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SELENIUM AND TRACE METALS AS POLLUTANTS

by

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B.Sc., M.Sc.

A Thesis submitted in partial fulfilment of the requirements for the degree of DOCTOR of PHILOSOPHY in the Department of Chemistry at the

UNIVERSITY OF BRISTOL

July, 1987

بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيْمِ

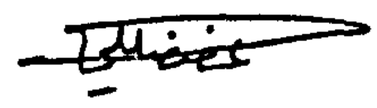
In the Name of Allāh, the All-merciful,

the All-compassionate

MEMORANDUM

The research described in this dissertation was carried out in the Department of Inorganic Chemistry at the University of Bristol, under the supervision of Dr. Graham Nickless, between February 1984 and September 1986. It was the independent work of the author and has not been described in any other dissertation except where otherwise stated.

Adly Fadel AL-ATTAR.



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To my:

FATHER	Fadel
MOTHER	Maryam
WIFE	Karama
SON	Khaled
DAUGHTER	Eman
SON	Muhammad

ABSTRACT

The dissertation is particularly concerned with the development, application and utilisation of an analytical chemistry method for the determination of selenium when present in a number of matrices. Since selenium is an important trace element which may act to ameliorate the effects of certain heavy metals such as mercury, the potential for such work was studied by observing the interaction of certain heavy metals plus selenium on plant growth.

The General Introduction is a survey emphasising the occurrence of selenium and tellurium in the environment, their biochemistry including toxicity and chemical forms as well as the uptake of these metalloids by plants. A section is also devoted to the possible interaction of other metals with selenium.

The second chapter is a review concerned with a series of comments concerning the suitability of a number of instrumental methods for the determination of trace metals and metalloids. The techniques covered include GLC with ECD, FAAS, GFAAS, CVAAS and DPASV with HMDE.

The next section of the dissertation is concerned with the Experimental work which was carried out in the laboratory. Here the dissertation consists of four major chapters entitled:-

1) 1,2-Diaminobenzene derivatives as reagents for determination of selenium by GLC;
2) Selenium determination;
3) Cadmium, mercury, thallium and tellurium determination and effects in plants;
4) Selenium and its interactions (via Response Surface Methodology).

Thus, in Chapter 3, selenium was reacted with 1,2-diaminobenzene

derivatives to form "piazselenols" and determined by GLC + ECD. The most sensitive reagent reported, namely 3,5-dibromo-1,2-diaminobenzene was synthesised and compared with commercially available reagents. An extremely detailed and lengthy series of syntheses were then carried out in order to prepare a number of new 1,2-diaminobenzenes, containing fluorine or the trifluoromethyl group. These reagents were characterised in terms of purity and spectroscopic properties and compared to those ligands already proposed. Fortunately these new reagents were very suitable for the determination of selenium as well as being extremely sensitive. Thus 3-bromo-5-trifluoromethyl-1,2-diaminobenzene affords a detection limit of a few picograms of selenium and was applied to the determination of selenium in a number of water and plant samples. In order to effect a suitable comparison and test, the selenium content of the plant tissue was determined by GFAAS and the results compared statistically with those obtained by GLC + ECD.

Water samples drawn from the River Avon were found to contain between 15-828 ng/L of Se(IV) with 52-2958 ng/L as total Se using the GLC + ECD system. The high levels were found in the section of river which was expected to be contaminated, with the very low values arising in the non-polluted sections, the much higher levels of total Se being a result of the conversion of Se(IV) to Se(VI) through the oxidising nature of the river system. The same water samples were used to estimate Cd, Cu, Pb and Zn with GFAAS and DPASV. Generally, the water samples were contaminated with Cu and Zn.

In order to prepare for an investigation of the effect of heavy metals with selenium on plant growth, methods for the determination of mercury and thallium were examined. Mercury in such plant samples was determined by CVAAS and GFAAS. The CVAAS technique was found to

be sensitive, faster and with high precision.

Similarly, in order to determine thallium in plant tissue, investigations were made of the techniques of GFAAS and DPASV. In particular the Tl content of some River Avon sediments was measured by DPASV. In order to complete the battery of techniques available Cd and Te in such plant tissue were determined by GFAAS.

The toxicity of Cd, Hg, Se, Te and Tl to Lolium perenne seedlings was investigated by means of their critical levels. The toxicity of these elements decreases in the following order:-

Hg > Cd > Tl > Te > Se (in shoots)

Hg > Tl > Te > Cd > Se (in roots).

The investigation into the interaction (if any) between toxic element uptake (Se, Cd, Hg, Tl and Te) by Lolium perenne seedlings was initiated. The data were fitted to second-order polynomials and analysed by means of the SAS and Minitab software packages. Contour and 3-D plots were used to display the surfaces produced and statistical regression tables were discussed.

Very detailed discussions of the results obtained are presented, especially of Experiment 2 (Se, Te, Tl), some indicating only additive effects of toxic elements, while others suggest a more involved system of responses. Also the uptake of some essential metals such as Cu, Fe, Mn and Zn in response to and possible interaction with the toxic element in the nutrient solution was studied and discussed in detail.

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
ASV	Anodic Stripping Voltammetry
BDH	British Drug Houses
conc.	concentrated
DC	Direct Current
DCP	Direct Current Polarography
DDW	double distilled water
DPASV	Differential Pulse Anodic Stripping Volammetry
DPP	Differential Pulse Polarography
$E_{\frac{1}{2}}$	Half wave potential
ECD	Electron Capture Detector
EDTA Na_2	Ethylene diamine tetra acetic acid disodium
FAAS	Flame Atomic Absorption Spectrometry
GCE	Glassy Carbon Electrode
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
GLC	Gas Liquid Chromatography
GTA	Graphite Tube Atomiser
HMDE	Hanging Mercury Drop Electrode
i.d.	internal diameter
i_d	Diffusion Current
ILL	Instrumental Laboratories Limited
IR	Infrared
MDE	Mercury Drop Electrode
M.wt.	Molecular weight
NBS	National Bureau of Standards
N.D.	Not Detected

NPP	Normal Pulse Polarography
PARC	Princeton Applied Research Corporation
pH	Activity of Hydrogen Ion ($-\log_{10} H^+$)
PIS	Piazselenol
PP	Pulse Polarography
RGCE	Rotating Glassy Carbon Electrode
RSM	Response Surface Method
SCE	Standard Calomel Electrode
SRM	Standard Reference Material
TFME	Thin Film Mercury Electrode
v/v	volume/volume (ratio)
vs.	versus
w/w	weight/weight (ratio)

UNITS

cm	centimetre	
L	litre	
ml	millilitre	
ul	microlitre	
g	gram	
mg	milligram	= 10^{-3} g
ug	microgram	= 10^{-6} g
ng	nanogram	= 10^{-9} g
pg	picogram	= 10^{-12} g
h	hour	
min	minute	
s	second	
mA	milliampere	
uA	microampere	
mV	millivolt	
ml/L	millilitre/litre	
ug/g	microgram/gram	
M	molar concentration	
°C	degrees Celsius	
%	percentage	
ppm	parts per million	
ppb	parts per billion	
rpm	revolutions per minute	
g/L	gram/litre	
ug/ml	microgram/millilitre	
ng/ml	nanogram/millilitre	

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

CONTENTS

1:1 ENVIRONMENTAL OCCURRENCE OF SELENIUM AND TELLURIUM

1:1:1 Chemistry of Elements

i) History

Selenium was discovered in 1817 by Jöns Jacob Berzelius, a Swedish chemist (1), whilst searching for tellurium in flue dust taken from lead chambers at a sulphuric acid plant. Berzelius noticed that the reddish colour sludge in these chambers became malodorous (a very strong odour of radishes) upon heating, and eventually isolated the cause, selenium (2). He was also the first to determine the atomic weight of selenium (3), and chose the name from the Greek word for the Moon, Selene, by analogy with tellurium from the Latin tellus, meaning Earth (1,4).

Tellurium was discovered first in 1782 by the Hungarian scientist, Franz Joseph Müller von Reichenstein (1). Until almost the end of the eighteenth century, the tellurides contained in the gold ores of Transylvania were thought to be compounds of antimony and bismuth (5). The discovery was confirmed by Martin Heinrich Klaproth (the leading analytical chemist of Germany) in 1798; he was able to separate this element, determine some of its properties, and called it tellurium (5,6). He mentioned that the original discoverer was von Reichenstein (1,4).

ii) Selenium and Tellurium in the Periodic Table

Selenium and tellurium are in Group VI-A of Mendeleev's Periodic table. They are positioned between sulfur, a typical metalloid, and polonium, a typical metal. Selenium is placed between arsenic and bromine in Period 4 and tellurium is between antimony

and iodine in Period 5 of the Periodic table. In its chemical properties and in the conditions under which it occurs in nature selenium resembles tellurium in many respects. Investigations, mainly on the application of semiconductors in new technical fields, have given much information on the nature of the similarity between selenium and tellurium, but have also revealed considerable differences in the properties of these elements (4,7). The electronic configurations in the atoms of the elements are:

$$Se = 1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 3d^{10}\ 4s^2\ 4p^4$$

$$Te = 1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 3d^{10}\ 4s^2\ 4p^6\ 4d^{10}\ 5s^2\ 5p^4$$

Thus, all the valence electrons in unexcited selenium and tellurium atoms are positioned in the s- and p-orbitals (5).

iii) Valence states

Because of the existence of six electrons in the outer atomic shell of selenium and tellurium, they are classed as metalloids. By the addition of two electrons, atoms of Se and Te turn into negative bivalent ions; in these forms Se and Te enter into compounds with metals to form selenides and tellurides which are similar to sulphides. In compounds with hydrogen also, Se and Te are negative-bivalent (5,7). The Se^{2-} and Te^{2-} ions are strong reducing agents. On oxidation the ions are converted first to the elements; while further oxidation leads to the formation of tetravalent Se and Te. For tetravalent sulfur compounds the reducing properties are more characteristic than the oxidizing properties, but it is just the opposite for Se(IV) and Te(IV) compounds; they are readily reduced to elemental selenium and tellurium. Tetravalent Se(IV) and Te(IV) may be converted into the hexavalent state by strong oxidizing agents only, but in turn

the Se(VI) and Te(VI) compounds are strong oxidizing agents, especially in acid media (7). Thus Se(VI) and Te(VI) are reduced to Se(IV) and Te(IV) by heating with hydrochloric acid at 100°C. Restrictions on acid concentration and temperature are necessary to avoid any loss of selenium or tellurium as volatile chlorides (8).

iv) The Isotope Composition of Selenium and Tellurium

There are six natural stable isotopes of selenium which exist under normal conditions, with atomic masses:

Se^{74}, Se^{76}, Se^{77}, Se^{78}, Se^{80} and Se^{82};

and more than ten unstable radioactive isotopes have been prepared. Table 1:1 lists the main stable and unstable selenium isotopes (15).

The isotope often used in medical and biological trace studies is Se^{75}, usually in the form of Se^{75} selenomethionine (2).

Tellurium offers a good example of the well-known rule, that elements with even atomic numbers have a larger number of isotopes than elements with odd atomic numbers. The neighbouring odd atomic number elements, antimony and iodine, have two and one isotopes, respectively. Eight stable isotopes have been established for tellurium, with mass numbers:

Te^{120}, Te^{122}, Te^{123}, Te^{124}, Te^{125}, Te^{126}, Te^{128} and Te^{130};

and eleven unstable isotopes with lifetimes from two minutes to 154 days. Table 1:2 gives the isotopes of tellurium, their abundance, type of transformation, and half-life (6).

Table 1:1. Radioactive and Stable Isotopes of Selenium (15,20)

Mass No.	Abundance, half-life	Particle energy, mev.
72	K, 8.5 days	-
73	β^+,K,γ, 7 hours	1.3
74	0.9%	-
75	K,γ, 127 days	-
76	9.1%	-
77	I.T.*, 17.5 sec	γ = 0.16
77	7.5%	-
78	23.6%	-
79	I.T.*, 3.9 min	γ = 0.096
79	β^-, 6 x 10^4 years	0.16
80	49.9%	-
81	I.T.*, 57 min	γ = 0.103
81	β^-, 17 min	1.38
82	9.0%	-
83	β^-, 67 sec	3.4
83	β^-, γ, 25 min	1.5

* I.T. - isomeric transition

Table 1:2. Isotopes of Tellurium (6)

5

Isotope	Abundance, %	Type of transformation	Half-life
Te^{117}	-	β^+	2.5 hr
Te^{118}	-	Electron capture	6 days
Te^{119}	-	Same	4.7 days
Te^{120}	0.089	Stable	-
Te^{121}	-	Electron capture	17 days
Te^{121}	-	Isomeric transition	154 days
Te^{122}	2.46	Stable	-
Te^{123}	0.57	Stable	10^{13}-10^{16} yr
Te^{123}	-	Isomeric transition	121 days
Te^{124}	4.61	Stable	-
Te^{125}	6.99	Stable	-
Te^{125}	-	Isomeric transition	58 days
Te^{126}	18.71	Stable	-
Te^{127}	-	β^-	9.3 hr
Te^{127}	-	Isomeric transition	115 days
Te^{128}	31.79	Stable	-
Te^{129}	-	β^-	72 min
Te^{129}	-	Isomeric transition	33.5 days
Te^{130}	34.49	Stable	-
Te^{131}	-	β^-	24.8 min
Te^{131}	-	Isomeric transition	30 hr
Te^{132}	-	β^-	77.7 hr
Te^{133}	-	β^-	2 min
Te^{133}	-	Isomeric transition	63 min
Te^{134}	-	β^-	44 min
Te^{135}	-	β^-	2 min

v) Properties of Selenium and Tellurium

a) Physical Properties of Selenium

The group VIA elements (sulphur, selenium and tellurium) show a great variety of allotropy. The allotropic changes in sulphur have been investigated thoroughly, but the modification of selenium has been studied less extensively (14). There are three allotropic forms of solid selenium:

1. "Metallic" hexagonal, crystalline-stable form, lustrous and silvery-grey to black in colour. The electrical conductivity, which makes grey selenium useful in photoelectrical and photochemical applications, is low in the dark but increases several hundredfold on exposure to light.
2. Crystalline red selenium exists in two monoclinic forms, which are obtained by cooling molten selenium or by evaporation of carbon disulphide extracts of amorphous red selenium. The α-Se monoclinic form is red and has a unit cell form of four Se_8 ring molecules. The β-Se monoclinic form is a deep red colour and is made up of puckered Se_8 rings.
3. Amorphous selenium can be black vitreous and is formed by rapid cooling of liquid selenium; the red amorphous and red colloidal forms are involved in reduction reactions (4,9,14). Heating and catalysts transform all the amorphous forms and both monoclinic crystalline forms to grey selenium. The solubilities of allotropic forms are indicated in Table 1:3. Liquid selenium is black in bulk and brownish red in thin films. The liquid probably contains chains and rings of variable numbers of atoms. Selenium vapour is also complex in nature. The most important species are

Se_2, Se_5, Se_6, Se_7, and Se_8. Se_2 is an important species at 900°C, whereas at 2000°C the vapour is mainly monatomic (9).

Table 1:3. Solubility of Allotropic Forms of Selenium (14)

Allotropic form	Water solubility	Other solvents
"Metallic" or grey	Insoluble	Insoluble in alcohol; slightly soluble in CS_2 (2 mg/100 ml); soluble in ether and chloroform
Crystalline monoclinic red	Insoluble	Soluble in CS_2
Amorphous	Reacts with water at 50°C forming H_2SeO_3 and hydrogen	Soluble in CS_2, methylene iodide, benzene, or quinoline

b) Chemical Properties of Selenium

The physical and chemical properties of selenium are intermediate between sulphur and tellurium. The following are the oxidation states in elemental and combined forms (4):

Oxidation State	Example
-II	Na_2Se
-I	Na_2Se_2
0	Se_8
+II	$SeCl_2$
+IV	SeO_2, K_2SeO_3
+VI	SeO_3, K_2SeO_4

The important oxidation states are -II, 0, IV and VI. As far as is known, the +II state does not occur in nature.

Selenium reacts with active metals and gains electrons to form ionic compounds containing the selenide ion Se^{2-}. Selenium forms covalent compounds with most other substances (9).

Selenium combines with metals and many nonmetals directly or hydrochemically. The selenides resemble sulphides in appearance, composition and properties. Selenium forms halides by reacting vigorously with fluorine and chlorine, and less so with interhalogen compounds and bromine; selenium does not react with iodine. It does not react with pure hydrogen fluoride or hydrogen chloride but decomposes hydrogen iodide to liberate iodine and form hydrogen selenide. Selenium combines with oxygen yielding a number of oxides, the most stable being selenium dioxide (9). Under proper conditions, selenium forms selenides with hydrogen, carbon (CSe_2), nitrogen, phosphorus and sulphur. Crystalline selenium does not react with water, even at 150°C.

Selenium remains unaffected by dilute sulphuric acid or hydrochloric acid, but dissolves in a nitric-hydrochloric acid mixture, concentrated nitric acid, or sulphuric acids (4,9,10).

Selenium is oxidized by ozone and solutions of alkali-metal dichromates, permanganates, chlorates and calcium hypochlorite. Selenium dissolves in strong alkaline solutions yielding selenides and selenites. It forms selenocyanates, MSeCN, with alkali-metal cyanides as well as many inorganic and organic derivatives of the corresponding acid, HSeCN. Selenium also dissolves in alkali-metal sulphites forming selenosulphates, M_2SSeO_3 and, because tellurium does not undergo this reaction, this method can be used to separate

the two elements. Selenium mixes in all proportions with sulphur and tellurium forming a continuous series of solid solutions and alloys (9).

Of special interest is the oxidizing and reducing action of selenium and its compounds with many organic compounds.

Chemical reactions are described in detail in references (4,8,10).

Organic reactions are reviewed in references (11,12,13).

Of particular importance for the present work is the method of selenium determination via gas-liquid chromatography (GLC) which uses the unique quantitative reaction between Se(IV) with a chosen 1,2-phenylenediamine (O-PDA) in acidic solution to form a piazselenol (see Chapter 2).

c) Physical Properties of Tellurium

Tellurium is a silvery-white substance with a metallic lustre (7). At ordinary temperatures and pressure, solid tellurium, unlike sulphur and selenium, exists in one modification only. Te crystallises in a hexagonal lattice system (16). Crystalline tellurium is made up of long spiral chains of Te- .... -Te atoms arranged in parallel. The structures of tellurium and selenium are identical, but with the difference that the Te- .... -Te chains are shorter than the selenium chains. Te is similar in structure not only to selenium but also to organic polymers (6).

Tellurium solidified from the liquid is crystalline, greyish-white with a metallic lustre resembling antimony, rather brittle, and tarnishing somewhat on exposure to air.

d) Chemical Properties of Tellurium

Tellurium resembles sulphur and selenium in chemical properties; but it is less active, and its metallic properties are markedly more pronounced (6); i.e. it is more basic, more metallic, and strongly amphoteric. The six electrons in the outer shell characterise tellurium as a metalloid.

In elemental and combined forms tellurium shows the following oxidation states (4):

Oxidation State	Example
-I	Na_2Te_2
-II	Na_2Te, H_2Te
0	Te
IV	TeO_2, $TeCl_4$
VI	TeO_3

The negative bivalent ions combine with hydrogen and some metals. When combined with oxygen, tellurium appears as positive bi-, tetra-, or hexavalent ions (6).

The most stable compounds of tellurium and selenium are the dioxides and the corresponding acids and salts, i.e. the tetravalent compounds. On the other hand, the most stable compounds of sulphur are the hexavalent sulphur trioxide, sulphuric acid, and the sulphates.

Dense tellurium resists oxidation at room temperature. When heated it burns with a greenish-bordered blue flame forming tellurium dioxide. In moist air and when powdered, tellurium is oxidized even at room temperature. Metallic tellurium reacts with water at 100-160°C, and when freshly precipitated even at 50°C (4).

$$Te + 2H_2O \longrightarrow TeO_2 + 2H_2$$

Tellurium reacts vigorously with halogens at room temperature. The halides are more stable than their selenium and sulphur analogues. When heated with numerous metals Te forms the corresponding tellurides, such as K_2Te, Na_2Te, CuTe, MgTe, Al_2Te_3 and Ag_2Te.

Tellurium mixes in all proportions with sulphur and selenium. Carbon disulphide does not dissolve tellurium. At ordinary temperature dilute hydrochloric acid dissolves Te very slowly. Tellurium dissolves in alkalis and in nitric and sulphuric acids to form the corresponding salts and acid. Tellurium reacts with concentrated (but not dilute) sulphuric acid to form sulphite:

$$Te + H_2SO_4 \rightleftharpoons TeSO_3 + H_2O$$

Dilution with water reverses the reaction.

Unlike selenium, tellurium is not soluble in aqueous sodium sulphite. This difference offers a method of separating the two elements. Elemental tellurium reduces chlorides such as $AsCl_3$, $AuCl_3$ and $PbCl_4$ to the element; $FeCl_3$ to $FeCl_2$ and SO_2Cl_2 to SO_2. Oxidation of metals by tellurium gives metallic tellurides, i.e.:

$$Te + 2Ag \longrightarrow Ag_2Te, \quad Te + Ca \longrightarrow CaTe$$

Tellurium itself is oxidized by strong reagents, e.g. $Na_2Cr_2O_7$, $KMNO_4$, $Ca(OCl_2)_2$ and $HClO_3$ to the hexavalent state Te(VI). Solutions of silver and gold salts oxidize tellurium metal to Te(IV):

$$3Te + 4AgNO_3 + 3H_2O \longrightarrow H_2TeO_3 + 2Ag_2Te + 4HNO_3$$

$$3Te + 4AuCl_3 \longrightarrow 3TeCl_4 + 4Au$$

Tellurium dioxide and tellurous acid and its salts are readily reduced to the element with $SnCl_2$, Na_3AsO_3, H_2S and $Na_2S_2O_4$ and are oxidized to the Te(VI) state with PbO_2, CrO_3, Cl_2^- and $KMnO_4$. But

selenium, H_2S and HCl reduce TeO_4^{2-} ions to TeO_3^{2-}.

