D.	Sr	S.D.
A1	0.001	0.000	0.044	0.002	0.002	0.004	0.123	0.002
B2	0.064	0.038	0.112	0.046	0.250	0.097	0.220	0.026
C3	0.067	0.001	0.137	0.019	0.348	0.056	0.125	0.001
A10	0.010	0.001	0.051	0.007	0.028	0.019	0.173	0.001
B11	0.017	0.018	0.038	0.022	0.028	0.015	0.253	0.002
C12	0.017	0.007	0.052	0.007	0.055	0.006	0.131	0.001
A20	0.015	0.004	0.039	0.007	0.071	0.018	0.230	0.002
B21	0.016	0.007	0.044	0.012	0.065	0.030	0.311	0.001
C22	0.021	0.004	0.081	0.054	0.018	0.013	0.186	0.002
A30	0.024	0.002	0.225	0.011	0.098	0.013	0.021	0.002
B31	0.007	0.007	0.443	0.061	0.023	0.031	0.017	0.002
C32	0.006	0.007	0.183	0.017	0.013	0.012	0.022	0.002

average normalized concentration (ppm) with standard deviation (S.D.)

sample no.	P	S.D.	S	S.D.	Zn	S.D.	Pb	S.D.
A1	9.779	0.477	6.978	0.378	0.128	0.011	0.068	0.070
B2	14.630	2.118	6.953	0.495	0.216	0.034	0.208	0.153
C3	12.118	0.551	7.866	0.289	0.266	0.010	0.340	0.034
A10	8.364	0.245	6.611	0.022	0.153	0.003	0.034	0.021
B11	13.204	0.036	5.861	0.017	0.216	0.002	0.002	0.004
C12	9.472	0.122	6.245	0.063	0.175	0.130	0.030	0.007
A20	8.080	0.172	6.907	0.094	0.133	0.003	0.077	0.006
B21	15.316	0.278	5.001	0.130	0.190	0.000	0.072	0.038
C22	9.824	0.127	6.335	0.217	0.282	0.034	0.105	0.003
A30	19.825	0.902	15.345	0.885	0.328	0.015	1.320	0.105
B31	19.006	1.005	15.078	0.036	0.443	0.012	0.843	0.097
C32	19.031	0.240	16.051	0.329	0.354	0.007	0.515	0.042

average normalized concentration (ppm) with standard deviation (S.D.)

sample no.	K	S.D.	Fe	S.D.	Mn	S.D.	Mg	S.D.	Si	S.D.
A1	48.859	0.338	0.599	0.152	2.346	0.094	14.495	0.825	0.548	0.021
B2	54.187	0.264	0.655	0.193	2.426	0.071	18.980	0.492	0.857	0.070
C3	50.378	0.432	0.824	0.068	3.773	0.057	14.192	0.383	1.192	0.254
A10	50.528	0.460	0.682	0.191	2.607	0.174	16.561	0.264	0.685	0.064
B11	59.900	0.190	0.561	0.010	2.555	0.128	19.857	0.250	0.720	0.154
C12	55.653	0.305	0.891	0.080	3.855	0.727	14.834	0.137	1.500	0.373
A20	59.380	0.395	0.955	0.017	3.154	0.013	22.454	0.427	1.576	0.187
B21	72.527	0.440	0.862	0.015	2.816	0.067	25.544	0.110	1.157	0.278
C22	67.576	1.820	1.124	0.040	5.526	0.113	19.653	0.236	1.418	0.451
A30	51.009	0.785	0.110	0.101	0.024	0.005	7.222	0.700	0.075	0.003
B31			0.292	0.196	0.034	0.008	8.167	1.191	0.080	0.010
C32	44.806	0.527	0.096	0.034	0.036	0.017	7.660	0.189	0.076	0.001