Solid tellurium oxides can be reduced by heating with hydrogen, carbon, carbon monoxide, and sulphur. The stability of organic chalcogen compounds decrease mostly in the order sulphur > selenium > tellurium.

Tellurium dioxide (TeO_2) can be used as an oxidant for acetoxymethylation reactions:

$$ArH \xrightarrow[LiBr,\ 120^\circ \text{ or } 160^\circ C]{TeO_2 \cdot CH_3COOH} ArCH_2\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3 \qquad (4,6,16)$$

Organic reactions of tellurium are described in detail and have been reviewed in References (11,17,18). Organotellurium compounds are of medical and biological interest, especially using radioactive isotopes as biological tracers (see Chapter 1:1:5) in mammalian myocardiac systems.

1:1:2 Geochemistry of Selenium and Tellurium

i) Selenium

a) Occurrence

Selenium is widely distributed on the earth's crust, but occurs in small quantities in igneous rocks in quantities about the same as silver, but more than platinum (10).

Selenium is the thirtieth element in cosmic abundance, while ranking about seventieth in the order of crustal abundance (9).

The content of selenium in the earth's crust is estimated to be 1×10^{-5} to 8×10^{-5} per cent (4), the average amount in the crustal rocks being 0.05 ppm (9,19).

The primary sources of selenium are volcanic emanations and metallic sulphides associated with igneous activity.

Secondary sources are biological sinks in which the element has accumulated. The selenium contents of black shales, coal, and petroleum is 10-20 times the crustal abundance (0.05 ppm). Seleniferous black shales are the parent materials of the widespread seleniferous soils, particularly those of the western plains of the United States.

When burned, coal and petroleum containing selenium gives rise to a redistribution of particulate Se and SeO_2 (19,20). Selenium occurs in coal in concentrations of the order of 0.5 to 12 ppm (9).

b) Minerals

Native selenium is uncommon, usually occurring in conjunction with native sulphur (8). Sulphur forms mineral compounds with some 40 chemical elements, but selenium with 16 only, mostly those with high atomic numbers (4). All of these minerals are rare, the most abundant ones being selenides of lead, copper, silver, mercury, nickel (8), bismuth, iron, thallium, palladium, arsenic, zinc (4), cobalt and cadmium. Compounds with light metals do not occur in nature (7). Table 1:4 contains selenium and tellurium as essential constituents (5,7).

c) Selenium in Rocks

1- Igneous Rocks

Sindeeva reported an average of 1.4×10^{-5} per cent (0.14 ppm) selenium in Russian igneous rocks (5).

Brunfelt and Steinnes determined the selenium content in standard rocks by neutron activation analysis, and found an average ranging from 0.004 to 0.110 ppm Se, in eight U.S. Geological Survey Standard igneous rock samples (21).

2- Sedimentary Rocks

Because sedimentary rocks cover more than three-quarters of the earth's surface, they are the major parent material of agricultural soils.

The concentrations of selenium in sedimentary rocks range from 0.08 to 1.0 μg/g and such levels are higher than the estimates for the earth's crust in total.

The selenium content of sandstone varies from 0.05 to 1.0 μg/g,

Table 1:4. Selenium and Tellurium minerals (5,7)

SELENIUM MINERALS			TELLURIUM MINERALS		
Name	Formula	Content %	Name	Formula	Content %
Elemental selenium	Se	100	Elemental tellurium	Te	100
Selenotellurium	TeSe	30	Selenotellurium	TeSe	70
Hydrogen selenide	H_2Se	98	Hydrogen telluride	H_2Te	97
Paraguanajuatite	Bi_2SeS	24	Vulcanite	CuTe	66
Laitakarite	Bi_4Se_2S	16	Rickardite	Cu_7Te_5	60
Guanajuatite	Bi_2Se_3	24	Weissite	$Cu_{2-x}Te$	50
Klockmannite	CuSe	56	Melonite	$NiTe_2$	80
Berzelianite	Cu_2Se	40	Montbrayite	Au_2Te_3	62
Umangite	Cu_3Se_2	54	Calaverite	$AuTe_2$	57
Naumannite	Ag_2Se	27	Krennerite	$(Au,Ag)Te_2$	56
β -Naumannite	Ag_2Se	27	Sylvanite	$(Au,Ag)Te_4$	62
Aguilarite	Ag_4SeS	6	Muthmanite	(Au,Ag)Te	Variable
Crookesite	$(Cu,Tl,Ag)_2Se$	32	Petzite	$(Ag_3Au)Te_2$	33
Eucairite	CuAgSe	30	Hessite	Ag_2Te	62
Eskebornite	Fe_3CuSe_4	52	Empressite	$Ag_{5-x}Te_3$	55
Tyrellite	$(Cu,Co,Ni)_4Se_4$	55	Tellurobismuthite	Bi_2Te_3	48
			Wehrlite	$Bi_{2+x}Te_{3-x}$	28
Stilleite	ZnSe	54	Hedleyite	Bi_7Te_3	20
Cadmoselite	CdSe	41	Tetradymite	Bi_2Te_2S	36
Tiemannite	HgSe	28	Csiklovaite	Bi_2TeS_2	20
Clausthalite	PbSe	27	Joseite A	$Bi_{4+x}Te_{1-x}S_2$	12
Freboldite	CoSe	57	Joseite B	$Bi_{4+x}Te_{2-x}S$	20
Trogtalite	$CoSe_2$	72	Gruenlingite	Bi_4TeS	Variable
Hastite	$CoSe_2$	72	Oruetite	Bi_8TeS_4	6
Bornhardite	Co_3Se_4	62	Coloradoite	HgTe	39
Blockite	$NiSe_2$	68	Altaite	PbTe	38
Ferroselite	$FeSe_2$	73	Frohbergite	$FeTe_2$	82
Achavalite	FeSe	58	Kotulskite	$Pd(Te,Bi)_{1-2}$	44
Palladium selenite	PdSe	-	Moncheite	$(Pt,Pd)(Te,Bi)_2$	33
Weibullite	$PbBi_2(S,Se)_4$	13	Michenerite	$(Pd_{0.75}Pt_{0.25})TeBi$	29-37
Platinite	$PbBi_2(S,Se)_3$	18	Nagyagite	$Pb_5Au(TeSb)_4S_{5-8}$	18
Wittite	$Pb_5Bi_6(S,Se)_4$	8	Goldfieldite	$Cu_6Sb_2(S,Te)_3$	17
Jeromite	$As(S,Se)_2$	7.5	Arsenotellurite	$Te_2As_2S_7$	40
Selenolite	SeO_2	71	Tellurite	TeO_2	80
Kerstenite	$PbSeO_4.2H_2O$	Variable	Paratellurite	TeO_2	80
Molybdomenite	$PbSeO_3$	"	Lead tellurate	$PbTeO_4$	Variable
Chalcomenite	$CuSeO_3.2H_2O$	49	Dunhamite	$PbTeO_3$	"
Iron selenide	-	-	Montanite	$Pb_2TeO_4(OH)_4$	26
Mercury selenide	-	-	Teineite	$Cu(Te,S)O_3.2H_2O$	48
Ahlfeldite	$NiSeO_3.2H_2O$	Variable	Emmonsite	$Fe_2(TeO_3)_3.2H_2O$	70
Cobaltomenite	$CoSeO_3.nH_2O$	"	Mackayite	$Fe_2(TeO_3)_3.nH_2O$	-
			Blakeite	$Fe_2(TeO_3)_3$	Variable
			Magnolite	Hg_2TeO_4	17

while that of shales averages about 0.6 μg/g selenium. Shales are the principal sources of selenium-toxic soils.

Carbonate rock ranges from 0.0 to 2.0 μg/g Se, but some carbonaceous limestones contain as much as 30 μg/g Se (22). Selenium is often present in phosphorites in relatively high concentration, up to a level of 300 μg/g (14). White phosphate rock (North African) contains 10.6 μg/g Se (23).

Coal has abundant amounts of selenium, ranging from 0.1 to 4.0 μg/g. When the seleniferous coal or oil is burned, selenium is introduced into the atmosphere from which selenium is redistributed to the earth's surface in rain and snow. Dust from air conditioner filters has been found to contain from 0.05 to 10.00 μg/g Se.

Selenium from these sources is probably in the form of insoluble oxides or in the elemental form and may not be of immediate value to plants and animals (22).

d) Selenium in Soils

Selenium is present in soils chiefly as the basic ferric selenite ($Fe_2(OH)_4SeO_3$) and calcium selenate ($CaSeO_4$); also in minor amounts as elemental selenium or organic compounds derived from plant tissue (25).

Of the rocks exposed at the weathering subsurface that may serve as parent materials, the shales represent about 40% of the parent rocks and are the most abundant. The sandstones, limestones, and igneous rocks are about equally abundant at about 20% each. The soils most likely to be uniformly deficient in selenium are those derived from igneous rocks (22).

The selenium content of soils ranges from 0.1 ppm in a selenium

deficient area (e.g. New Zealand) (19) to 1200 ppm in an organic-rich soil of a toxic area in County Meath, Ireland. Three areas exist where vegetation is especially toxic to animals; these areas are low-lying, poorly drained and rich in organic matter (26). Areas containing more than 2 ppm Se have been located in the U.S., China and Turkestan (U.S.S.R.). In many of these areas farmers avoid cropping the selenium-toxic land or mix the crop with crops from low-selenium areas (30).

Soils of Hawaii (U.S.) that contain 6-15 ppm Se do not produce toxic seleniferous vegetation (27). In contrast, soils of South Dakota, Wyoming, Nebraska, Kansas and Colorado that contain less than 1 ppm Se do produce seleniferous toxic vegetation (19,22).

The forms and concentrations of selenium in soil solution available to plants growing in the soils are governed by the various physico-chemical factors, expressed in terms of pH, solubility products, and oxidation-reduction potentials.

Elemental selenium is a stable form in soils and is not available to plants (29).

Gissel-Nielsen and Bisbjerg added selenium to soils in the forms of Se^{o}, K_2SeO_3, K_2SeO_4 and $BaSeO_4$. During a two year study, they found that for mustard plants the total uptake as a percentage of the added material was 0.01%, 4%, 30% and 30% respectively. With lucerne, barley and sugar beet, the uptake was one-third or less of that obtained with mustard (31).

Selenium can be easily oxidized from Se^{o} (elemental selenium) to Se^{4+} (SeO_3^{2-}) and to Se^{6+} (SeO_4^{2-}). Selenites (SeO_3^{2-}) are stable in alkaline to mildly acid conditions and should be found in nature. In general, the more alkaline soil contains less selenium. Selenium

might be most readily oxidized to the more soluble selenate at pH values above 6.5. The reaction $2H_2SeO_3 + O_2 \longrightarrow 2H_2SeO_4$ takes place most easily in an alkaline environment. Alkaline soils can occur in either low-rainfall or poorly-drained areas.

Reduction of selenate to selenites or even to selenides probably takes place in the horizons which are rich in acidic organic matter (32).

Selenium is mobile under oxidizing, alkaline conditions, but immobile under reducing and neutral to acid conditions. Depending on the temperature, pressure, pH and other factors, selenium enters the soil as:

1. elemental selenium by reduction of selenites;
2. water-soluble selenites and selenates;
3. organic selenium compounds; and
4. adsorbed selenium (4).

The availability of selenium to plants is a function of the pH of the soils as well as the total selenium content of the soils. In acid soils (pH 4.5-6.5) selenium is usually bound as a basic ferric selenite of extremely low solubility which can be classified as practically unavailable to plants.

In alkaline soils (pH 7.5-8.5), selenium may be oxidized to selenate ions and become water-soluble; this form is readily available to plants (19).

Certainly the natural source of selenium for agriculture and most other biological uses is the soil, from which the element is accumulated in varying amounts by plants which are ultimately consumed by animals (22).

A fairly straightforward chain of reactions takes place, allowing the diagrammatic presentation of a selenium cycle in nature (Figure 1:1) (20,22,28,33) and Figure 1:2 shows a skeletal geochemical cycle

FIGURE 1.1 SOIL-PLANT ANIMAL CYCLE FOR SELENIUM

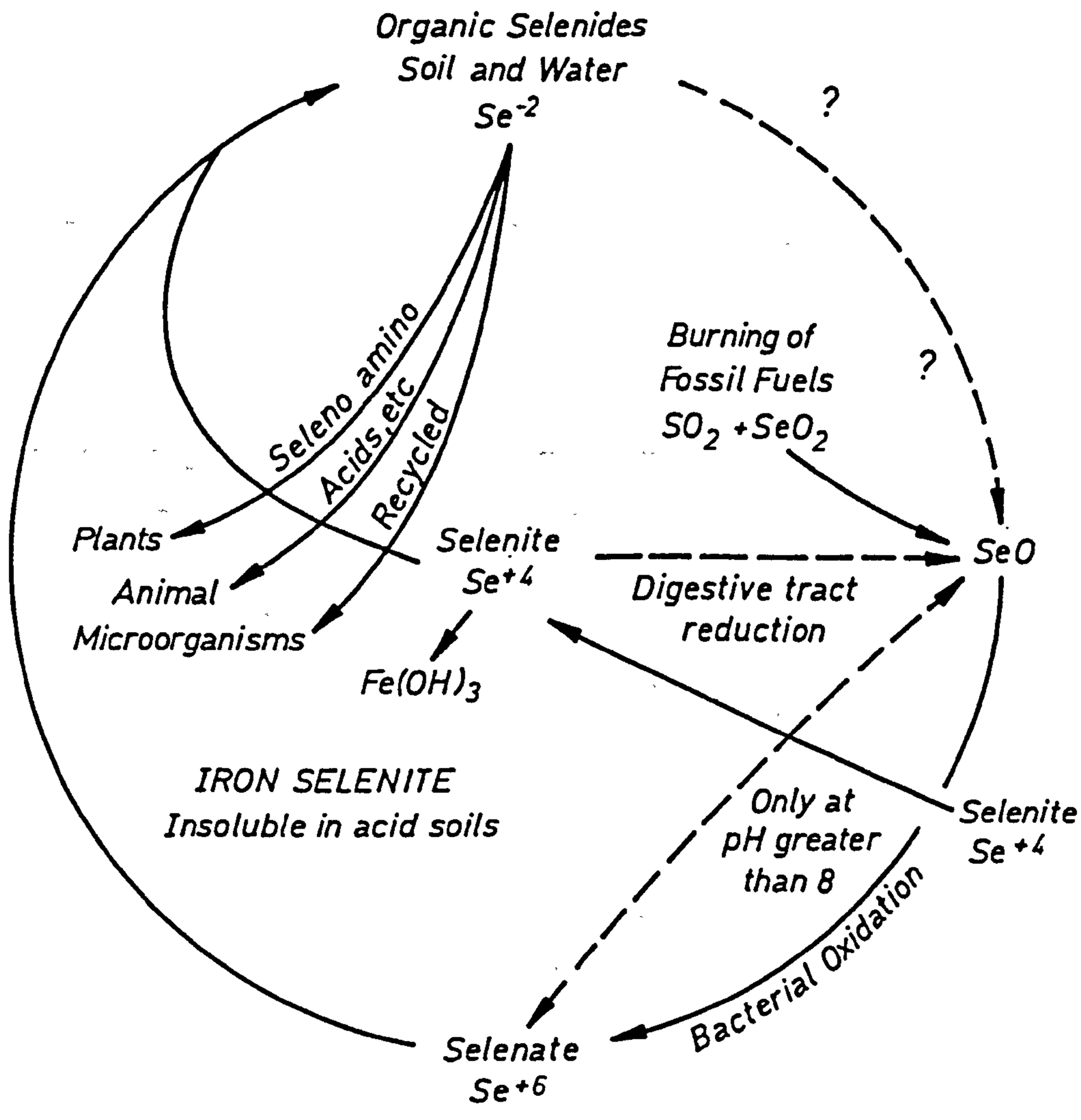
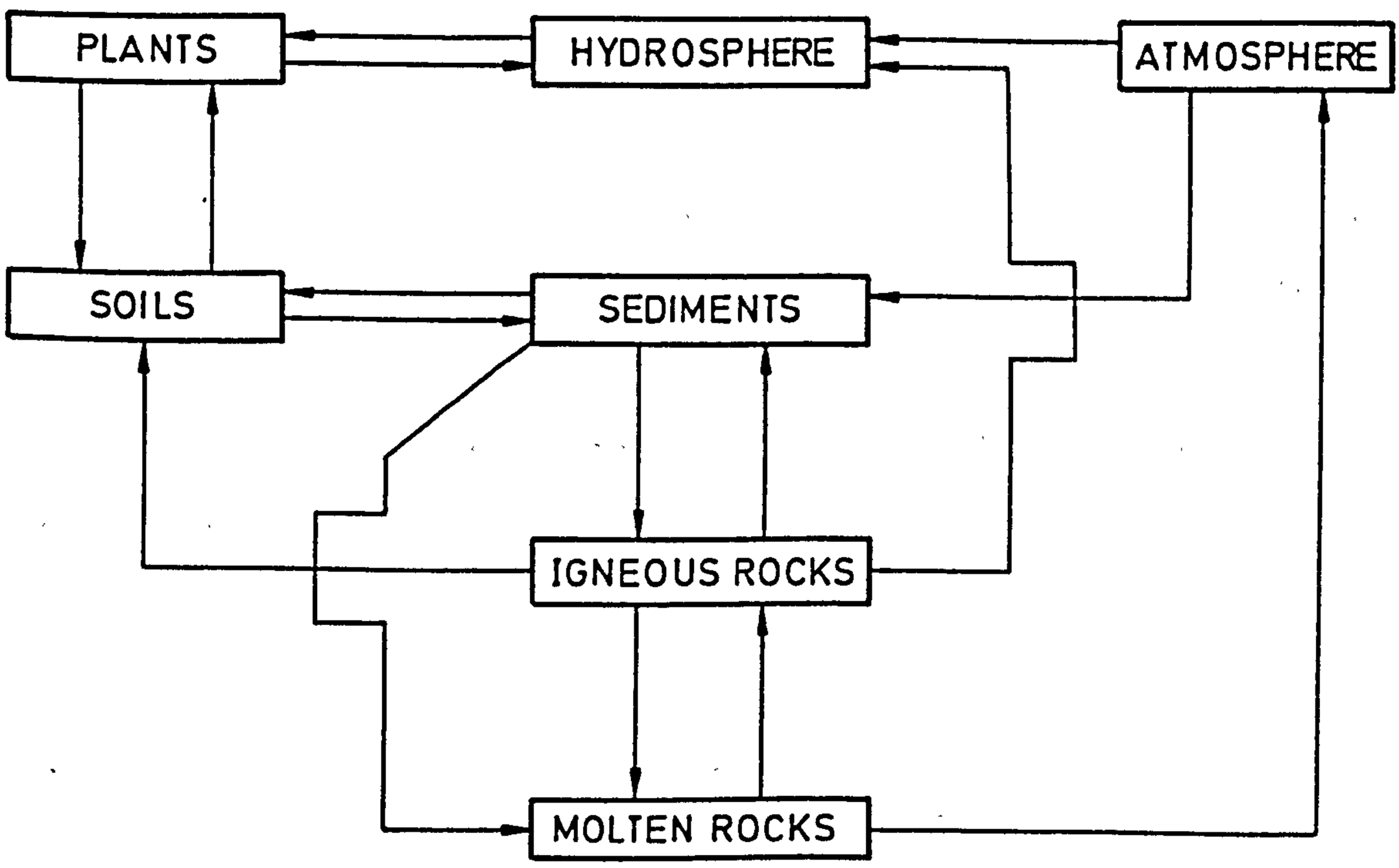


FIGURE 1.2 GEOCHEMICAL CYCLE OF SELENIUM



of selenium (22,32).

In Northern Europe the general picture is inadequate; Figure 1:3 (selenium levels in crops) shows the selenium concentration in crops for some European countries (30).

e) Selenium in Water

Because of the crucial importance of water, its quality must be managed and preserved. Thus the water pollution problem may reach crisis proportions.

Quality Criteria: Whether the water of a given source is suitable for a specific use depends on the criteria or standards for that use (44).

The most recent drinking water guidelines recommended by the World Health Organisation (WHO) were released as a draft document in 1982, which, when adopted, will replace both the European and International WHO standards for potable water, which currently recommend heavy metal concentrations which should not be exceeded in potable waters. Table 1:5 shows the metals included in the WHO International and European standards for drinking water quality (WHO, 1970, 1971, 1982) (45).

The safe upper limit for the selenium content of drinking water is considered to be 10 µg Se/litre i.e. 0.01 mg/L ≡ 0.01 ppm (19,22,34). This limit is for total selenium regardless of the valence form, selenite Se(IV) or selenate Se(VI), which means that the maximum allowable concentration of selenium in a drinking water supply is 10 µg/L. If the selenium concentration exceeds this value, treatment will be required to reduce the level to the maximum permissible concentration (MP) or an alternative source of water must be sought (34).

FIGURE 1.3 SELENIUM CONCENTRATIONS IN CROPS FOR SOME EUROPEAN COUNTRIES

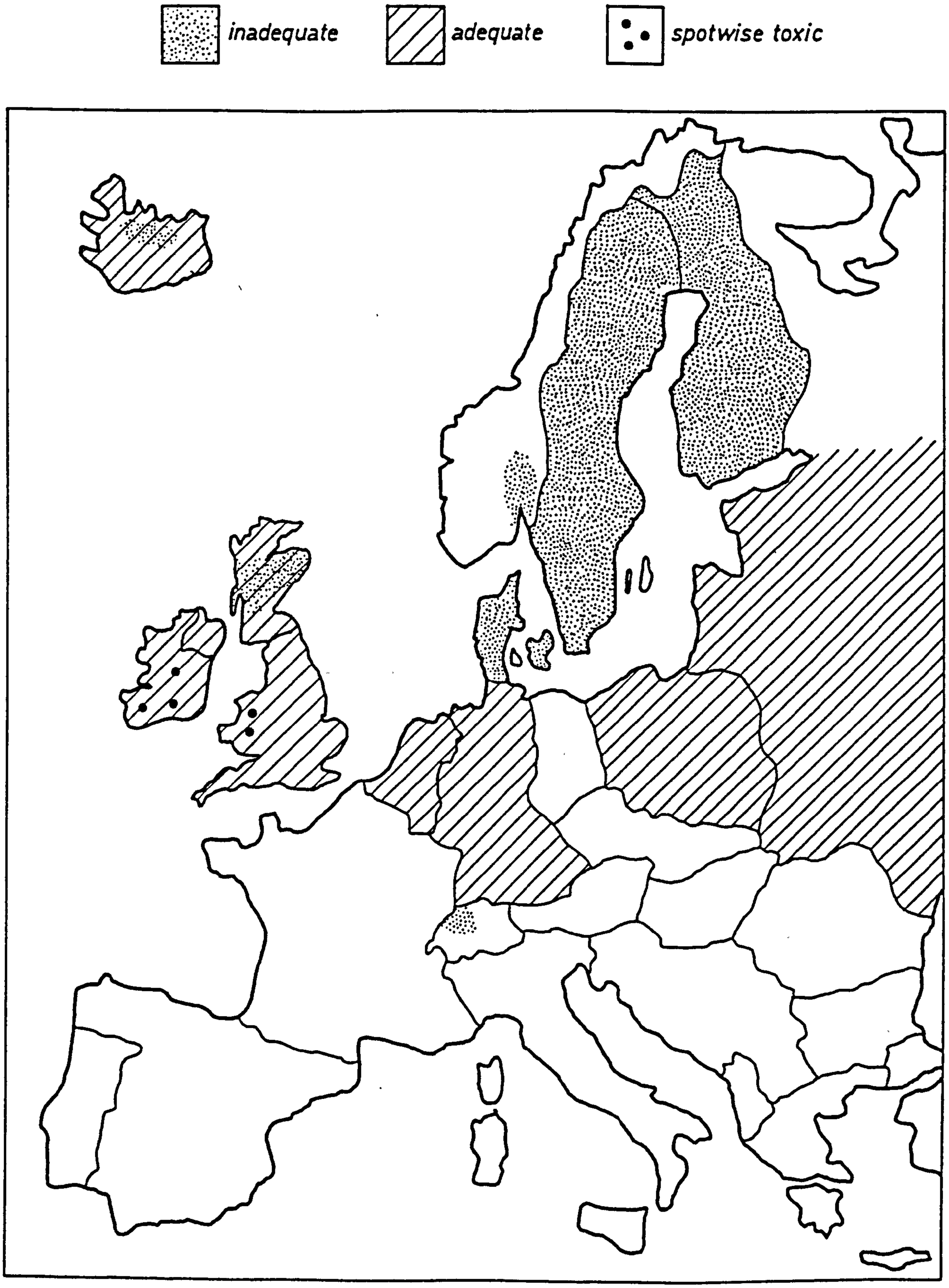


Table 1:5. Metals Included in the WHO and International Standards for Drinking Water Quality (WHO, 1970, 1971, 1982) (45)

Metal	International MP (mg/L)	European MP (mg/L)
As	0.05	0.05
Cd	0.01	0.01
Cr	-	0.05*
Hg	0.001	PC
Pb	0.1	0.1
Se	0.01	0.01
Ag	-	PC
Cu	1.5	0.05
Fe	1.0	0.1
Mn	0.5	0.05
Zn	15.0	5.0

MP = maximum permissible concentration

PC = presence should be controlled

* = Cr(VI)

Fortunately, no evidence has been obtained up to the present time that selenium occurs in water in sufficient amounts to produce selenosis in man or animals except in very isolated cases. Occasionally water from seleniferous areas may contain high concentrations of selenium, but this water is generally unpalatable to livestock (14).

i) Surface waters

The average selenium content of nine rivers in the U.S., Russia, France and Brazil was given as 0.2 µg Se/Litre (35); while the range in the Thames (England) was 0.13-0.41 µg/L (51).

Se(IV) exists as the weak selenious acid, H_2SeO_3, and as a number of inorganic selenites. In aqueous solution (pH range 3.5-9.0), dissolved selenites exist predominantly as the hydrogen selenite ion (51).

Information in the literature on the speciation of selenium in environmental water is very confusing. The findings of Measures and Burton (52) indicate that Se(IV) is present in some rivers (England) as a minor fraction of the total dissolved selenium (less than 10%). Hiraki et al. reported that Se(IV) accounts for 2-16% of the total selenium in samples from two rivers in Japan, whereas it comprises 75% of the dissolved selenium in the Kuji river (51).

Shimoishi et al. (53,54) found the percentage of selenite in seawater and river water varied from 35 to 70%; but Sinemus et al. (55) studied the influence of the oxidation state on the determination of selenium in lake water and found that element was present only as Se(VI).

ii) Marine waters

Schutz and Turekian (1965) estimated an average value of 0.09 µg/litre for selenium in the major oceans of the world. In acid water having a pH of 2.4 to 3.0 or in weakly alkaline waters having a pH 7.4 to 8.0, however, higher concentrations of selenium have been observed. In general, all waters contain less than 1 µg/litre (36).

Chau and Riley reported 0.50 µg Se/litre in the English Channel and 0.34 µg/litre for the Irish Sea (37).

Water from the North Sea contained 3.8 µg Se/litre. Nine deep sea samples from the Bering Sea and the Arctic Ocean off the coasts of Alaska and Siberia contained 0.03 to 0.7 µg Se/litre. Surface core samples from the Atlantic Ocean contained about 0.2-0.6 µg Se/litre; the content usually increased with depth of the core to a maximum of 2 µg Se/litre.

Marine waters of Japan contained 4 to 6 µg Se/litre (14).

f) Selenium in the Atmosphere

The natural crustal sources of atmospheric selenium are either the physical breakdown of selenium bearing soil, or the mobilisation via volatility. It is possible that the vapour pressure (as minute as it is) of selenium minerals such as those present in copper ores could be a significant atmospheric source. The relatively volatile compounds H_2Se and SeO_2 have been observed to be concentrated in volcanic gases. It has been estimated that approximately 1 kg Se/m^3 has been deposited onto the earth's surface by volcanic gases (38).

Selenium is an air pollutant that has been generally overlooked in spite of the fact that its distribution is widespread (39). The atmospheric concentrations vary from several ρg/m^3 to ng/m^3 (38). Total selenium vapour in ambient air has been reported to span a wide range, 0.006-5 ng/m^3 (41), while other workers have quoted values of 0.1-10 ng/m^3. Much of this selenium is derived from the combustion of fossil fuels (38).

Unfortunately, most data available on the distribution and amount of selenium present in the atmosphere are misleading because of

erroneous sampling methods used. Because in the air selenium probably exists as SeO_2 or H_2SeO_3, it is important that the volatility of these species are kept in mind. These, and most other selenium compounds, are so volatile that filtration by means of high-volume samplers cannot be used because the selenium is vaporised as fast as it is collected (39).

Lakin and Byers found a range of 0.05-10 ppm selenium in atmospheric dust collected on air-conditioning filters in 10 U.S. cities (19); selenium from these sources is probably in the form of insoluble oxides or in the elemental form and may not be of immediate value to plants and animals (22).

At least 50 per cent of the selenium present in urban air passes through a filter designed to collect all particles greater than 0.1 μm in diameter. However, irrespective of whether selenium is inhaled as vapour or as very small particles, its toxicology will undoubtedly involve the pulmonary region of the lung (40).

Volatile selenium is also released by non-accumulator plants such as alfalfa, and the amounts released are quantitatively related to the amounts of selenium within the plant (19). Another study identified biological alkylation as the main source for volatile organic Se in air (41). Dimethyl diselenide CH_3-Se-Se-CH_3 has been demonstrated as a volatile product of Astragalus racemosus (42). Abu-Erreish and others (43) noted that soil bacteria expel a gaseous form of selenium. Thus from biological processes small amounts of selenium can enter the atmosphere in a truly gaseous form.

Until the nature of the form of Se in the atmosphere and the form and biological availability in which it is returned to the ground is known, large and important gaps remain in the knowledge of the selenium cycle in nature (28), as shown in Figure 1:1.

ii) Tellurium

a) Occurrence

Tellurium is about the fortieth element in the order of cosmic abundance. Along with platinum, palladium and ruthenium, Te ranks about the seventy-first in the order of crustal abundance. The average amount in crustal rocks is 0.01 ppm (16), 1 x 10^{-7}% (6,7), and 1 x 10^{-6}% (4).

The geochemistry of tellurium is determined by its behaviour during mineral formation. Te is a typical chalcophile anion, a heavy analogue of sulphur and selenium.

Tellurium is a typical trace element, occurring chiefly in the sulphides, in association with other chalcophile elements such as copper (6) [copper ores from various deposits contain between 0.001-0.01% Se and 0.0001 to 0.0008% Te (5,6)].

The chalcogen elements, sulphur, selenium and tellurium, are primary components of intrusive or extrusive magmas and volcanic gases, and hence of volcanic sulphur deposits. Nevertheless, selenium and tellurium are not essential components of the common igneous rock-forming minerals. Te is widely distributed in the earth's crust in deposits of many different types, from magmatic and pegmatitic to hydrothermal forms, especially where these deposits are associated with epithermal gold and silver deposits (5,16).

b) Tellurium Minerals

Tellurium present in the earth's crust is either scattered, or found as tellurium minerals. Higher Te concentrations are characteristic of gold deposits. In many cases the occurrence of selenium and tellurium is strongly correlated (7).

In the naturally occurring minerals, it is associated chiefly with the heavy nonferrous and noble metals as tellurides Cu_2Te, Ag_2Te, PbTe, $PbTe_2$, AuTe, Bi_2Te_3, etc.

Tellurium minerals do not form commercial ore concentrations. Te is a typical trace element (6). At present more than 40 tellurium minerals are known, including 24 tellurides, two tellurates, native tellurium, a selenium-tellurium alloy (16), oxides and tellurites. At least ten tellurium minerals occur with gold and silver, ten with bismuth, and six with iron (7). Table 1:4 (page 15) contains selenium and tellurium minerals (5,7).

c) Tellurium in Rocks

1- Igneous Rocks

Beaty (1973) studied and detected tellurium in 20 different rocks of the United States using atomic absorption spectroscopy. He reported the average tellurium content of 12 igneous rocks was between 0.082 to 0.210 ppm (24).

Sindeeva (1964) reported the average tellurium content of igneous rocks of the Soviet Union was less than 0.100 ppm (5).

2- Sedimentary Rocks

Little is known about tellurium in sedimentary rocks, though some shales contain 0.1-2.0 ppm (16).

Beaty observed that some carbonates showed the highest tellurium content of 1-2 ppm, while the tellurium concentrations in 6 sedimentary rocks decreased in the following order:

Carbonates > Shales > Sandstones (24).

Nazarenko et al. reported tellurium is not present in high concentrations in sedimentary rocks, while in soils it is practically absent (7).

Kudryavtsev (1974) mentioned that the levels of Te in sedimentary rocks does not show an increase over that in igneous rocks and was practically undetectable in soils (4).

d) Tellurium in Water

The marine geochemistry of tellurium is unknown, being one of the few elements whose concentrations in seawater had not been reported until the 1980s. The major problem when studying Te stems from its rarity and the poor sensitivities of most analytical methods (even the crustal abundance of Te is still controversial).

Tellurium and selenium both occur throughout the oceans in two oxidation states; the major component of each is the oxidised form, but whereas for Se this is the thermodynamically stable species, the reverse is true for Te. The vertical distributions of the two elements are very different in shape, with Se displaying a nutrient-type distribution resulting from biological uptake at the surface and scavenging at depth. The deep water distribution of Te is controlled primarily by scavenging, as opposed to water mass advection, owing to a residence time much shorter than the ocean turnover time, whereas that of Se reflects the advective processes. The high geochemical reactivity of Te resembles more closely polonium (^{210}Po) than Se (49).

Although Se(VI) and Te(VI) have the same oxidation state, they have different chemical structures; selenate, like sulphate, has tetrahedral coordination, SeO_4^{2-}, whereas tellurate is octahedral, $Te(OH)_6$. Thus from the structural point of view, tellurate should behave like Sn (tin) or Po, which exist in seawater as $Sn(OH)_4$ and $Po(OH)_4$ respectively (50).

Andreae (1984) determined tellurium by GFAAS on several samples of seawater and rainwater; he found only Te(IV), with an average from 1.9 to 6.8 picomole/litre; and reported that there was no evidence for the presence of Te(VI) in these samples at the level of the precision obtainable for the difference between Te (IV+VI) and Te(VI) (46).

Lee and Edmond studied the concentration and speciation of tellurium in seawater of the eastern South Atlantic and the western North Atlantic. They reported that total Te decreases smoothly from a surface maximum of 1.30 picomole to ~ 0.6 picomole at 2500 m, but below this the concentration levels were constant. Te(VI) had a mixed layer concentration of 0.8 pM and showed a slight maximum in the shallow oxygen minimum. Below 400 m the Te(VI) levels parallel closely the total Te distribution, dropping to Deep and Bottom water values of 0.43 pM. The thermodynamically stable reduced form Te(IV) had a surface concentration of 0.36 pM, the levels dropping slightly in the oxygen minimum to 0.12 pM (49).

e) Tellurium in the Atmosphere

Since Te and Se occur in association with sulphur in many natural materials, including fuels, ores and soils, processes that release sulphur to the atmosphere may also release selenium and tellurium. These two heavy chalcogen elements may be used as probes for gaining information about atmospheric sources and processes.

Information on the concentrations of Te, its chemical forms, and its distribution as a function of particle size is also needed for an assessment of problems posed by suspended chalcogen elements in air, although there is no clear indication that current levels of Te in air pose a health hazard (47).

Many measurements of atmospheric sulphur are made routinely each year, atmospheric levels of Se receive much less attention, and the first reports of Te in atmospheric aerosols were by Chiou et al. (1984). They determined Te by GFAAS in atmospheric aerosols; the average was about 0.3 ng Te/m^3 (48).

The concentrations of Te and Se in 12 atmospheric aerosol samples collected during a 4-month period, July-October, was from 0.26 to 0.71 ng Te/m^3. The average weight ratio Te/Se = 0.13 $\pm$ 0.03, shows that Se is more abundant than Te in aerosols, which is also the case for average crustal material, where Te/Se = 0.02. It is possible that Te is enriched in fuels or other major sources of the chalcogen elements found in present day aerosol particles but proof is not yet forthcoming.

The chalcogen elements show concentration maxima in atmospheric particles with diameters of 0.01-1.1 μm. The fractions of Te, Se, and S in fine particles (<2 μm) are 75%, 79% and 85% respectively (47).

1:1:3 Selenium Biochemistry

i) Selenium as a Trace Element of Life

Many mineral elements occur in living tissues in such small amounts that in the past, workers were unable to measure the precise concentrations with the analytical methods then available. They were therefore frequently described as occurring in "traces" and the term trace elements arose to describe them (56).

At the present time 26 of the 90 naturally occurring elements are known to be essential for animal life (56). (Six elements - carbon, nitrogen, hydrogen, oxygen, phosphorous and sulphur make up the molecular building blocks of living matter (57).)

The essential elements consist of 11 major elements; exclusive of the six elements above, the others are calcium, potassium, sodium, chlorine and magnesium (56).

The other 15 elements are generally accepted as trace elements; they are iron, zinc, copper, manganese, nickel, cobalt, molybdenum, selenium, chromium, iodine, fluorine, tin, silicon, vanadium and arsenic. In addition, boron is essential for the higher plants, but has not yet been shown to be necessary for animals.

The concentrations of these elements are usually expressed as parts per million (ppm) = μg/g, or with some, such as iodine, chromium, nickel and vanadium, as parts per billion (ppb) = ng/g (56).

It has been known for more than 100 years, that iron and iodine are essential to man. In the rapidly developing period for biochemistry between 1928 and 1935 four more elements, all metals, were shown to be essential; copper, manganese, zinc and cobalt.

In the next 30 years, however, three further elements were shown

to be essential; chromium, molybdenum and selenium, while fluorine, silicon, tin and vanadium have been added since 1970 (57).

The toxic effects of selenium have been recognised much longer than the nutritional ones. The discovery in the 1930s that certain geographical areas were seleniferous and produce plants with high selenium content, and that food grown in these areas caused sickness in man and beasts ingesting such vegetation (58) focused attention on the biochemical properties of selenium compounds (59).

In 1957 selenium was identified as the essential element of a dietary factor that protected rats from severe necrotic degeneration of the liver, heart, kidney and muscle; similar results were found for the mouse, while Se was found to give protection against oxidative diathesis in the chick (60).

Selenium has also been shown to be an essential micronutrient for animals and bacteria. There are only three enzyme-catalysed reactions that have been shown to require the participation of selenium-containing proteins (59).

Since the discovery of Se as an essential element (60) and its role in the enzyme glutathione peroxidase which has been demonstrated by the isolation of glutathione peroxidase from human erythrocytes and human placenta (61), there has been an increased interest in this element. Initially interest in Se was caused by its potential toxicity, but subsequently the emphasis has shifted because of four significant observations:-

1) Se acts as an anticancer agent in chemically or virally induced tumour formation in experimental animals.
2) The important role of Se in the prevention of cardiovascular diseases and myocardial infarction.

3) Se acts as an antagonist against various toxic metals like mercury, cadmium and lead.

4) In hospitalised patients receiving long-term parenteral alimentation, Se deficiency may become important (62).

ii) Selenium in Human Tissues and Fluids

Selenium occurs in all the cells and tissues of the animal body in concentrations that vary with the tissue and the level and chemical form of Se in the diet. The liver and kidney usually carry the highest Se concentrations, with much lower levels in the muscles, bones and blood, with very low levels in adipose tissue (56).

The human tissue in which Se has been most frequently measured is blood. A full understanding of Se blood levels is not presently possible due to lack of knowledge of the nature of Se in human blood (58).

The Se levels in whole human blood were reported to range from 0.10 to 0.36 μg/ml (63). Some evidence was obtained of a geographic pattern reflecting established regional differences in the Se levels in crops. But Hamilton et al. obtained a lower level of 0.08 μg/g for the U.K. (56).

A study of human plasma showed that most Se was present in the α- and β-globulins, with average concentration level of 0.144 μg/ml (64).

iii) Selenium in Milk and Food

The concentration of Se in cow's milk varies greatly with the Se intake of the animal. In a study of pasteurised milk from different areas of New Zealand, the actual range reported was from 2.9 ± 0.7 to 9.7 ± 0.7 ng Se/ml, with a mean value from eight areas

of 4.9 ng Se/ml (56).

Allaway et al. reported that cow's milk from a low-Se area in Oregon contained less than 20 ng Se/ml, compared with 50 ng Se/ml from a high-Se area of South Dakota in the U.S. (56,63). Higher levels, ranging between 160 and 1270 ng Se/ml, have been reported for cow's milk from other high-Se rural areas in the U.S. (56).

Dried skimmed milk powder samples ranged from 95 to 240 ng Se/g (22,65).

Human mature breast milk contains about twice as much Se as normal cow's milk, in New Zealand ranging from 11.5 to 14.5 ng Se/ml, compared to 5 ng Se/ml for cow's milk (56). The mean Se value for human milk is reported to be 18 ng Se/ml (range of 7 to 33 ng Se/ml) (66). However, the amount of Se in human milk varies geographically. The Se content of human milk is a function of dietary Se (Shearr and Hadjimarkos, 1975 (66)).

Food Se content is related to protein content and geographical origin. In most biological material, Se is found largely in the protein fraction; indeed, food low in protein, such as fruits, have been shown to contain very little Se (58). Morris and Levander (1970) determined (by fluorimetry) the Se content of a wide variety of foods representing a cross-section of the U.S. diet. Most fruits and vegetables contained less than 10 ng Se/g; some exceptions were garlic, mushrooms and radishes containing 250, 130 and 40 ng Se/g respectively (65). Geographic areas have been delineated where the soil content of Se is high, intermediate or low; Se content of plant and animal products from those areas generally corresponds to the soil Se levels (58).

Grain products vary widely in their Se content with cornflakes

containing as low as 25 ng Se/g, with barley cereal as high as 660 ng Se/g. Whole wheat flour (660 ng Se/g), whole wheat bread and brown sugar (11 ng Se/g) contains two to four times more Se than did white flour, bread or sugar (22,58).

Lindberg found samples of Swedish wheat to contain 7 to 22 ng Se/g. These are similar to the levels found in wheat from Se-deficient areas in New Zealand (56).

Egg yolk contains about 180 ng Se/g, and egg white about 50 ng Se/g (65). Under normal dietary conditions a hen's egg contains a total of 10-12 ug Se, most of which is present in the yolk (67).

Meat samples ranged from about 100 ng Se/g for chicken muscle to as much as 1900 ng Se/g for pork kidney, with most values between 200 and 500 ng Se/g. The content of seafood was generally higher, ranging from 400 to 700 ng Se/g (65).

iv) Selenium and Vitamin E

The nutritional and metabolic interrelationships between Vitamin E and Se, and indeed the status of Se as an essential nutrient have remained ambiguous and contentious. While Se was reported to be useful in the prevention of several diseases of livestock, there has been no unequivocal demonstration that Se was essential under conditions of adequate Vitamin E nutrition.

Se administration was potentially more economical as a prophylactic measure but field cases of so-called "Se-Vitamin E Deficiency" have occurred (68).

1:1:4 Essentiality and Toxicity of Selenium

i) Selenium Deficiency

Biological function is thought to be dependent on the tissue concentration or the intake of a nutrient. The severity of deficiency signs and the effects of resupplementation depend on the degree of deficiency. This dependency has been formulated mathematically by Bertrand (1912). According to Bertrand's rule, a function for which a nutrient is essential and the nutrient is low or absent results in a theoretical deficiency, but the function increases with increasing exposure to the essential nutrient. The increase in function is followed by a plateau representing the maintenance of optimal function through homeostatic regulation, and a decline of the function toward zero as the regulatory mechanisms are overcome by increasing concentrations that become toxic (22).