sample no.		average normalized concentration (ppm) multiplied by Schroeder's value									
food	value		Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A1		1.07	0.050	50.817	0.219	0.383	0.073	0.002	0.047	0.002	0.132
B2		1.07	0.069	81.907	0.377	0.498	0.325	0.068	0.120	0.267	0.236
C3		1.07	0.072	47.936	0.289	0.630	0.322	0.072	0.146	0.372	0.133
residuals											
A10		1.07	0.085	61.874	0.401	0.416	0.085	0.011	0.054	0.030	0.185
B11		1.07	0.089	86.687	0.417	0.520	0.079	0.018	0.041	0.030	0.271
C12		1.07	0.097	48.020	0.300	0.841	0.127	0.018	0.056	0.059	0.141
feces											
A20		0.82	0.098	63.307	0.319	0.589	0.096	0.013	0.032	0.058	0.188
B21		0.82	0.041	84.691	0.428	0.618	0.093	0.013	0.036	0.053	0.255
C22		0.82	0.037	52.234	0.370	0.720	0.080	0.017	0.066	0.015	0.153
larva											
A30		0.16	0.012	3.102	0.001	0.011	0.036	0.004	0.036	0.016	0.003
B31		0.16	0.013	3.316	0.001	0.018	0.019	0.001	0.071	0.004	0.003
C32		0.16	0.012	3.798	0.001	0.016	0.026	0.001	0.029	0.002	0.003

sample no.		average normalized concentration (ppm) multiplied by Schroeder's value									
food	value		P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A1		1.07	10.464	7.466	0.137	0.073	52.279	0.641	2.510	15.509	0.587
B2		1.07	15.655	7.440	0.232	0.222	57.980	0.701	2.596	20.309	0.917
C3		1.07	12.966	8.417	0.285	0.364	53.904	0.882	4.037	15.186	1.276
residuals											
A10		1.07	8.950	7.074	0.163	0.036	54.065	0.730	2.789	17.721	0.733
B11		1.07	14.128	6.271	0.231	0.002	64.093	0.600	2.733	21.247	0.771
C12		1.07	10.135	6.682	0.187	0.032	59.548	0.953	4.125	15.872	1.605
feces											
A20		0.82	6.626	5.664	0.109	0.063	48.692	0.783	2.586	18.412	1.292
B21		0.82	12.559	4.101	0.156	0.059	59.472	0.707	2.309	20.946	0.948
C22		0.82	8.056	5.195	0.231	0.086	55.412	0.922	4.531	16.115	1.163
larva											
A30		0.16	3.172	2.455	0.053	0.211	8.161	0.018	0.004	1.155	0.012
B31		0.16	3.041	2.412	0.071	0.135		0.047	0.005	1.307	0.013
C32		0.16	3.045	2.568	0.057	0.082	7.169	0.015	0.006	1.226	0.012

budgets for each bug

specimen	Ti	Ca	Ba	Al	As	Cr	Cu	Se	Sr
A	2.19	1.31	1.46	1.57	1.80	10.73	1.44	33.98	1.45
B	0.79	1.07	1.14	1.28	0.34	0.21	0.89	0.21	1.09
C	0.68	1.17	1.28	1.17	0.33	0.25	0.65	0.04	1.17

specimen	P	S	Zn	Pb	K	Fe	Mn	Mg	Si
A	0.94	1.09	1.18	3.78	1.09	1.25	1.03	1.26	2.22
B	1.00	0.88	0.98	0.87		1.08	0.89	1.10	1.05
C	0.86	0.92	1.01	0.46	1.16	1.06	1.12	1.14	0.92

	Ti	S.D.	Ca	S.D.	Ba	S.D.	Al	S.D.	As	S.D.
	1.22	0.84	1.18	0.12	1.29	0.16	1.34	0.20	0.82	0.85
after q-test									0.34	0.011

	Cr	S.D.	Cu	S.D.	Se	S.D.	Sr	S.D.
	3.73	6.06	0.99	0.40	11.41	19.55	1.24	0.19
after q-test	0.23	0.03			0.13	0.12		

	P	S.D.	S	S.D.	Zn	S.D.	Pb	S.D.
	0.93	0.07	0.96	0.11	1.06	0.11	1.71	1.81

Appendix C

K	S.D.	Fe	S.D.	Mn	S.D.	Mg	S.D.	Si	S.D.
1.12	0.05	1.13	0.10	1.02	0.12	1.17	0.09	1.40	0.72

The following values were not considered in calculations, due to the Q-test:

11,1 S 0.0330 Q=0.9959 @99%n=3
11,1 Zn 0.0114 Q=0.98788 @ 96%n=3
11,3 Fe 0.8394 Q=0.9486 @ 90%n=3
31,2 P 4.329004 Q=0.864@99% n=9