Bertrand's work has been graphically interpreted by Mertz (1981) as illustrated in Figure 1:4 (69).

Recent work has demonstrated that several of the trace elements including Se possess the characteristics outlined by Bertrand and Mertz. It is likely that each essential nutrient has its own curve which differs from that of other nutrients, i.e. the width of the plateau (22,69).

Selenium is necessary for growth and fertility in animals and for the prevention of various disease conditions which show a variable response to Vitamin E (56).

Human dietary Se deficiency is more likely to occur in certain types of cancer patients than in healthy individuals. The role of Se in the causation and prevention of cancer has been widely debated, and a recent review is available (58).

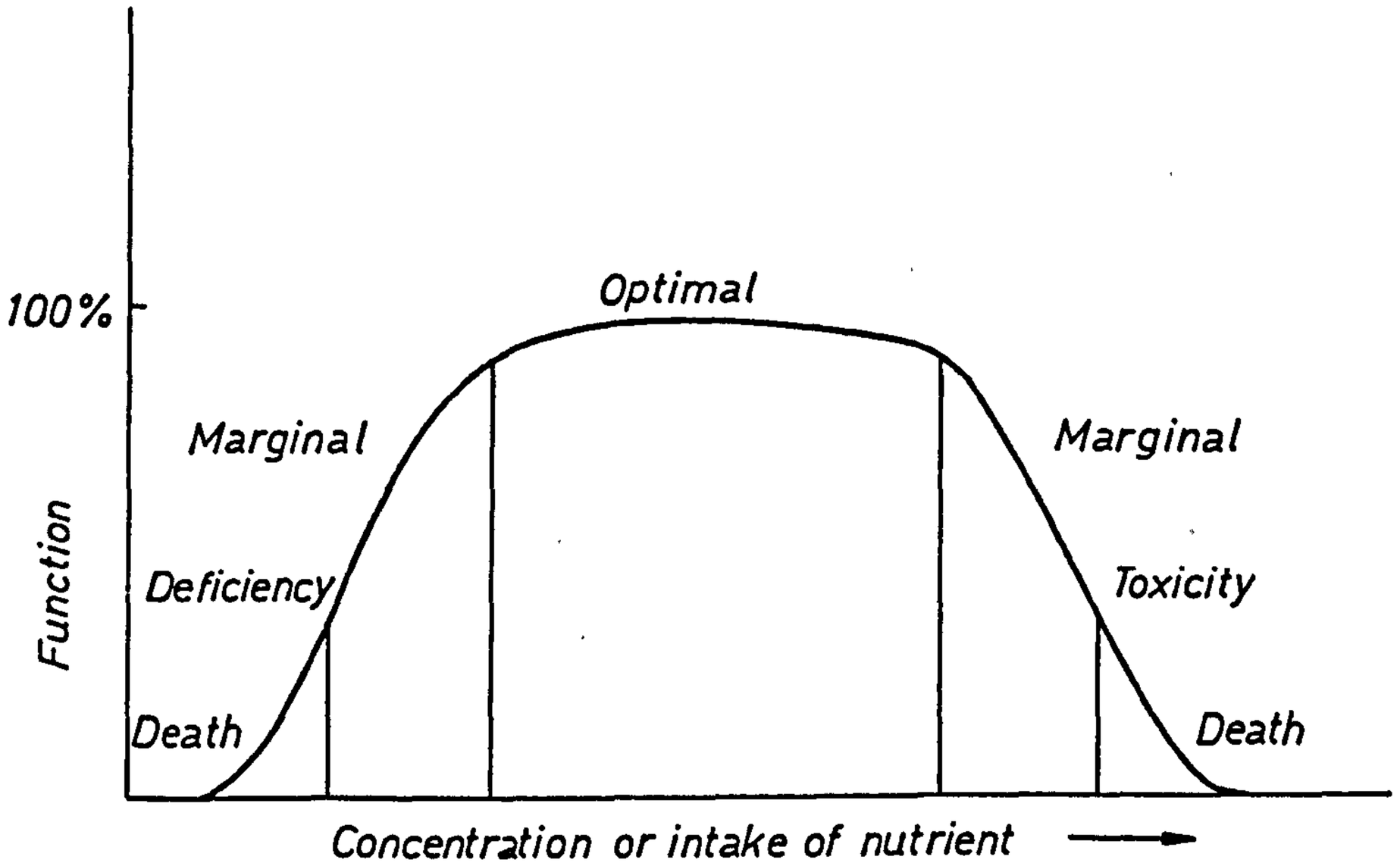


Figure 1:4. Dependence of biological function on tissue concentration on intake of nutrient (22,69)

Several investigations have shown that Se is involved in the processes of the male reproductive system. After administration of Se^{75} in the form of sodium selenite to mice, rats, bulls and rams, a considerable part of the Se^{75} activity accumulated in the testes, where it was incorporated into developing spermatozoa. Retention was especially high in selenium-deficient animals (70).

In humans and in several other mammalian species in which the male gonads do not represent such a large percentage of the body mass, the Se amount retained in the testes is a much smaller part of total body Se than in the rat. However, due to the influence of gonadotropic hormones on Se, there is a possibility that in these cases, too, the Se metabolism may be affected during puberty (70).

ii) Toxicity of Selenium

Selenium is an essential nutrient for humans and animals and is required at a concentration of about 40 ppb (40 ng Se/g) and is beneficial to 100 ng Se/g; at levels above, however, it becomes toxic to animals (19,60).

Elemental Se is relatively inert and is not thought to be a health hazard. The same applies to the stable heavy metal selenides, such as those of copper, zinc, cadmium, mercury, lead and bismuth. All other Se compounds should be treated with respect and handled with care (4).

Elemental selenium is relatively inert and can be handled without special precautions. The toxic forms include the reactive selenides; the gaseous; volatile and soluble compounds; but particularly hydrogen selenide, the halides and oxyhalides and organic derivatives. Some of these can enter the body through the lungs or the skin, especially if the tissue is damaged, and may affect the body organs. Because of the rare incidence of occupational poisoning, the pathology of Se in humans has not been studied adequately (9). Nevertheless, many Se compounds are believed to cause damage to some body organs when absorbed through the skin or when inhaled or ingested.

Liquid solutions of Se compounds can also pass through the skin (22).

Contact with elemental Se does not injure the skin, but some of its compounds may cause dermatitis. SeO_2 and selenious acid attack the skin and may cause local irritation followed by dermatitis; the effects resemble those produced by hydrofluoric acid. Selenium oxyhalides are extremely vesicatory and, by hydrolysis to selenious acid and halogen acid, cause slowly healing burns. Hydrogen selenide

affects the mucous membranes of the upper respiratory tract and the eyes.

Industrial precautions include proper ventilation, personal cleanliness, provision of dust masks where needed, gloves and either safety glasses or chemical goggles. Calamine lotion, calamine ointment and various creams have been used to protect the skin (9).

Se toxicity in animals has been a major problem in the past, and a few cases of Se toxicity in human beings have been reported. Although large land areas contain excessive quantities of the element and Se is extensively used in industry, there is no firm evidence of a significant Se toxicity problem in human beings (58).

iii) Selenium as Carcinogen and Cancer

One of the more exciting effects of Se on health is its anticarcinogenic effect against experimentally-induced cancer in several animal systems (22), espcially cancers of the:

1. Skin
2. Liver
3. Colon
4. Breast
5. Trachea

The carcinogen potential has been claimed by a few researchers, but challenged by others. In general, flaws in the experimental design or non-physiological nature of feeding markedly toxic amounts over a long period of time have clouded the results (22).

1:1:5 Tellurium Biochemistry

i) Introduction

Since the discovery of tellurium, Te has found numerous commercial applications as an additive element. Te is added to Pb, Cu and steel for increased resistance to corrosion and stress, as well as for workability and machinability. When added to cast iron, Te is a powerful carbide stabilizer and provides extra surface resistance to wear and corrosion (71).

Other uses of Te include as a colouring agent in glass (73), as a catalyst in ceramics and as a fuse for explosives (71). The diethyl- complex with Te is the fastest known accelerator for curing butyl rubbers (73).

Tellurium has also been used in bacteriological procedures for a rapid diagnostic test, especially for diphtheria, by virtue of the fact that bacteria reduced tellurite solutions to black amorphous Te.

Since 1950 there has been much interest in the use of Te in the formulation of semiconductors, especially bismuth telluride and lead telluride for thermoelectric applications. Lead-tin-telluride has found increasing use in sophisticated infrared detection systems (71).

ii) Effects of Tellurium on Metabolism

The metabolism of Te in the mammalian body is not well understood.

Te can be absorbed into an animal body by inhalation, ingestion and by absorption through the skin. In experiments in which tellurate was administered in the feed of rabbits, 30 per cent of Te was absorbed,

10 per cent was eliminated in the urine, but 60 per cent was eliminated in the faeces (71).

When Te is administered orally, renal excretion is very low in the first hour, but increases to a maximum between the seventh and twenty-four hours, and then decreases (25). When the amounts given are small, only a trace is detectable in the urine. Te salts ingested orally are quickly reduced to elemental Te by intestinal bacteria; in fact all living cells rapidly reduce all Te compounds to the metallic state (71).

Soluble tellurites (i.e. Na_2TeO_3) are absorbed into the body after oral administration, which are then reduced to tellurides, partly methylated, and then exhaled as dimethyl telluride. The latter is responsible for the garlic odour on the breath of persons exposed to Te compounds (72).

The concentration of Te in the kidneys was three to ten times that in the heart, lungs and spleen, while the liver level was a factor of 1.5 to 2 lower. The heart muscle contains a concentration 20 times greater than the skeletal muscle, a fact which relates to the probability that stimulated muscle reduces Te compounds more rapidly than when the muscle is at rest (25,71). The concentration of Te in the blood is much less than that in the urine (71).

iii) Tellurium and Medicine

Organotellurium chemistry has grown rapidly over the last few years as a result of development of Te "reagents" that have unique and subtle applications in organic synthesis. In addition a variety of new organotellurium compounds of biological interest have been synthesised and evaluated because of interest in the potential use

of ^{123m}Te-labelled agents for diagnostic applications in clinical nuclear medicine, in external scanning or imaging techniques (74). Tellurium-123m labelled amino acid has been used as a new class of pancreatic imaging agents (75).

Recently, the development of radio-labelled long-chain fatty acids for the evaluation of heart disease is of interest because of their potential use for measurement of myocardial fatty acid metabolism in relation to various states of the disease. Because of the attractive physical properties of iodine-123 (13.2 hours physical half-life) and the versatility of iodine chemistry, this radioisotope has been used to radio-label a variety of fatty acids. Fatty acids containing the divalent tellurium heteroatom in the fatty acid chain show pronounced heart uptake in experimental animals and exhibit the unique property of slow myocardial washout. Te can be readily incorporated while maintaining the linearity of the fatty acid molecule. The effects of chain length and Te position on the heart uptake of ^{123m}Te fatty acids in rats have also been investigated (76).

iv) Tellurium Toxicity

Te is not an essential element; relatively little is known about its distribution in soil and atmosphere. However, since very low levels of intake cause a strong garlic odour of dimethyl telluride in the breath, excess environmental pollution should be easily detected (77).

Early observations on the toxic effect of Te in animals emphasised especially two phenomena, the garlic odour and a deep bluish black colouration in the tissue (71). The signs of poisoning with Te and its derivatives are headache, increased respiratory rate and pulse

rate, premature fatigue and vertigo. Nausea, garlic odour of breath and sweat, vomiting, renal colic, cyanosis, dyspnea, and haematurea are observed in poisoning with sodium tellurite (6).

The only account of severe Te intoxication in man was the tragic case reported in a British military hospital (1946). Sodium tellurite was administered by mistake (instead of sodium iodide solution) by catheter into the uretha, which caused three cases of severe poisoning, two of them fatal. The fatal dose was estimated to be about two grams. Garlic breath was noted after a short time. All three cases experienced cyanosis. The two who died experienced pain in the loins, some nausea and vomiting, followed by stupor, loss of consciousness, and death within six hours. The third, who did not receive such a large dose, recovered without symptoms other than the garlic breath (78).

Elemental Te and the stable metallic tellurides are relatively inert and are not thought to be a health hazard. All other Te compounds should be handled with caution, including reactive tellurides, the volatile and the soluble compounds, such as hydrogen telluride, tellurium hexafluoride and the organic compounds (4).

Some of these compounds can enter the body by absorption through the skin or inhalation and ingestion of dust, fumes, vapours or sprays. Thus hydrogen telluride is a very toxic gas. The toxic effects of the compounds all arise through reduction to the relatively harmless elemental Te and to dialkyl (chiefly methyl) tellurides (16).

Te oxides and oxy-acids are less reactive than their Se analogues. However, sodium tellurite is somewhat more toxic than sodium selenite and sodium arsenite because of its ease of reduction, while sodium tellurate is less toxic than sodium selenate and somewhat less toxic than sodium arsenate (4).

1:1:6 Selenium and Plants

i) Introduction

The study of Se in organisms began in the 1930s with the discovery that the disorders of livestock known as alkali disease, ill thrift and blind staggers were associated with the high Se content of certain plants. These species commonly accumulate 1 to 10 mg Se/g dry weight basis from seleniferous soils which typically contain 2 to 14 µg Se/g (14). Plants possessing these characteristics are known as Se-accumulators and have been reported in various parts of the world, e.g. Astragalus racemosus in the U.S. and Neptunia amplexicaulis in Australia (80), indeed some Se-tolerant plants may contain up to 1.5 wt % Se. Other vegetable matter grown on seleniferous soils can have a sufficiently high Se content to be toxic when ingested by animals or humans (9).

Following the discovery of the Se-accumulator plants it was soon established that Se inhibited plant growth. The Se-accumulators are less sensitive to Se toxicity and most of the Se present in both accumulators and non-accumulators was contained in various seleno amino acids, principally as Se analogues of intermediates or simple derivatives of the methionine cycle. They include Se-methylseleno cysteine, selenocystathionine and Se-methylselenomethionine. In non-accumulators, Se also occurs in proteins. Accordingly, there is the possibility that Se is subject to the same or similar metabolic processes as sulphur (80).

Se was found essential for the expression of activity of several enzymes in certain anaerobic bacteria (79,80). Se was also found to be an essential trace element for the nutrition of animals (Schwarz and Foltz (60) and Stadtman (59)). The phenomenon raises the question

whether plants, including accumulators and non-accumulators, also have an essential requirement for selenium.

It is evident that Se is metabolised in organisms in two fundamental ways:-

i) Se accumulator and non-accumulator plants require non-specific metabolism of Se in place of sulphur, resulting in the formation of Se isologues of sulphur-containing metabolites. This process is especially evident when Se is supplied at relatively high concentrations (millimolar).

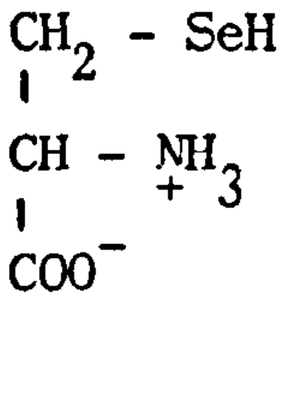
ii) The requirement for trace amounts of Se for the nutrition of animals and its incorporation into specific proteins, but do not involve metabolism of the more abundant element, sulphur (80).

ii) Chemical Forms of Selenium in Plants

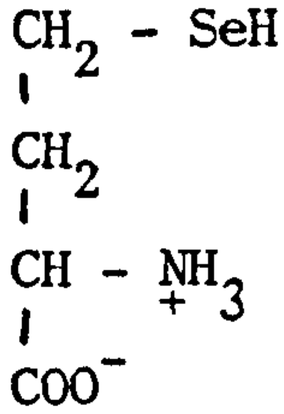
Most of the Se found in plants (accumulator and non-accumulator) occurs in selenoether amino acids of the form R -Se-R', where R and R' are amino acid residues. A difficulty when characterising the Se-containing compounds in plants is that the Se isologues of the sulphur amino acids are relatively labile. For example, selenocysteine and selenocystine are destroyed under conditions used for the acid hydrolysis of proteins; derivatisation by alkylation or some other agent must be employed prior to hydrolysis to stabilise these compounds (80).

Only the two main classes of compounds are summarised below (80):

1. Derivatives of selenocysteine and selenohomocysteine



Selenocysteine (I)



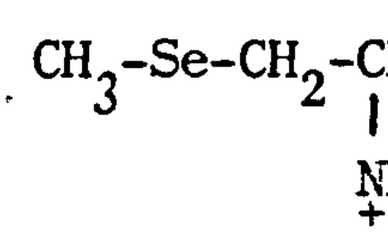
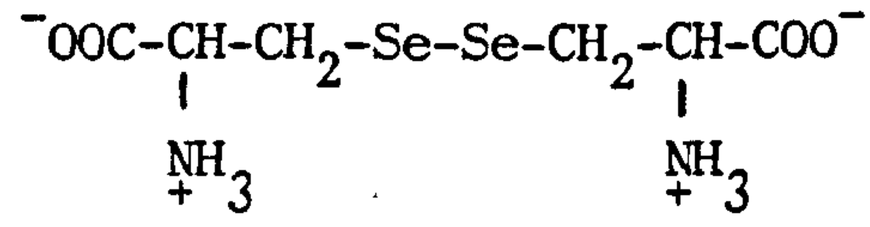
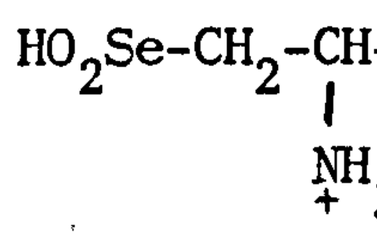
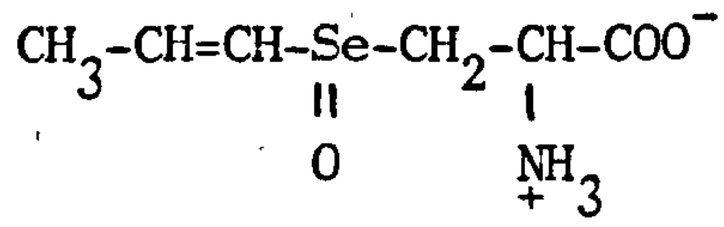
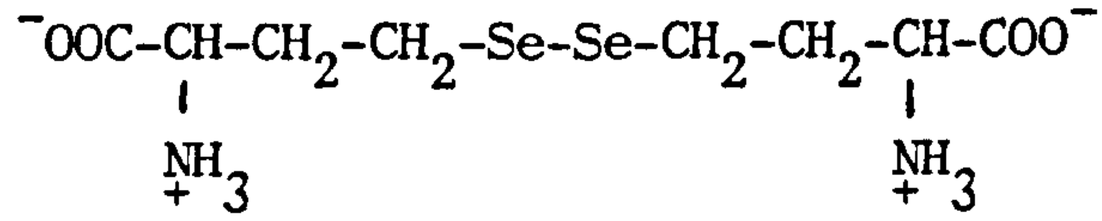
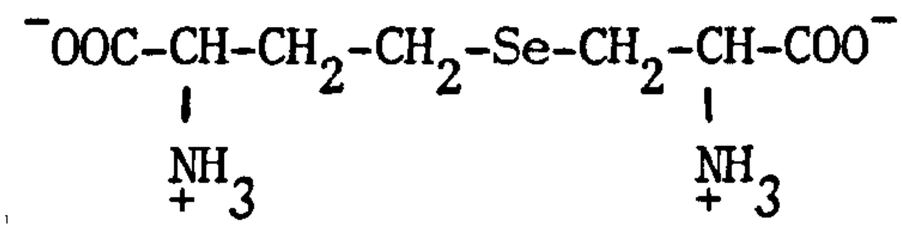
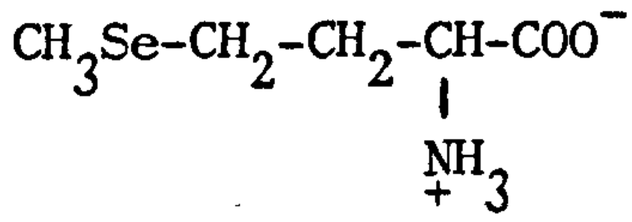
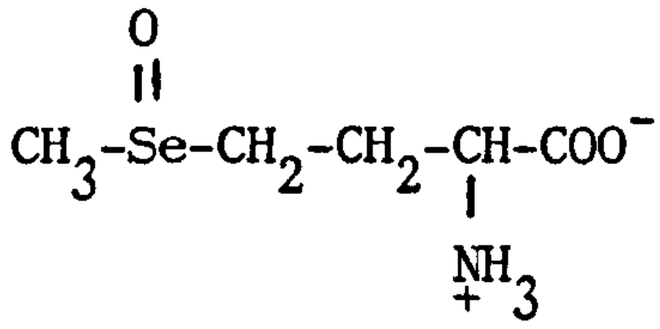
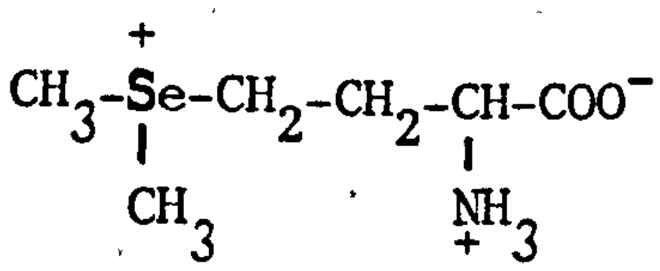
Selenohomocysteine (II)

Free selenocysteine (I) has not been reported or detected in plants, but Brown and Shrift (1980) raised mung beans in the presence of selenate and isolated a Se-protein. After treating the protein fraction with iodoacetate and subjecting it to acid hydrolysis, carboxymethylselenocysteine was identified. This suggests that the protein contained selenocysteinyl residues in peptide linkages (81).

Several other compounds structurally related to selenocysteine which have been recorded in plants are listed in Table 1: 6. Of these, Se-methylselenocysteine is a major form of Se in some Se accumulators. On the other hand, the oxidised forms of selenocysteine, selenocystine, and selenocysteine selenic acid have been reported in trace amounts from non-accumulators.

Free selenohomocysteine (II) has not been reported in plants. The same type of problems associated with the identification of selenocysteine in plants would also apply here. Selenohomocysteine has been isolated from Astragalus and is presumably subject to diselenide interchange with plant thiols or selenols as described above for selenocysteine. The selenoether, selenocystathionine, is much more stable than the corresponding selenol amino acids (80).

Table 1:6 . Some Selenoamino Acids found in Plants; Major Forms of Se in Accumulators[a] and Non-accumulators[b]

Derivatives of selenocysteine: Se-Methylselenocysteine[a]	
Selenocystine	
Selenocysteine seleninic acid	
Se-propenylselenocysteine selenoxide	
Derivatives of selenohomocysteine: Selenohomocystine	
Selenocystathionine[a]	
Derivatives of selenomethionine: Selenomethionine[b]	
Selenomethionine selenoxide	
Se-Methylselenomethionine	

2. Derivatives of selenomethionine

Selenomethionine, like selenocystathionine, is a relatively stable selenoether amino acid.

When non-accumulators are grown on seleniferous soil or supplied with high concentrations of Se, a considerable proportion of the Se taken up by the plant becomes associated with the protein fraction. Following acid hydrolysis most of the Se was found in selenomethionine suggesting that selenomethionyl residues are present in peptide linkage.

Se isologues of various other sulphur-containing plant products are known. They include the isothiocyanate in which presumably one or both the sulphur atoms are replaced by Se. The Se isologue of S-propenylcysteine sulphoxide is the precursor of the lachrymatory factor found in onions (80).

iii) Selenium uptake and transport

Many plants have been shown to concentrate Se to levels in excess of the level initially present in the external medium. The degree of concentration depends on the form of the Se supplied and on the plant species under investigation. In general, a higher concentration of Se has been found in plants when selenate was added to the soil than when the same amount of selenite was supplied (79).

Selenate is taken up through the same binding sites in the plant roots as sulphate. The two ions are taken by the same active absorption process in competition with each other, while selenite is taken up through other sites (30). Ulrich and Shrift (82) and Gissel-Nielsen (31) reported that selenite uptake was slower than for selenate.

Johnson et al. have reported that Se is taken up by plants either as selenate, selenite, or organic selenium (22).

Gissel-Nielsen (1976) has studied the uptake and translocation of selenite by means of Se^{75} in water culture experiments with barley and maize. After 10 minutes the Se^{75} activity was found in the green part of the plant. This corresponds to a translocation speed from the roots of 1-2 cm/min. After 30 min, the experiment showed that more than 15% of the Se absorbed by the root was translocated as selenite, and greater than 80% was present in the amino acid fraction, probably as selenomethionine (30). The Se concentration in the xylem sap ranged from 1 to 5 ug Se/ml. After fractionation of the Se in the leaves, the results showed that 25% - 30% of the Se was water soluble, while 10% of the total was present as selenite. If the protein was hydrolysed with pronase, the water-soluble fraction of Se^{75} increased to 96% of which 73% - 76% were in the amino acid fraction. The predominant Se-containing compound was selenomethionine (22).

Maize grown in culture solutions containing 5 ppm of selenite or organic Se accumulated 200 and 1000 ppm of Se, respectively. When the Se level was increased to 10 ppm, maize accumulated 300 ppm from the selenite form and more than 1500 ppm from the organic form. Ganje (1966) has reported that the organic forms of Se and selenate were the most available forms for maize grown in culture solutions. In general, resistance to Se toxicity among plant species varies so widely that a general toxicity level cannot be reliably estimated (22).

Se uptake by plants also depends on the sulphur level. The most important factor affecting the Se content of a given species is the sulphur/selenium nutrition (S/Se ratio). Thus the Se content increases with the level of Se available and decreases with the sulphur supply (80).

Other factors in addition to those described above also affect the

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selenium content of plants, i.e. the calcium, phosphorus and nitrogen concentrations present in the culture media. In calcium-rich soils the mobility of Se increases because of its oxidation and greater solubility. Se is also more available for plant consumption in calcium-rich soils. Several Se poisonings have been observed in cattle that have consumed seleniferous plants grown in calcareous soil areas (22).

The influence of phosphorus on the uptake of toxic levels of Se by different plant species was studied by Fleming (26) in a pot experiment. Three differing amounts of superphosphate were added to the plants, and for all crops increasing amounts of phosphorus caused a decrease in the Se concentrations. Nitrogen also plays a role in decreasing the Se content in crops (22).

iv) Metabolism of Selenium in Plants

A considerable amount of the evidence discussed above suggests that macro concentrations of Se are metabolised by at least some parts of the pathways of sulphur metabolism. This generalisation is probably not correct for all aspects of Se metabolism, especially the assimilation of inorganic selenite (80).

a) Assimilation of inorganic Se by plants

Studies on the incorporation of Se^{75} selenate and Se^{75} selenite into the various Se-containing metabolites of whole plants have confirmed that macro concentrations of these forms of inorganic Se are subject to similar metabolic fates as inorganic sulphur (80).

Peterson and Butler (83) examined whether inorganic selenite was incorporated into the Se isologues (and/or their derivatives) of intermediates in the pathway, like sulphate into methionine and other

amino acids. They raised the pasture plants, Lolium perenne (perennial ryegrass) for 10 days in nutrient solution containing Se^{75} selenite. Approximately 20-30% of the Se^{75}-label in the pasture plants was associated with selenocystine, selenocysteic acid, selenomethionine, and selenomethionine selenoxide in the ethanol-soluble fraction (83).

Nigam and McConnell confirmed these early experiments in a study of Se^{75} selenate metabolism in wheat and bean non-accumulators and accumulator . However, they found relatively similar proportions of Se^{75}-label in the soluble protein fractions of all three species and concluded that differences in the sensitivity of the species to Se could not be attributed to differences in the incorporation of Se into soluble protein (84).

b) Metabolism of selenocysteine

In general, most of the inorganic Se incorporated by Se accumulators occurs as selenocystathionine and/or Se-methylselenocysteine. In non-accumulators, most of the Se is incorporated into proteins where it is associated with a selenomethionyl residue or is found as free selenomethionine or its Se-methylated derivatives (80).

Several lines of evidence suggest that those accumulators which contain Se-methylselenocysteine synthesise this compound from selenocysteine in a manner analogous to the synthesis of S-methylcysteine from cysteine. Most species which contain Se-methylselenocysteine also contain S-methylcysteine which is synthesised by methylation of cysteine (80).

c) Incorporation of selenium into protein

As noted previously, inorganic Se is incorporated into the protein of non-accumulators where it occurs as selenomethionine, and possibly as selenocysteine in Vigna radiata. On the other hand only trace amounts of Se are incorporated into the protein of the accumulator (83). One explanation could be that accumulators possess mechanisms which select the sulphur amino acids for incorporation into protein but not their Se isologues. Thus, provided that the later stages of amino acid incorporation do not distinguish between the two substrates, these results demonstrate that selenocysteine competes with cysteine for incorporation into protein (80).

v) Is Selenium Essential for Plant Growth?

a) Se requirements of organisms other than plants

The essential nutritional requirements for Se of the rat, certain birds, and various livestock has been reviewed by Stadtman (59). In summary, Se is required only in trace amounts and can be supplied in a variety of inorganic or organic forms. In animals, four gram atoms of Se are associated with each mol of the enzyme glutathione peroxidase. Since conditions such as necrosis of the liver and sensitivity of erythrocyte membranes to oxidative damage are associated with Se deficiency in animals, it is assumed that the reduction of lipid peroxides by glutathione peroxidase forms the basis of essentiality (80).

Se is also required for the expression of several enzymes in some anaerobically grown bacteria. They include glycine reductase from several species of Clostridium. Three other Se-containing enzymes are also known in Clostridia (Stadtman (85)).

b) The function of Selenium in Se-specific proteins

Glutathione peroxidase has been purified to homogeneity and its three dimensional structure determined by X-ray analysis. Enzymes from bacterial sources have been studied less extensively presumably because of their extreme sensitivity to oxygen. Nevertheless, it seems that the Se-containing enzymes studied to date undergo redox changes at the Se centre during enzyme catalysis.

The mode of action of clostridial glycine reductase is thought to parallel that of ribonucleoside diphosphate reductase which involves the interaction of redox-active disulphides with iron and a tyrosinyl free radical.

The inhibition of the enzyme by hydroxylamine, an inhibitor of free radical enzymes, is consistent with this proposal (80).

c) Studies of Selenium essentiality in plants

Essentiality of Se for growth of Se-accumulators was first put forward by Trelease and Trelease (79) when they demonstrated a Se-induced growth stimulation in greenhouse trials. Nine accumulators were reported to have been stimulated and six non-accumulators to have been severely poisoned, but no attempt was made to examine if it was possible for the plants to complete their life cycle in the absence of the Se (79).

Broyer et al. (86) while working with two accumulators and two non-accumulators (grown hydroponically in Se-free and Se-supplemented solutions) demonstrated that the Se-induced growth increases noted by Trelease and Trelease (79) were probably related to an amelioration of the phosphate toxicity present in the hydroponic solutions.

An alternative technique for investigating the essentiality of

Se is to determine whether any of the proteins reported to contain Se in other organisms are present in plants. It is necessary to establish also that the Se cannot be replaced by sulphur and that the protein(s) do not function without Se (80).

Glutathione peroxidase activity has also been postulated as a component process in the dark deactivation of light-modulated enzymes in chloroplasts (87).

The report of selenocysteinyl residues in the protein fraction by Brown and Shrift gives some cause for encouragement, for although it is likely that the selenocysteinyl residues represent non-specific replacement of cysteine, the Se present in the Se-specific proteins examined to date occurs in this form (80,81).

vi) Selenium Toxicity

There are many reports in the literature on the comparable toxicities of selenate, selenite and other seleno compounds towards plants, animals and microorganisms and of the amelioration of these toxic effects by other ions [Rosenfeld and Beath (14)]. Some of these early reports are conflicting, and no useful purpose can be served by detailing these effects yet again. Se toxicities are not confined to whole plants. Stewart et al. reported that Se inhibits organogenesis in detached leaves grown in sterile culture (88). The mechanism whereby the element exerts its toxic effect is not known, but it is believed to involve competition with sulphur metabolism (88). For example, the presence of seleno amino acids completely inhibited the incorporation of sulphur amino acids into proteins, but the biochemical effects are not known in detail (79).

vii) Selenium Accumulators

Ordinarily, plants grown on soils containing high concentrations of Se exhibit selenosis, but Se accumulator species are able to successfully tolerate the element as well as accumulate it to high levels - even up to 1% (dry weight). Miller and Byers reported that the Se-accumulating plants Astragalus and Stanleya contained 4000 and 300 ppm Se respectively, while the non-accumulator grasses grown in the same soil nearby contained only 2-4 ppm Se (79).

Beath and his co-workers classified American Se-tolerant plants according to their Se contents:

Group 1: Primary accumulators which grow only on seleniferous soils and accumulate Se to very high levels. The four major genera are Astragalus, Xylorhiza, Conopsis and Stanleya; these species are Se endemics. These plants accumulate Se in the range of thousands of ppm.

Group 2: Secondary accumulators are plants which accumulate Se to lower levels but are not restricted to seleniferous areas. Some important genera are Aster, Atriplex, Castillega and Grindelia. Plants in this group accumulate Se in hundreds of ppm.

Group 3: Weed and grass species which occasionally grow on these areas. These plants contain relatively low levels of Se, up to 30 ppm (79).

1:2 ENVIRONMENTAL POLLUTION BY HEAVY METALS

1:2:1 Heavy Metals in the Environment

During the last twenty years there has been increasing interest in environmental contamination by heavy metals such as cadmium, chromium, cobalt, copper, lead, mercury, nickel and zinc, as a result of increased industrial activity (89,90).

Heavy metals may be broadly defined as metals with atom weights higher than scandium, and about 95% of these elements are distributed in igneous and metamorphic rocks while the other 5% exist as sedimentary formations (91,112). In nature, heavy metals are compounded as relatively inert ores such as sulphides, carbonates and silicates, and without man's interference will remain so for long geological periods of time; redistribution only being caused by natural processes such as earthquakes, volcanic activity and erosion. Heavy metal deposits in contact with air and water are naturally recycled by chemical and physical action, but their ultimate fate is usually back to inert minerals by their subsequent redeposition in oceanic sediments (92).

The major natural sources originate from:-

i) geological weathering of rocks and minerals;

ii) animal and human excretion (which contains heavy metals).

The important anthropogenic sources for the environment are generally classified as:-

1. those involved with metal smelting and refining
2. emissions from coal and oil fired power generating plants
3. solid waste (disposal metal)
4. burning of leaded petrol (motor vehicle traffic)
5. chemical processing operations
6. cement manufacture (95,96).

In general, interest arose in heavy metals because some are essential for maintenance of normal growth and development, while others are toxic. Also, as a result of modern food technology there is a direct industrial effect exerted on metal concentrations in man by food processing and food preparation techniques (90). Heavy metals can enter the food chain via plant uptake or contamination of fresh waters (93). There has been recognition of a significant association between excessive heavy metal accumulation in man and the incidence of diseases (94).

Table 1:7 gives an estimate of the emission of heavy metals from the combustion of fossil fuels and the production of cement.

Table 1:7 . Metal Emissions (Tons/year) (97)

	Fossil Fuel Combustion		Cement production
	Coal	Oil	
Cobalt	700	30	-
Chromium	1,400	50	-
Copper	2,100	23	-
Nickel	2,100	1,600	-
Vanadium	3,500	8,200	-
Mercury	400	1,600	100
Cadmium	140	2	80
Selenium	420	30	700
Arsenic	5,000	10	3,200
Zinc	7,000	40	30,000
Lead	3,500	50	30,000

1:2:2 Heavy Metals in Fresh Water and Sediments

Heavy metals may exist as a number of different chemical entities in natural waters. Different species can be distinguished in solution and the solid phase. The following may be present in the dissolved state:-

1. as hydrated ions i.e. $Cd(H_2O)_6^{+2}$
2. as a complex with inorganic ligands, such as chloro, carbonato, and hydroxo complex, i.e. more or less labile complexes, i.e. $Pb(H_2O)_4Cl_2$
3. chelated in rather stable and in inert complexes with inorganic ligands such as amines, humic acid, and proteins, i.e. Cu-glycinate (98).

Metal ions can be associated with colloidal particles, or occur adsorbed at or incorporated into suspended matter or sediments. The suspended and colloidal particles may consist of individual or mixed hydroxides, oxides, silicates, sulphides, or other compounds, or they may consist of clay, silica, or organic matter to which metals are bound by adsorption, ion exchange, or surface complexation (98).

In river systems, heavy metals originate to a large extent from:-

a) the abundance of metals in the rocks and soils of a river's catchment area and by their geochemical mobility.

b) mining and smelting activity; because of river run-off over waste tips, and through mines, the heavy metals discharge to the waters. The pollution by such metals may continue long after the mine and smelting activities have ceased (99).

c) wastewater treatment has resulted in detrimental effects on drinking water in some areas, due to the traditional role of the surface waters as receiving bodies for wastewater effluents (97).

Heavy metals in river water reach the sediment in three ways:-

1. in or on particles which settle to the bottom;
2. in or on particles which are transported along the bottom;
3. by the sorption of dissolved metals from waters in contact with the sediment.

The sedimentation of particles is usually the most important route to the bottom sediments. Three classes of particles may be distinguished:

i) Detrital particles - heavy metals contained within the crystal lattice, adsorbed on the surface, i.e. surface coatings formed by hydrous oxides or organic matter.

ii) Biogenous particles - heavy metals contained within inorganic material, adsorbed on surface.

iii) Precipitated particles - e.g. calcium carbonates, hydrous oxides and sulphides which carry heavy metals adsorbed on their surface as co-precipitated material.

As such particles settle through the water column they may be partially dissolved by bacterial attack or by changes in the chemical environment. In addition to physical or hydraulic factors there are several chemical factors which influence the depositional environment; thus pH, salinity, temperature, redox potential, nature and amount of organic matter are the most important (100,101).

Sediments are deposited under either oxidising or reducing conditions. Brownish sediments containing iron oxides are indicative of a fully aerated environment, whereas grey or black sediments containing iron sulphides signify a reducing medium (100).

1:2:3 Toxicity of Heavy Metals

Although heavy metals were formerly important as therapeutic agents, present interest lies primarily with the toxic reactions they are capable of inducing. The problems created by water and air pollution, food contamination, and the widespread use of agricultural chemicals are largely concerned with these toxicants (102).

The toxicity of heavy metals is not restricted to those metals thought to be non-essential. All elements, including the essential ones, are toxic at high concentrations. The toxicity leads to abnormal growth, disease or even death (100).

For all these elements there exists a fairly narrow "concentration window" between the essential and toxic levels. Some essential elements, for example selenium and vanadium at high concentrations, are much more toxic than some non-essential elements such as mercury and thallium (103).

The various factors influencing the toxicity of trace metals in aquatic environments are listed in Table 1:8. . It shows that the potential toxicity of heavy metals is controlled to a very large extent by their physico-chemical forms, and environmental factors such as temperature, pH and salinity (97).

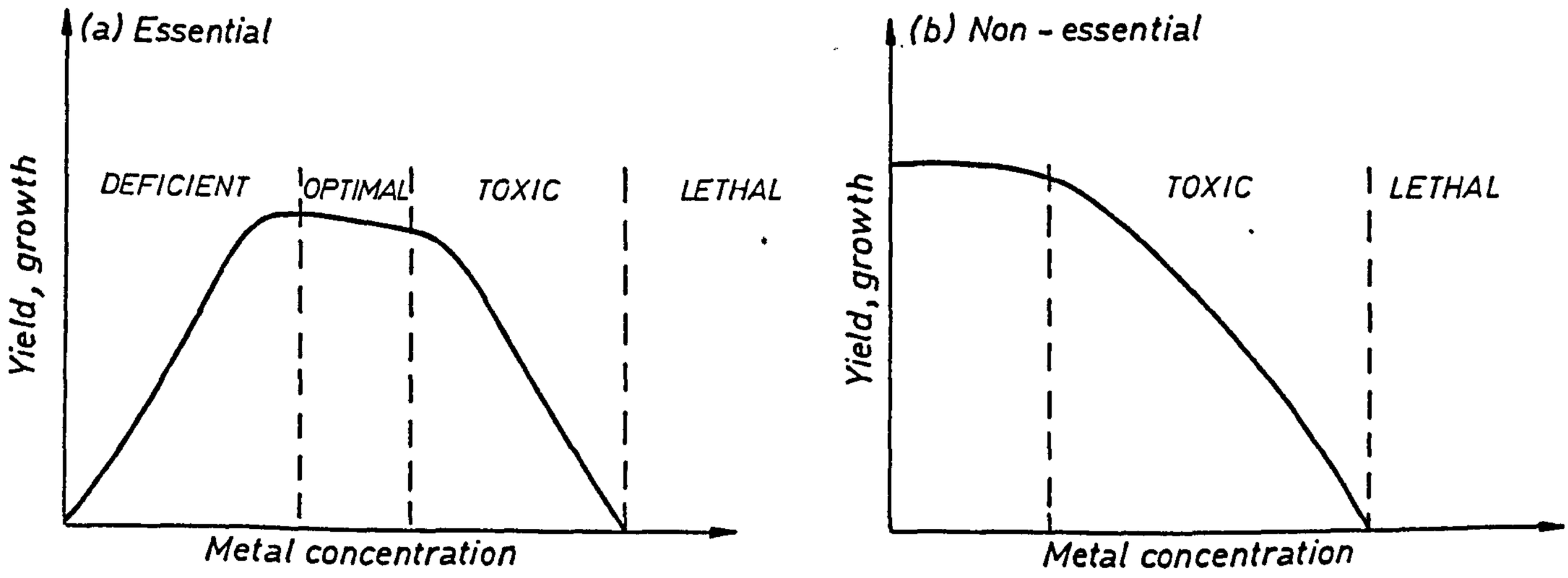
Variation in the speciation of trace elements can dramatically change their bioavailability or toxicity. Cobalamin (Vitamin B12) is the only assimilable chemical form of cobalt, and the group of chromium amino-acid complexes, known as the glucose tolerance factor, provides most of the usable chromium. The alkyl compounds of mercury and lead are especially dangerous because they are lipid-soluble and enter via absorption, while materials such as the lead halide aerosols emitted by automobiles can enter the lungs and be absorbed directly into the blood stream (103).

Table 1:8. Factors influencing the toxicity of heavy metals in solution (97)

- 1. Form of metal in water
  - inorganic
  - organic
  - soluble
    - ion
    - complex ion
    - chelate ion
    - molecule
  - particulate
    - colloidal
    - precipitated
    - adsorbed
- 2. Presence of other metals or poisons
  - joint action
  - no interaction
  - antagonism
- 3. Factors influencing physiology of organisms and possibly form of metal in water
  - temperature
  - pH
  - dissolved oxygen
  - light
  - salinity
- 4. Condition of organism
  - stage in life history (egg, larva, etc.)
  - changes in life cycle (e.g. moulting, reproduction)
  - age and size
  - activity
  - additional protection (e.g. shell)
  - adaptation to metals
  - altered behaviour

Essential trace metals such as zinc become toxic when the nutritional supply becomes excessive. A metal in trace amounts (smaller than 0.01% of the mass of the organism) is essential when an organism fails to grow or complete its life cycle in the absence of that metal. However, the same trace metal is toxic when concentration levels exceed those required for correct nutritional response by factors varying between 40- and 200-fold (97). The toxicity of trace metals follows the general trend that an undersupply leads to a deficiency, sufficient supply results in optimum conditions, while an oversupply results in toxic effects and lethality in the end. These facts are graphically displayed in Figure 1:5a, which illustrates the essentiality of trace metals as a dose-response curve ranging from deficiency to oversupply. Figure 1:5b shows the biological response for non-essential heavy metals; it indicates that the curve does not follow the first part of the curve for the essential metals, but depicts a tolerant range followed by the toxic and lethal regions (97).

Figure 1:5. Deficiency and Toxicity of Essential and Non-essential Heavy Metals



Change in the oxidation state of an element can have a profound effect on bioavailability and toxicity. Chromium (III) is an essential element; but chromium (VI) is highly toxic. Arsenic (III) is much more toxic than arsenic (V).

Heavy metals are toxic to a wide range of aquatic animals. Based on the free metal ion concentration, the order of toxicity to a marine diatom was found to be (103):

$$Hg^{2+} > Ag^{+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Tl^{+}$$

The metal ions used by biological systems must be both abundant in nature and readily available as soluble species. Abundance generally restricts the available metals to those of atomic number below 40. Some of these are virtually unavailable due to the low solubility of their hydroxides, e.g. aluminium and titanium. Viewed from the standpoint of environmental pollution, metals may be classified according to three criteria: (1) noncritical, (2) toxic but very insoluble or very rare, and (3) very toxic and readily accessible. Such a classification is listed in Table 1:9 (97).

Table 1:9. Classification of Elements according to Toxicity and Availability

Noncritical (1)			Toxic but very insoluble or very rare (2)		Very toxic and relatively accessible (3)		
Na	C	F	Ti	Ga	Be	As	Au
K	P	Li	Hf	La	Co	Se	Hg
Mg	Fe	Rb	Zr	Os	Ni	Te	Tl
Ca	S	Sr	W	Rh	Cu	Pd	Pb
H	Cl	Al	Nb	Ir	Zn	Ag	Sb
O	Br	Si	Ta	Ru	Sn	Cd	Bi
N			Re	Ba		Pt	

Some of the non-essential trace elements are used therapeutically, like Al, Au, Bi, Ga, Li and Pt (104).

The safe upper limit for heavy metals and selenium content of drinking water is listed in Table 1:5 (see page 20) (45).

1.2.4 Uptake of Heavy Metals by Plants

Green plants are the miners of the earth's crust, being the major selective accumulators of inorganic nutrients upon which other life forms are directly or indirectly dependent. The initial accumulation of ions by terrestrial plants is a function of the root system which exhibits an extensive ramification through the soil.

Plants will absorb to some extent any element presented to them in nutrient media but the ionic content of plants will vary, reflecting selectivity and species variation, the availability of nutrients in different rooting media, or the stage of development of the plant (105).

The mechanism of uptake of elements by plants is not known, however it has been proposed that:-

1) plant roots excrete hydronium ions H_3O^+ at their surface so that exchange with cations can occur. Similarly it would be expected that roots would excrete hydroxyl or bicarbonate ions to exchange with anions.
2) metal ions move to the root surface by diffusion so that adsorption of ions on the root surface occurs whether it is dead or alive (106).

However, when the metal ion is taken up by the roots, it can either remain at the roots or is translocated to the shoots. The distribution

of cations within the plant may be heterogeneous; certain elements such as lead, nickel, copper, zinc, iron, manganese, chromium and vanadium being preferentially retained by the root system, whereas calcium and potassium are transported in major part to the shoot system. The pattern of distribution of these elements may be considerably affected by their concentration in the external medium (105).

The availability of heavy metals to plants in a soil-water system is dependent on a number of factors such as inorganic and organic complexing agents, adsorptions onto clays and organic matter, metal-metal interactions, coprecipitation with other elements, formation of sparingly soluble salts and chemical species (107).

Plant-soil relationships have been proposed, and mechanisms of adaptation to toxic metals; three basic concepts should be mentioned:-

a) Accumulators - these plants are defined as those concentrating metals within their above ground plant parts from low or high soil levels; in certain cases "hyperaccumulators" if the plant exceeds 1000 ug metal/g dry weight of plant.

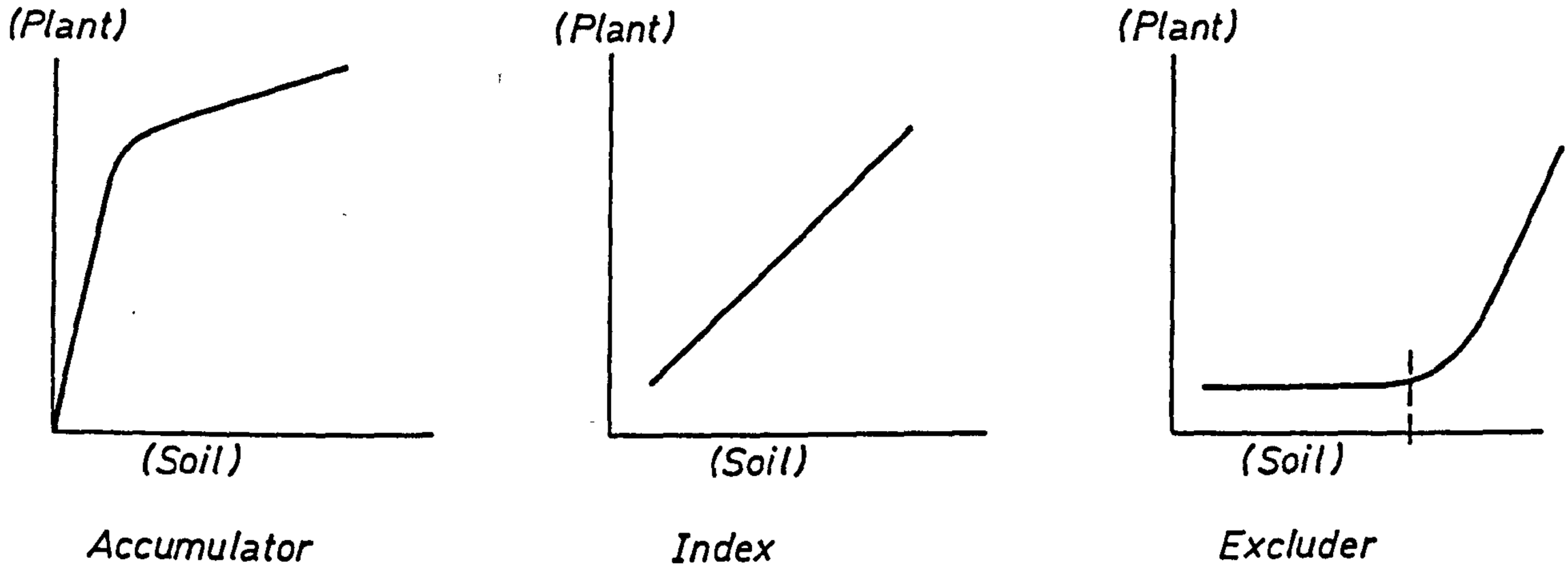
b) Index plants whose tissues reflect soil concentrations.

c) Excluders, or plants which restrict transport of metals into their tissue (108).

These three plant-soil relationships are demonstrated in Figure 1:6 (100).

Heavy metal tolerance in plants has been demonstrated. The mechanisms of tolerance, whilst widely discussed, are still not fully understood, but are better understood in animals, where greater tolerances can be induced by prior treatment by exposure of the animal to, say, cadmium at rather low levels.

Figure 1:6 . The Response of Plants to Increasing Soil Metal Levels



However, detailed investigations of the tolerance of Holcus lanatus L. to cadmium, lead and zinc have been carried out. Experiments were conducted with this species to test if increased tolerance may also be induced in plants (109). The tolerance of plants to toxic metals is frequently measured by comparing the rates of root growth in culture solution with and without the addition of the metal (110).

Regarding the adaptive mechanisms of plants to the toxic effects of metals, three basic concepts must be mentioned:-

1. Accumulators: Studies have revealed the presence in "accumulator" plants of high concentrations of some inorganic ions, low molecular weight organometallic compounds, as well as high molecular weight metal-containing biopolymers. The following tolerance mechanisms have been proposed:-

(a) Low molecular weight compounds (108):-

i) Seleno-amino acids (see Se and plants, 1:1:6) (80).

ii) Nickel-organic acid: Ni is complexed with malic and malonic acids.

iii) Chromium-organic acids: Cr complexes such as trioxalato chromate (III) ion, $[Cr(C_2O_4)_3]^{3-}$, Cr being transported in the xylem sap as chromate, indicating that metabolism to the complex took place within the leaf tissues. Presumably the function of Cr-organic acid complex would be to reduce the cytoplasmic toxicity associated with Cr^{3+} and chromate ions.

iv) Zinc tolerance where the zinc is complexed as Zn-malate (108).

(b) High molecular weight compounds:-

i) Metallothionein: Cd-thionein and Cu-thionein; metallothionein-like proteins exist in plants.

ii) Cell wall adaptations: the reduced transport of Cu from roots to shoots of some grasses has been studied. The tolerance mechanism in both Zn- or Cu-tolerant grasses has been shown to be of a similar type (108).

2. Index: "index" plants do not control their metal uptake and transport processes, they merely reflect soil concentrations.

3. Excluders: the concept of metal exclusion from plants is not well established although there are several examples where metals are restricted to varying degrees in the roots. Lead is normally considered to be accumulated in roots with little transported to the leaves (108).

Heavy metals uptake and distribution between tolerant and non-tolerant populations of plants depends on the species and the metal. Coughtrey and Martin (111) reported that the root/shoot ratio concentration of cadmium in tolerant Holcus lanatus is significantly higher than

in the non-tolerant form. It appears that Cd is readily taken up from solution but that 85-91% is then localised within the roots, resulting in low concentrations in the shoots relative to the roots (8.6-12%). This localisation of Cd within the roots forms the basis of Cd tolerance in the tolerant population of Holcus, so preventing the toxic effect. For example, with Lolium perenne, the proportion of Cd in roots remained constant throughout a wide range of solution concentrations (111).

1:3 BIOLOGICAL INTERACTION OF SELENIUM WITH OTHER ELEMENTS

1:3:1 Introduction

Selenium does not ameliorate the toxicity of all metals by a common mechanism. One way selenium apparently counteracts arsenic toxicity is by increasing the biliary excretion of this element (113). Se does not alleviate the effect of heavy metals such as Hg, Cd, Ag, Te or Tl by decreasing tissue retention, but instead Se results in an increased tissue deposition of these metals. When injected, Se appears to counteract the toxicity of inorganic mercury and cadmium by diverting their binding in low molecular weight proteins to higher molecular weight ones where the metal is less available. Since Se does not divert the binding of these metals when given orally, another mechanism of biological counteraction is apparently in operation under environmental conditions. Silver promotes the development of liver necrosis in Se and vitamin E deficient rats, but this effect is not shared with other heavy metals such as Hg, Pb, Cd or Tl. Dietary Ag and Hg (but not Pb or Cd) significantly depress the activity of glutathione peroxidase and Se concentrations in tissues of rats. The level of vitamin E, cystine, or arsenic are some of the dietary factors influencing the effects of Se against heavy metal toxicity, as shown in Table 1:10 (22). Thus, the mechanism in which Se counteracts heavy metal toxicity under environmental conditions has largely yet to be determined (22,113).

1:3:2 Cadmium

One of the most extensively studied interactions is that of selenium with cadmium. Se, like cysteine, attains the highest concentration in the kidneys, yet unlike cysteine does not increase the kidney uptake of cadmium, nor the damage by Cd when in this organ. Se, like cysteine, protects the testes against Cd-induced damage in spite of the fact that Se is able to increase the testicular level of Cd up to six times the level of the control and nearly three times that of cysteine-treated mice (114).

Se is also effective in protecting against lethal doses of Cd. Therefore, Se appears to have a general detoxifying effect on this element. The interactions between Se and Vitamin E with heavy metals are outlined in Table 1:10 (22).

Although Se protects effectively against Cd-induced testicular injury, it actually increases the Cd content in the testis, indicating that Se causes a redistribution of Cd. Marked conversions of Cd from its presence in 10,000- and 30,000-molecular weight proteins to large-molecular weight proteins were also observed (Chen et al., 1975) (115).

Se was found to give a 22-fold increase in the Cd content in the blood, while decreasing that in the liver by 48% and 12% in the kidneys. The conversion in the binding of Cd in the soluble fraction to a higher-molecular weight protein was also observed in the kidneys and plasma (22). This process may be a second mechanism involved in the protection of these organs against Cd (by Se) (115).

Cadmium treatment can potentiate drug response and inhibit hepatic microsomal drug metabolism in male rats. Studies have attempted

Table 1:10. Relative Effectiveness of Selenium and Vitamin E against Heavy Metal Effects (22)

Metal	Influence of selenium and Vitamin E
Arsenic	Arsenic reduces selenium toxicity.
	Vitamin E has not been tested.
Bismuth	Selenium affects tissue distribution of bismuth.
	Vitamin E has not been tested.
Cadmium	Selenium is highly effective against cadmium damage.
	Vitamin E has not been tested.
Cobalt	Affects selenium absorption.
	Vitamin E has not been tested.
Copper	Affects selenium release indirectly.
	Vitamin E has not been tested.
Lead	Selenium has little effect on its toxicity.
	Vitamin E is highly protective.
Manganese	Deficiency parallels selenium deficiency.
	Vitamin E has not been tested.
Inorganic mercury	Selenium is effective against its toxicity.
	Vitamin E has very little influence on its toxicity.
Methylmercury	Selenium is highly effective against its toxicity.
	Vitamin E is effective but not as good as selenium.
Silver	Selenium is effective as excessively high levels.
	Vitamin E is highly effective against its toxicity.
Tellurium	Enhances selenium deficiency.
	Vitamin E has not been tested.
Thallium	Selenium protects against toxicity.
	Vitamin E has not been tested.
Vanadium	Enhances selenium deficiency.
	Vitamin E has not been tested.

to clarify the role of Se on this hepatic mono-oxygenase system (22).

Early and Schnell (1981) have examined the effect of Se on Cd-induced inhibition of drug metabolism in male rats. Prior administration of sodium selenite (1.6 mg Se/kg) blocked the Cd-induced (0.84 mg Cd/kg) inhibition of hepatic microsomal biotransformation (detoxification) of ethylmorphine or aniline (22).

1:3:3 Mercury

It has now become clear that the distribution and toxicity of both inorganic and organic mercurials in mammals, including man, are affected by, and can be obviated by, the presence of ligands which interact with the mercury. Thus, Se or Se compounds protect animals from lethal doses of mercuric compounds (116).

Parizek and Ostadalova (1967) first demonstrated that selenite, and to a lesser extent selenomethionine, markedly decreased the acute nephrotoxicity of mercuric mercury in rats, as long as the Se compound was administered after the mercury compound. If, on the other hand, Se was given before mercuric mercury, the males showed an increased mortality (22). The physiological significance of this interaction was not fully realised until Ganther et al. (117) found the Hg in tuna fish to be less toxic, possibly because of its Se content (113).

The protective effect of Se against Hg poisoning in the rat has since been confirmed by several investigators. Burk et al. (1974)

made similar observations of the antagonism of Se on $HgCl_2$ poisoned animals. On the other hand, mercuric mercury did not affect the toxicity of selenite. In a similar way, Se has been observed to reduce the toxicity of methylmercury when administered at the same time (22).

The effect of Se on the tissue distribution of mercury is dependent on the dose of Se given: maximum uptake of mercury by blood and liver was produced by approximately equimolar doses of Se. Contrary to Se which exerts its protective effect by decreasing the rate of mercury uptake in the target organ, cadmium pretreatment increases the kidney uptake of mercury (114).

Cappon and Smith (1981) have studied the content, chemical form and distribution of Hg and Se for selected human and animal tissue samples by GLC. Methylmercury averages for human brain, heart, spleen, liver, kidney and placenta were 38.7%, 40.2%, 57.0%, 39.6%, 6.0% and 57.1% respectively of the total mercury content. The amounts of Se paralleled the amounts of Hg (22).

In general, the counteraction of Hg toxicity by Se has been proved; although Se causes more Hg to accumulate in all tissues (except possibly the kidneys) in animals exposed to Se + Hg rather than Hg alone, the form of the mercury is less toxic, possibly due to the formation of higher molecular weight proteins. The effect of Se on the amount of Hg deposited in tissues is dependent upon the chemical form of Hg and also upon the chemical form of Se (113).

The dietary cystine, Vitamin E and arsenic content are other factors which apparently influence the effectiveness of Se against Hg toxicity. The combination of Se and cystine produced a greater additive effect against Hg toxicity than either one alone.

Since Se diverts the binding of Cd, studies were designed to determine whether Se would also act in a similar manner for the binding of inorganic Hg. Injection of Se, 30 minutes before the injection of an Hg salt, resulted in almost complete diversion of Hg binding from low molecular weight (MW) proteins (about 10,000) to larger MW ones (about 150,000) in kidneys, liver, plasma and testes.

Since Se causes Hg to accumulate in tissues, the protective action of this element against heavy metals is obviously not related to lower tissue accumulation of these metals. Thus, the effects of Se on inorganic Hg metabolism are similar to those observed for Cd. Se causes diversion of metal binding when injected, but not when given orally (113).

1:3:4 Tellurium

In a study on ducklings, tellurium (Te) toxicosis resulted in the development of cardiac lesions that resembled those of Se-Vitamin E deficiency. Ducklings fed 500 ppm $TeCl_4$ developed characteristic clinical signs and the pathologic alterations of Se-Vitamin E deficiency, in spite of being fed diets adequate in Se and Vitamin E. Affected birds had anorexia, slowed growth, a reluctance to stand, and eventually many died (22). The birds fed Te had marked vascular injury with prominent epicardial and myocardial congestion, edema, and haemorrhage. Myocardial necrosis involved all chambers and tended to involve the full wall (118).

Clinical signs and lesions were prevented completely in ducklings fed Te with supplements of Se at 5.0 ppm as sodium selenite or Vitamin E as α-tocopherol. Clinical signs and lesions were partially protected by addition of Se at 1.0 or 0.1 ppm as sodium selenite, Vitamin E as α-tocopherol (22).

1:3:5 Thallium

Hollo and Zlatarov (1960) reported that death in rats due to thallium poisoning could be prevented by the parenteral administration of selenate. This observation was later confirmed by Rusiecki et al. (1966), who showed that oral administration of selenate prevented the toxicity of thallium. The content of thallium in liver, kidneys and bones was markedly increased by the selenate treatment. Conversely, the subcutaneous injection of thallium acetate increased the retention of selenium in the liver and kidneys and also diminished pulmonary and urinary excretion of selenium. In Vitamin E and Se-deficient rats, 10 ppm of dietary thallium did not promote liver necrosis (22,113).

It can be assumed that the interrelationship between thallium and Se is similar in character to that between Se and Cd or Hg and it is possible that the same applies to the interrelationship between Se and silver (119).

1:4 OBJECTIVES

The objectives of the present work were:-

1. to determine selenium using GLC-ECD at high sensitivity with comparable precision and accuracy;
2. to synthesise the most sensitive reagent reported, namely 3,5-dibromo-1,2-diaminobenzene and the recently prepared 4-fluoro-0-phenylenediamine and 4-trifluoromethyl-0-phenylenediamine;
3. to synthesise, characterise and investigate similar fluorinated ligands;
4. to compare the synthesised ligands in terms of sensitivity;
5. to assess the applicability of the best reagent:
   a) for the determination of the total level of Se in water, sediment and plant samples,
   b) for the speciation of the Se forms in river water samples,
   c) to compare such results with graphite furnace AAS;
6. to investigate the effect of Se, Cd, Hg, Te and Tl on Lolium perenne by measuring critical and lethal levels;
7. to determine the mercury in plant samples by GFAAS and CVAAS;
8. to determine thallium in plant and sediment samples by GFAAS and DPASV;
9. to investigate the interactions (if any) between the toxic element uptake (Se, Cd, Hg, Te and Tl) by plants using response surface methodology.

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CHAPTER 2

DETERMINATION OF SELENIUM AND TRACE METALS

CONTENTS

2:1 INTRODUCTION

Any work involved with environmental pollution analysis should properly begin with a definition of "pollutant". The term has been defined as any substance which changes the natural composition of the environment. However, the environment has been changing since the world began, and its natural composition is difficult to identify. Man has played, and continues to play, a major part in bringing about changes and for the purpose of this discussion the term pollutant means a material that enters the environment primarily as a result of man's activities (1).

Environmental pollution is currently creating problems on a global scale, and one of the major factors contributing to this pollution is the ever-widening use of industrial and agricultural chemicals and their eventual introduction into the human and wildlife food chains (2).

The determination of traces of elements such as Se, Cd, Pb, Cu, Zn, Ni (3), Hg, Tl (4) and Te (5) in biological materials or in environmental samples, at ng/g (ppb) and lower levels relevant materials, assumes greater importance as more and more elements are recognised to exert adverse or beneficial effects on life. Consequently, the question of what may be a normal concentration in the pertinent matrix and what may be a toxic or deficient concentration is of the utmost relevance? In order for these inherently difficult analytical problems to be solved, new procedures have to be matched to the new requirements, as regards high sensitivity, accuracy, ease of handling and economy (6).

For example, selenium occurs naturally in biological materials with a content that varies from a few parts per billion to a few percent. Methods for the determination of selenium, therefore, have

to cover a wide range of concentrations. As a result a large number of procedures for selenium determination have been developed.

Because of selenium's toxicity, as well as its occurrence in a variety of valence states (such as Se^{-2}, Se^{o}, Se(IV) and Se(VI)), great interest has also been shown in the determination of these valency states (7).

Many analytical methods based on a variety of principles are available for the determination of Se and other toxic metals; these include spectrophotometry, fluorimetry, atomic absorption spectrometry, gas chromatography, polarography, voltammetry (ASV, CSV), neutron activation analysis, X-ray fluorescence and gravimetry.

Spectrophotometry, X-ray fluorescence and gravimetric techniques are usually insufficiently sensitive for biological analysis or environmental samples. Neutron activation analysis has the required sensitivity, but is expensive, slow and not available to many laboratories. Therefore attention will be paid to those techniques which have application to environmental situations (3,8).

2:2 METHODS FOR THE DETERMINATION OF Se AND TRACE METALS

2:2:1 Gas Chromatography

i) Introduction

Chromatography is a process for the separation of a mixture of compounds. It involves the transport of the solute compounds past a stationary phase by means of a mobile phase. By definition, gas liquid chromatography (GLC), therefore, is a method of isolating compounds which are, or can become, gaseous. The stationary phase is a viscous liquid and the mobile phase is a gas. The stationary phase is held and spread homogeneously onto the surface of a particulate material packed into the column. The technique is often known as partition chromatography since the separation is based on the partition of sample molecules between the stationary phase (liquid) and the mobile phase (gas). During elution, wherever a solute molecule is in the liquid phase, it is essentially stationary, and hence no migration towards the end of the column takes place. Components which are more soluble in the liquid phase (probably the solute and stationary phase are of similar polarities) will be retained longer than those which are relatively less polar and more soluble in the gas phase. Hence a separation is achieved (9).

ii) Theory

It is advantageous to offer a brief discussion of the theory of chromatography, because it is necessary for an understanding of the GLC technique.

a) Partition Coefficient

When a solute enters a chromatographic system, it immediately distributes between the stationary and mobile phases. The equilibrium distribution between the two phases is reflected by the ratio of the weights of solute in equal volumes of both phases (liquid and gas) (9):

$$K = \frac{\text{concentration per unit volume liquid phase}}{\text{concentration per unit volume gas phase}}$$

Therefore, by careful manipulation of the parameters affecting K, namely column temperature, nature and amount of the stationary phase, flow rate, etc., a reasonable value of K is attained. If K is too high (> 8), the solute takes too long to elute, whereas if K is too small (say < 2) then the solute is eluted too quickly and great demands are made on the GLC system. When interfering peaks are present, say from real samples, it may be necessary to vary the conditions slightly so as to move the solute peak to a clear region of the chromatogram.

b) Number of Theoretical Plates and Plate Height

The quantity that measures the column efficiency and is related to the peak width is called the plate height, sometimes known as the height equivalent of a theoretical plate (HETP), which has the dimension of distance. A high plate number usually indicates a minimum of band spreading (narrow peaks) and, therefore, better resolution. N is defined as:

$$N = 16\left(\frac{t_R}{W_b}\right)^2 = 5.54\left(\frac{t_R}{W_{\frac{1}{2}}}\right)^2 \qquad (2.1)$$

where N is the number of theoretical plates
t_R is the retention time of the solute
W_b is the peak width at the base
$W_{\frac{1}{2}}$ is the peak width at half height

The height equivalent to a theoretical plate, HETP, or, more simply, H, is the parameter most often used to compare band spreading in different columns. It is defined as:

$$H = \frac{L}{N} = \frac{L}{16}\left(\frac{W_b}{t_R}\right)^2 \qquad (2.2)$$

where L is the column length (10,11).

Plate height, rather than plate number, is a more meaningful measure of the column efficiency since it is independent of the column length. More importantly, from a theoretical point of view, plate height can be directly related to the experimental conditions and parameters (10).

c) Van Deemter Equation (10,12)

For GLC progress to be made the influence of the column on the eluted peak width had to be ascertained in order to minimise the extent of spread and maximise the efficiency of chromatographic separation of the columns.

The Van Deemter equation (also known as the rate theory) considers the chromatographic process as a chemical reaction moving along the length of the column and consists of the mathematical solution to a series of partial differential equations. The Van Deemter equation, although one of the earliest, still remains most popular because of the ease of explanation of the observed results in terms of a theoretical basis. The plate height is a function of thermodynamic and kinetic processes that take place in the column, which include:

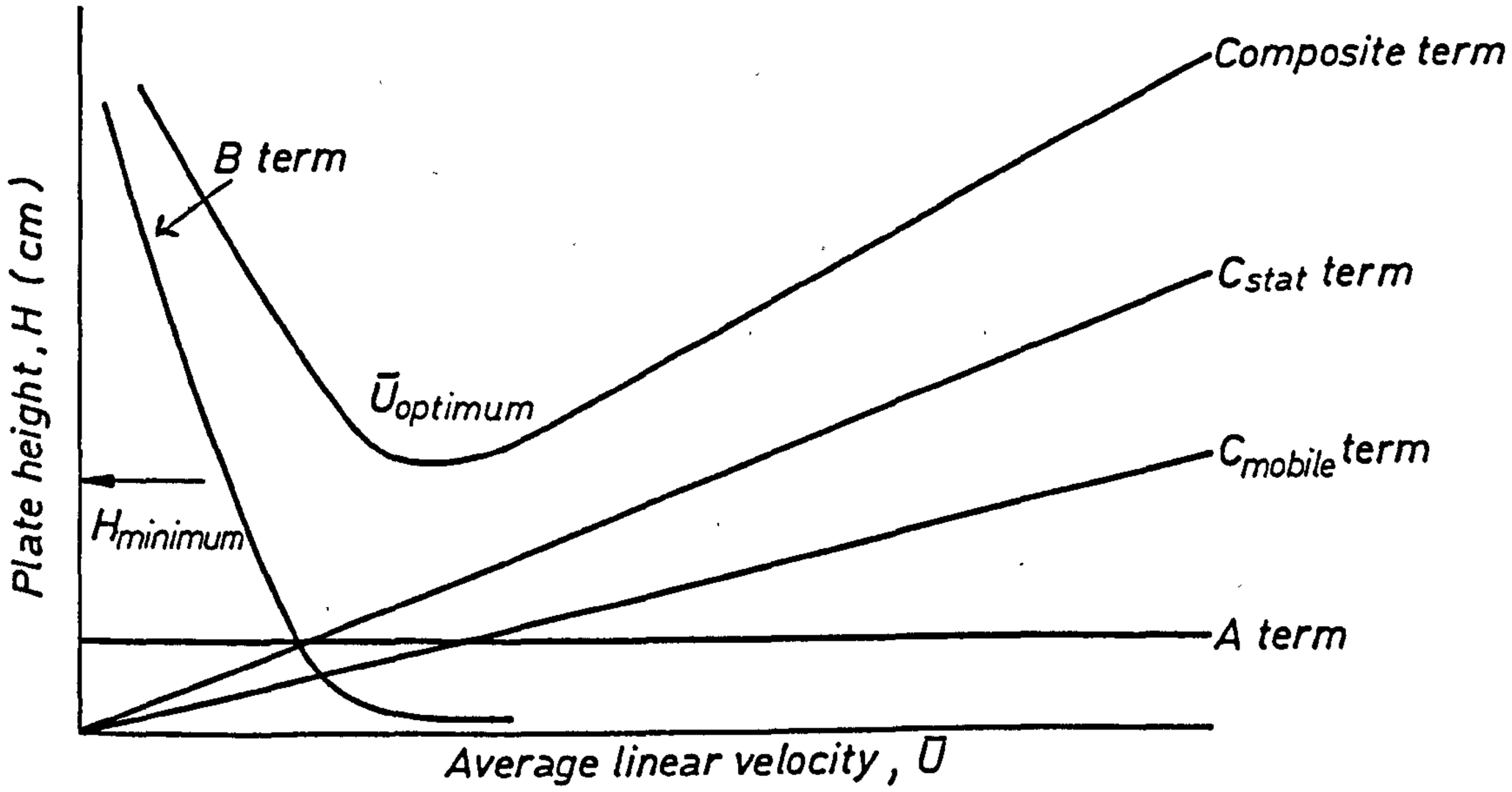
i) transverse and longitudinal diffusion in the mobile phase,

ii) a finite rate of equilibration of solute between the stationary and mobile phase (mass transfer),

iii) diffusion in the liquid stationary phase, and

iv) flow irregularities leading to convective mixing.

Plate height is an effective way of expressing in simple terms the extent of band broadening.



Typical H/U curve for GL-column

For the sake of simplicity, the Van Deemter equation can be abbreviated to:

$$H = A + B/U + C_L U \qquad (2.3)$$

The A term is referred to as the Eddy Diffusion term and signifies the peak broadening due to the packing of the column. The B term arises from the molecular or axial diffusion of the solute within the carrier gas superimposed upon the general transport. The final term, C, relates to the lack of equilibrium and can be regarded as the resistance to mass transfer in the liquid phase (9,10).

For a packed column in which the stationary phase is coated onto small inert particles packed in a column:

$$H = 2\lambda d_p + \frac{2\gamma D_G}{U} + \frac{8}{\pi^2} \cdot \frac{K}{(1+K)^2} \cdot \frac{d^2 f}{D_L} \cdot U \qquad (2.4)$$

where:

U is the linear carrier gas velocity (flow rate),

d_p is the average particle diameter,

d_f is the thickness of the liquid layer on the particles,

D_G is the diffusion coefficient of the solute in the carrier gas,

D_L is the diffusion coefficient of the solute in the liquid phase,

γ is the tortuosity factor, the ratio of the straight path length through the column to the average real path travelled,

λ is a geometric factor indicating the packing uniformity of the column, and

K is the partition ratio = $\frac{\text{time spent in liquid phase}}{\text{time spent in gas phase}}$

Since H changes inversely with U in the B term and directly in C terms, the optimum value of U is defined as the highest possible efficiency of a column at a given partition ratio of a solute.

A plot of H against U gives a curve, the minimum of which corresponds to the optimum carrier gass velocity referred to as U_{opt}. Thus, column efficiency diminishes as the carrier gas velocity is increased or decreased relative to U_{opt} (9).

iii) Electron Capture Detector (ECD)

a) Theory of Operation

The ECD measures a loss of signal rather than an increase in electrical current. As the nitrogen carrier gas flows through the detector, a radioactive foil, usually nickel-63, ionizes the nitrogen molecules, and slow electrons are formed. The electrons migrate to the anode, which has a potential of about +90 V. When collected, the slow electrons produce a steady current, which is amplified by an electrometer. The background current is about 0.01 μA. If a sample containing electron-capturing molecules is introduced into the detector, this current will be reduced because these molecules attract electrons and thus cause a change in background current. The loss of current is a measure of the amount and electron affinity of the sample. Appropriate calibration can relate the loss in current to the concentration of the sample (11).

b) Detector Designs

The ECD was first described in 1961 by Lovelock (13,14); he used a parallel-plate design, in which the two electrodes are flat surfaces parallel to each other. Varian Aerograph developed the concentric-tube design in 1962, where one electrode is a metal cylinder. As the column effluent flows through this cylinder, the charged particles are exposed to the field created by the cylinder. The concentric-tube design has proved to be simple to operate and works well in the simple DC mode (11) and in the pulse mode. Analyses in the pulsed mode are normally carried out with the pulse space "μs" (usually 150 μs) and the typical standing current at this pulse euqal to 1.7 x nA (36). Both DC and pulse modes can be operated especially with dry nitrogen as the carrier gas (11).

Ni-63 is now used mainly as the radioactive source, supplying the primary ionization of the carrier gas by bombardment with beta particles and can be used up to 400°C. The ^{63}Ni source can be used for months without cleaning of the foil (10,11). Figure 2:1 shows the ECD design.

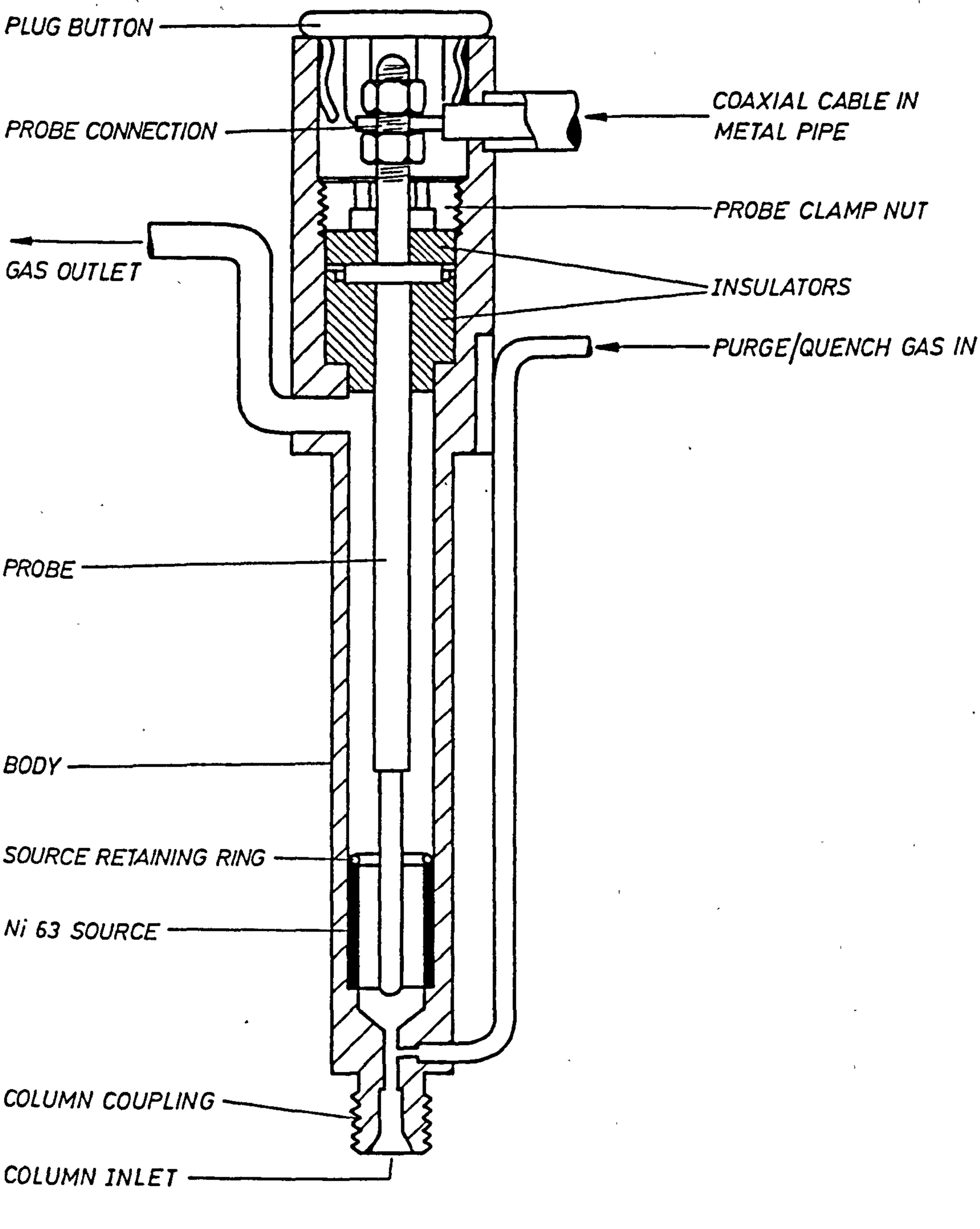
c) Selectivity and Sensitivity

Thus the ECD is extremely sensitive to certain molecules, such as alkyl halides, nitriles, polyhalogenated aromatics and organometallic compounds (11) such as selenium (15). The ECD selectively responds to molecules containing electronegative atoms. The order of increasing response is F < Cl < Br < I. These atoms easily attach or attract an electron and thus cause a change in the background current. The extreme sensitivity at picogram (pg) level to halides makes this detector especially valuable for the analysis of pesticides. Other substances can also be determined by an ECD at extremely low concentrations if they may be derivatised or eluted from the column in the form of electron-capturing derivative (10,11), such as selenium (IV) with a chosen orthophenylenediamine (ligand) (15). The ECD is virtually insensitive to saturated hydrocarbons, alcohols, and ketones (11).

d) Linearity

All electron-capture detectors suffer from the disadvantage of a narrow linear range of response (about 1.7 nA); while the flame ionization detector FID has a wide linear dynamic range (10^{-14} A to 2×10^{-7} A) (11,36). As ECDs are easily saturated, very small samples should be injected. Also, the sample must be dry, because traces of water destroy the normal detector response (11).

FIGURE 2.1 SECTION THROUGH LENGTH OF ELECTRON CAPTURE DETECTOR



Nitrogen as a carrier gas is very popular and it is recommended by many workers (8,25-27,29-34) but argon mixed with 5-10% methane gives a signal about ten times greater than nitrogen (10).

iv) Measurement of Selenium by GLC + ECD

Estimation of selenium by GLC is based almost exclusively on measurement of the amount of piazselenol formed by the reaction of Se(IV) with a chosen o-phenylenediamine (o-PDA), in an acidic solution (usually HCl) (15). Piazselenols are easily extracted with organic solvents (most frequently toluene) in which they can be subsequently determined by spectrophotometric, fluorimetric, or chromatographic methods. In GLC, piazselenols are usually estimated using ECD due to its extremely high sensitivity and selectivity with respect to these compounds (Table 2:1). Apart from the superior sensitivity and selectivity, the GLC method allows for the elimination of many interferences from the matrix (16).

Historically, the parent piazselenol was first synthesised in 1889 by Hinsberg (17) who demonstrated that only Se(IV) reacted with o-PDA to form a Se complex. However, it was not until 1948 that Hoste (18) used 3,3′-diaminobenzidine (DAB) as reagent for selenium, while Hoste and Gillis (19) in 1955 used DAB to form an Se complex for analytical purposes. Among the first to employ this reagent were Cheng (20) in 1956 in a spectrophotometric method, and Watkinson (1960) in a fluorimetric method (21). Yet, it was apparent at that time a better (on toxicity grounds) as well as a more sensitive reagent was needed and this led to the introduction of o-PDA and 2,3-diamino-naphthalene (DAN). Thus, in 1960, Ariyoshi et al. (22) described a spectrophotometric method using o-PDA and Parker et al. (23) in 1962

Table 2:1. Analytical Methods Using Piazselenols and ECD for Se Determination in Various Materials

Piazselenol (Reagent)	Materials	Detection limit in Sample	Gas Chromatography Conditions	Reference
5-Chloro-	Marine biological	27 ng	Glass column, 150 x 0.32 cm 2% DEGS at 170°C or 2% SE-30 at 163°C column temperature	32
5-Bromo-	Marine biological	13 ng	As for Ref. 32 above	32
5-Nitro-	Human blood, urine, hair, placenta	1×10^{-12} g (as detection limit)	1.5 ft x 0.25 in column with 7% OV-225 on Suprasorb (AW-HMDS), 210°C	8
5-Nitro-	Marine sediments	20 ng Se/g or 0.2 pg	200 cm borosilicate glass column 3% OV-225 on Chromosorb W, at 200°C	25
4,6-Dibromo-	River water and sea water	2 ng Se/L	Glass column 100 x 0.3 cm, 15% SE-30 on Chromosorb W, 200°C	30
4,6-Dibromo-	Human blood	2 ng	As for Ref. 30 above	31
5,6-Dichloro-	Food, plants, tissues	10 ppb	a) Pyrex column, 150 x 0.3 cm, 1.5% OV-17 + 1.95% QF-1 on Chromosorb W-H.P, at 200°C b) As (a) except 2% DEGS + 0.5% H_3PO_4 at 170°C	34
5-Trifluoromethyl-	Biological	5 ppb	Glass column 200 x 0.2 cm, 5% or 10% SE-30 on Chromosorb W-AW-DCMS at 148°C	26
5,6-Benzo-	Blood, urine, river water	5×10^{-10} g (as detection limit)	Stainless steel 6 ft x $\frac{1}{8}$ in, 3% SE-30 on Chromosorb G at 165°C	35

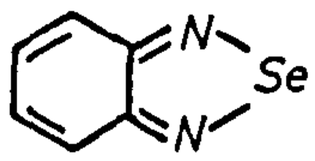
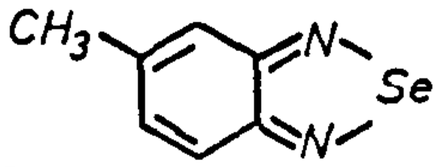
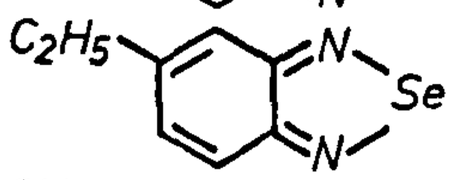
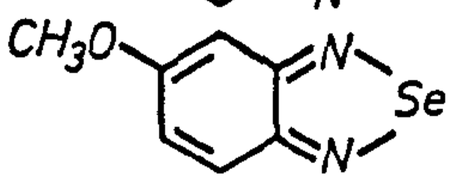
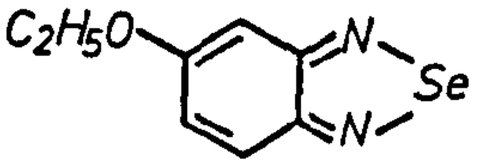
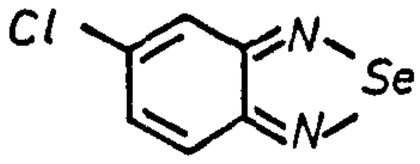
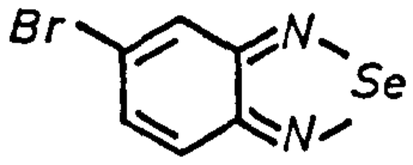
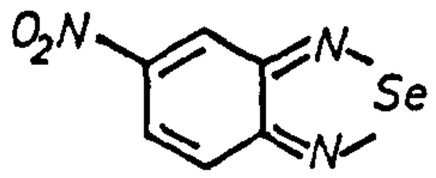
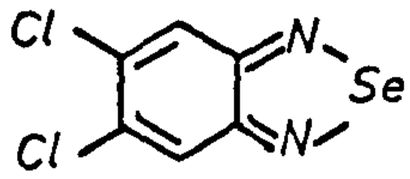
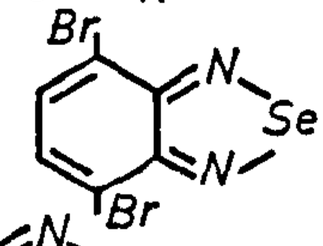
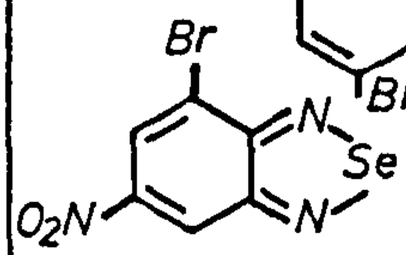
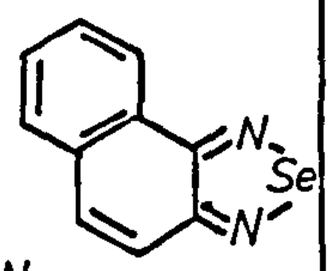
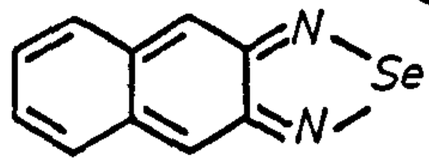
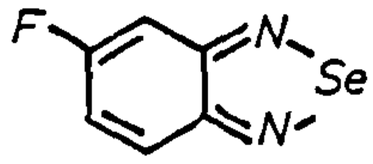
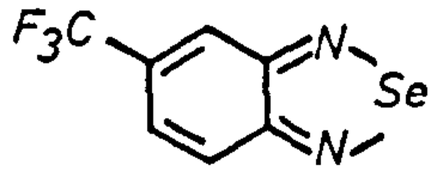
used DAN in a fluorimetric method. Subsequently, from this point the development of spectrophotometric and fluorimetric methods diverged, with the former leading to GLC methods whereas the latter, through many improvements (15), exists today in recognised procedures such as those suggested by the Official Methods of Analytical Chemists (37). The introduction of a second electrophore into the molecule, such as the fluoro, chloro, bromo or nitro group, considerably improves the sensitivity allowing picogram (10^{-12} g) amounts of selenium to be determined (8,24,25,26,27).

Tanaka and Kawashima (1965) reported that o-PDA and its 4-substituted derivatives, 4-methyl-, 4-chloro- and 4-nitro-, all react with Se(IV) in acid solution to form complexes which can be extracted into toluene (28).

However, in 1968 Nakashima and Toei first used the GLC + ECD method for the determination of Se with 4-chloro-o-phenylenediamine, achieving a sensitivity of 40 ng Se (29). Shimoishi (1977)(27), looking for increased sensitivity in the determination of Se, studied 13 derivatives of 1,2-diaminobenzene, and found the sensitivity of the substituents was H < Cl < Br < NO_2. Of the compounds studied 4,6-dibromopiazselenol had the highest sensitivity and was capable of detecting a level of 1 ng Se, while the 5-nitropiazselenol was the most effective of the commercially available reagents(27). The sensitivity values reported relative to the sensitivity for the parent piazselenol are given in Table 2:2.

Uchida et al. (1980) used 3,5-dibromo-1,2-diaminobenzene to determine Se in natural waters. He also reported that the reagent only reacted quantitatively with Se(IV), but not with Se (-II, 0, and VI) in acidic solution to form the piazselenol. The oxidation state

Table 2:2. Substituted Piazselenols in Analytical Methods Using GLC + ECD. Relative Sensitivity compared with the Parent Piazselenol (ECD Response)

Piazselenol	Structural Formula	Relative Sensitivity			
		Ref. (27)	Ref. (26)	Ref. (32)	Ref. (8)
Unsubstituted		1	1	1	1
5-Methyl-		1.4	1.1		
5-Ethyl-		1.2			
5-Methoxy-		1.7			
5-Ethoxy-		1.6			
5-Chloro-		17	7.4	8.2	
5-Bromo-		30		14.2	
5-Nitro-		128	27.6	38.8	
5,6-Dichloro-		102	25.9		
4,6-Dibromo-		363			
4,7-Dibromo-		172			
4-Bromo-6-nitro-		255			
4,5-Benzo-		25	11.6		
5,6-Benzo-					2-4.5
5-Fluoro-			3.6		
5-Trifluoromethyl-			23.6		

of Se can therefore be determined by successively converting Se (-II, 0, and VI), into the quadrivalent state by suitable oxidation and reduction reactions. These workers estimated directly Se(IV) and total Se in fresh waters at a level of 2 ng Se/L by GLC + ECD (30).

Kurahashi et al. (31) determined Se(IV) in blood and plasma with a practical detection limit of 2 ng Se. The level in blood was determined by GLC + ECD by forming the 4,6-dibromopiazselenol after reduction of Se(VI) to Se(IV) by heating with concentrated HCl and extraction into toluene.

In 1984 Dilli and Sutikno (26) prepared and investigated the two new fluorinated reagents, 4-fluoro- and 4-trifluoromethyl-o-phenyl-diamine. They examined and compared these reagents with other o-PDA systems previously reported for analytical purposes. So far some sixteen reagents have been examined for GLC applications as shown in Table 2.2.

Many methods have been described for the digestion of samples (biological or plant or sediments) prior to selenium analyses, and in most cases wet acid digestion is employed. Decomposition of organic matter is usually accomplished with HNO_3, ($HNO_3/H_2SO_4/HClO_4$) or HNO_3 and $Mg(NO_3)_2$ mixtures. Hydrochloric acid must be avoided because of the possible loss of Se as the volatile chlorides, such as $SeOCl_2$ and $SeCl_4$. Nitric acid is the most common oxidising agent, but high concentrations of acid remaining after the digestion interfere with the action of the diamine reagent (8,32,33).

Cleaning-up or washing of the toluene extract for the removal of excess reagent is necessary and usually requires perchloric acid or hydrochloric acid, but $HClO_4$ is the best (26,30,31) and is preferred if at all feasible.

2:2:2 Atomic Absorption Spectrometry (AAS)

i) Introduction

Since the pioneering paper by Walsh (38) in 1955, the AAS method of analysis has proved to be a very versatile analytical tool and is currently one of the most used analytical methods for trace metal determination. The popularity of this method can partly be attributed to its sensitivity and relative simplicity as it is based on the selective absorption of radiation by atoms in the vapour phase and is highly selective with respect to elements. However, this method depends totally on the creation of a cloud of neutral, free atoms in the vapour phase. The most widely used method for production of this cloud of free, neutral atoms is the introduction of a fine mist of the analyte solution into a chemical flame at a temperature of ca. 2000-3000 K (39). However, the flame method has its limitations. In an effort to improve the sensitivity, applicability and reliability of the method, in 1961 L'vov (40) proposed the use of the method of electrothermal atomization.

AAS is the determination of absorption of radiation by free atoms. A given population of free atoms exists at various electronic energy levels (41). The distribution of atoms in the energy levels is given by the Boltzmann distribution equation as follows:-

$$\frac{N_2}{N_1} = \frac{g_2}{g_1} e^{-\Delta E/kT} \qquad (2.5)$$

where

N_1 : number of atoms per unit volume in ground state

N_2 : number of atoms per unit volume in excited state

g_1, g_2 : statistical weight of atoms in ground and excited state

ΔE : energy difference between excited and ground states

T : absolute temperature of the system

k : Boltzmann distribution constant (1.38×10^{-16} erg/°K)

Numerically, with ΔE in electron volts:

$$\frac{\Delta E}{kT} = 11600 \frac{\Delta E}{T}$$

The numerical values given in Table 2.3 have been calculated by inserting into the formula the values for ΔE applicable to the particular resonance level and ground state. It is evident the population of the atoms in the resonance level is an extremely small fraction of that of the ground state in each case. This fraction must be even smaller for other excited levels. It follows that for all practical purposes all the atoms are in the ground state (10,42).

Table 2:3. Relative Populations of Ground State and Resonance Levels (10,42)

Element	Response line λ (nm)	ΔE (eV)	g_2/g_1	N_2/N_1	
				2000 °K	3000 °K
Cs	852.1	1.45	2	4.44×10^{-4}	7.24×10^{-3}
Na	589.0	2.10	2	9.86×10^{-6}	5.88×10^{-4}
Ca	422.7	2.93	3	1.21×10^{-7}	3.69×10^{-5}
Sr	460.7	2.69	3	4.99×10^{-7}	9.07×10^{-5}
Fe	372.0	3.33		2.29×10^{-9}	1.31×10^{-6}
Mg	285.2	4.35	3	3.35×10^{-11}	1.50×10^{-7}
Cu	324.8	3.82	2	4.82×10^{-10}	6.65×10^{-7}
Zn	213.9	5.80	3	7.45×10^{-15}	5.50×10^{-10}

The atom is called in the ground state when all the valence electrons are in orbitals with low energy level (E_1); when the valence electrons move to higher energy levels (E_2), the atom is deemed to be

in an excited state. The difference in energy between the ground and excited states is called the energy of excitation (resonance level). Normally atoms will remain in the ground state unless they absorb energy and are converted to the excited state (41,42). In atomic absorption technique, the sample is converted from an ionic form to atomic vapour by an atomiser system (normally by flame or electrothermal energy) and irradiated (to cause absorption) at a specific wavelength by light from a source which is often a hollow cathode lamp (HCL) or electrodeless discharge lamp of the specific metal being determined. The level of absorption of radiation absorbed from the light source such as HCL is measured,

$$T = \frac{I}{I_o} \tag{2.6}$$

where

T : transmittance

I : the radiation (intensity) from HCL with the sample in the sample cell

I_o : the radiation from HCL without sample (blank) in the sample cell

$I_o - I$: amount of radiation absorbed by the sample

Absorbance, A, is the base-ten logarithm of the reciprocal of the transmittance:

$$A = \log_{10}\frac{1}{T} = -\log_{10}\frac{I}{I_o} \tag{2.7}$$

and where

$$A = abc \tag{2.8}$$

where a = absorptivity constant

b = cell path length

c = sample concentration

which is the Beer-Lambert law stating that the relationship between concentration or path length and absorbance is linear (10,41):

$$A = abc = -\log_{10} T = -\log_{10}\frac{I}{I_o} \tag{2.9}$$

101

The attraction and importance of AAS technique to analytical chemists is because it allows the determination of over 70 elements of the Periodic Table at low levels with high sensitivity and very good reproducibility at modest cost, since commercial instrumentation is readily available. It is a rapid, versatile technique for the determination of metals in water. Consequently, it is highly suitable for use in a quality-control laboratory situation. Since aqueous samples can be introduced directly into the spectrometer, often only a minimum of pretreatment is necessary. Single or multi-element analysis can be automated or computer controlled, giving good selectivity. Solid sampling may be possible. A further advantage of AAS is that interferences, though important, are few and well documented and therefore it is ideally applicable to the analysis of the wide range of samples likely to be encountered (43,44).

The major disadvantages of AAS are the matrix or inter-element interferences, while slurry or solid sampling can result in poor precision and destruction (44).

Since AAS is an indirect procedure, absorption signals measured for samples must be directly compared with those from prepared standards. These standards should match as closely as possible the chemical and physical properties of the samples and have been subjected to the same level and type of treatment, e.g. acid dissolution, etc. As a consequence, it is essential to calibrate the instrument for each element being determined, measuring the absorbance of a number of standard solutions containing known concentrations of the analyte, then drawing a calibration graph by plotting absorbance against concentration. A linear regression relationship between the absorbance and known concentration is normally used to find the concentration of the analyte in the unknown, which should be within the working range of standard solutions (43).

ii) Flame Atomic Absorption Spectrometry (FAAS)

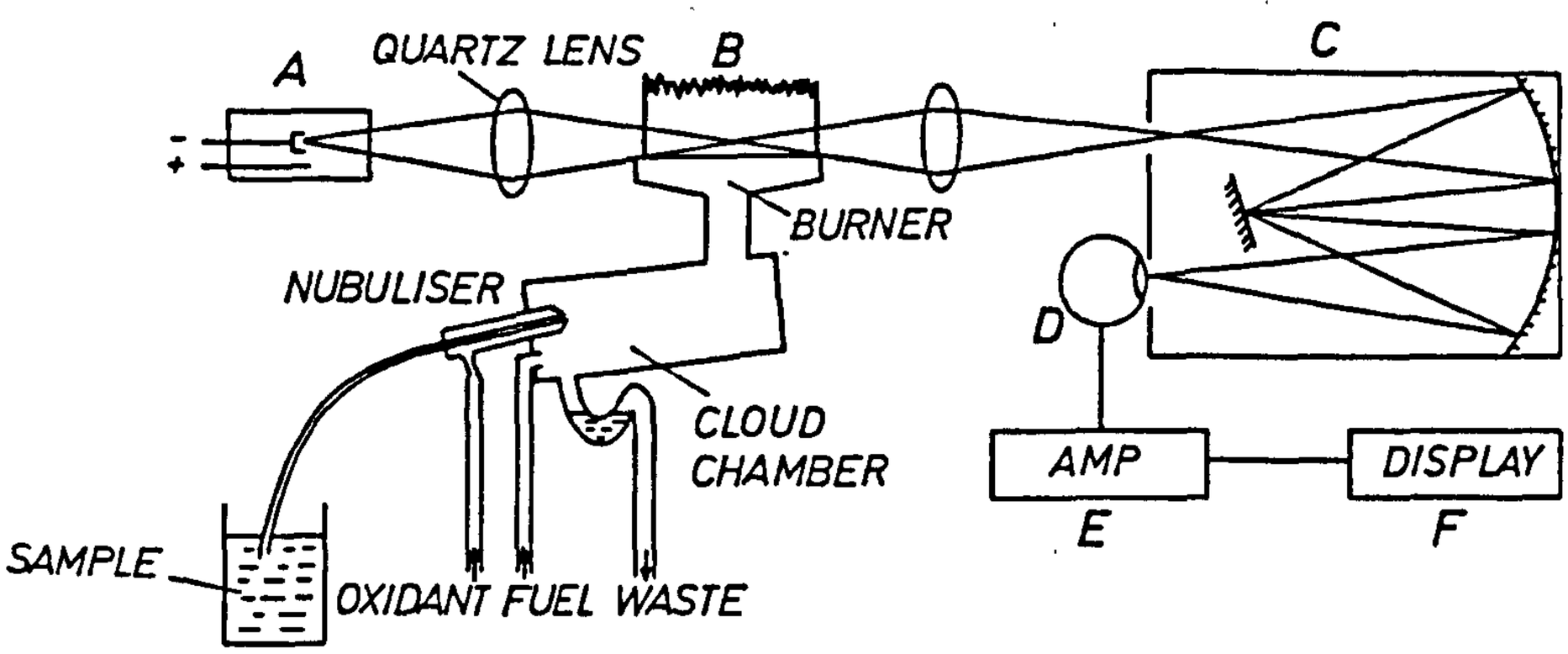
The first paper demonstrating the use of AAS as an analytical tool was published in 1955 by Walsh (38). Standard commercial equipment became available about 1960 and since that time the use of this technique in routine laboratories has become widespread (45). Figure 2.2 depicts a typical flame atomic spectrophotometer.

Since the introduction of AAS, the main developments have been in the design and performance of the atomisation system. When a flame is used in AAS, the technique is said to be FAAS. The most commonly used atomiser is the chemical flame, based upon the combination of a fuel gas (e.g. acetylene) with an oxidant (e.g. air or nitrous oxide) (46). Air is blown through a venturi, and the sample solution is drawn into the throat of the venturi and the air stream where it forms a spray of fine droplets. A large fraction (~ 90%) of the drops fall out of the air stream in the cloud chamber and go to waste but the very small droplets remain suspended. The air stream plus droplets are mixed with a fuel gas and the mixture passes through a narrow slot where it is ignited to form a flame (1).

The flame is a chemical reaction which takes place in the gas phase (46). An air-acetylene flame is used for determination of more than 30 elements, while a hotter N_2O-C_2H_2 flame is required with refractory elements such as Al, As, B, Ba, Be, Cr, Ge, Mo, Si, Sn, Sr and V. Under certain conditions, the latter flame is hazardous and the use of an automatic gas control box is recommended (47).

In the flame, the sample vaporises, the solvent is largely evaporated, eventually forming a cloud of atoms in the gaseous state. Through the flame is passed a light beam composed of the wavelength corresponding to the energy required to raise the atoms of the

FIGURE 2.2 GENERAL SCHEMATIC OF A SIMPLE FLAME ATOMIC ABSORPTION SPECTROMETER



A Hollow cathode lamp
B Flame
C Monochromator
D Photomultiplier detector
E Amplifier
F Readout

particular element being determined from their ground state to an excited state. The intensity of this beam is observed via the monochromator and detector with the amount of energy absorbed by the flame being ascertained, hence the number of atoms in the ground state in the flame can be determined. A hollow cathode lamp may be used to obtain the light of the required wavelength. The lamp is an evacuated silica envelope containing a cathode which is composed of a hollow chamber containing the element being determined, so that the emission radiation of the element is obtained. Electrodeless discharge lamps are also used for some elements (1).

Before the determination of the total metal content of a sample (at the trace level), it is normally necessary to bring the metal into solution, exceptions being the use of neutron-activation analysis, X-ray fluorescence spectroscopy and, in some situations, electrothermal atomic absorption spectrometry (48).

The total concentration of metals in samples can be found by dry ashing followed by acid dissolution, or via wet digestion with acids individually or in mixtures. Dry ashing might cause loss of metals by volatilisation, e.g. cadmium and lead, and does not always yield complete recovery of metal. Most mineral acids are utilised for wet digestion, namely nitric, sulphuric, perchloric, hydrochloric and hydrofluoric acids, individually or as mixtures, have been widely used in atomic absorption spectrometry determinations (49). Hydrochloric acid dissolves complex absorbed and precipitated metals, but has a minimum effect on the more inert silicate metals. Sulphuric acid has the disadvantages of the possibility of the formation of insoluble sulphates plus the interferences that it can cause in the FAAS determination due to the tendency of the sulphate anions to form

salts which are very difficult to dissociate and atomise in the flame (45). Perchloric acid is a very good oxidising agent and forms soluble perchlorates, but the acid may cause explosions when it is used to digest samples which contain high levels of organic materials. Hydrofluoric acid dissolves metal silicates and releases metals by volatilisation of the major elements of the matrix (50):-

$$SiO_2 + 4HF \longrightarrow SiF_4 + 2H_2O$$

Hydrofluoric acid has the property of decomposing silica and has been used in conjunction with nitric, hydrochloric or perchloric acid in the total decomposition of silicates. Nitric acid has been recommended, and is the most attractive acid for the extraction of the trace metals from matrices, mainly due to the fact that most nitrates are soluble and the acid is a good medium for AAS, causing few interferences (45).

The chloride, fluoride and iodide ions in major amounts tend to interfere in FAAS determination of metals by forming molecules, with alkali and alkaline earth elements, which can give rise to background effects when vaporised in the flame, either by their particulates scattering light or by molecular absorption within the bandpass of the monochromates (51).

Interferences

Among the problems encountered in atomic absorption spectrometry, the most important arise from disturbances due to the substances associated with an element in the sample to be analysed. Interference is the influence of one or more elements present in the sample on the element to be determined. This interaction may induce a systematic or random error in the determination of an element. Interference is one of its principal limiting factors, in particular limiting accuracy, sensitivity and precision (52).

Interferences encountered in FAAS can be separated into the following categories:

i) Spectral
ii) Flame emission
iii) Chemical
iv) Matrix
v) Non-specific scatter
vi) Ionisation

The majority of difficulties arise from categories (iii) to (vi) (53).

i) Spectral interference

In multi-element, or even supposedly single-element, hollow cathode lamps may contain two elements which emit radiations of nearly the same wavelength which can both be absorbed in the flame by atoms of a single element. It has been reported that a cerium cathode also emits a calcium line which is absorbed by cerium atoms in the flame (at 422.6 nm); iron can similarly interfere with nickel (at 232 nm); and also lead (the 216.9 line) absorbs antimony 217 nm radiation and even the 217.5 nm as well. These are, however, interferences due to the characteristics of the particular apparatus involved (52).

In the past the interferences generally experienced were in a

given solution, where element A was being determined in the presence of element B. If the source contained both elements and the absorption lines of these elements could not be resolved by the monochromator, element B would cause an interference. In some early HCLs this was a a well-known phenomenon (53), and is still a very serious problem in flame emission and requires narrow slits to overcome the problem; additionally, a very good monochromator to minimise the interference (54), or the use of an alternative absorption line, the probability of two lines coinciding again being extremely remote. Atomic absorption now suffers almost no spectral interference problems because of improvement in the purification techniques of the hollow cathode sources and to some extent in monochromator quality (53,54).

ii) Emission interference

Emission interference was common in the early AAS instruments which were often accessories for UV/visible spectrophotometers, which operated in most instances on a d.c. system. The interference was caused by emission of the element at the same wavelength as that at which absorption was occurring. All modern instruments use a.c. detection systems which are, of course, 'blind' to the continuous emission from the flame. However, if the intensity of the emission is high, the 'noise' associated with the determination will increase, since the noise of a photomultiplier detector varies with the square root of the radiation falling upon it.

The emission effect can be reduced by either increasing the source current or by closing down the slit, both methods resulting in an increase in the signal-to-noise ratio (53).

iii) Chemical interference

The phenomenon of chemical interference is by far the most frequently encountered interference in AAS (53). Chemical interference arises when the fraction of the element forming atoms in the ground state in the flame is different with the sample than with standard solutions used for calibration. This may result in a depression or an enhancement of the signal. Where the element forms a compound that is not dissociated readily in the flame the signal is depressed (1). Such a situation arises, for example, in the effect produced by aluminium, silicon, and phosphorus on the determination of magnesium, calcium, strontium, barium and many other metals, being due to the formation of aluminates, silicates and phosphates. In most cases, the moieties are refractory in the flame being used for analytical purposes (53,55).

Three basic approaches have been used to remove this type of interference and can be applied to almost any metal in some form or another. Thus calcium phosphate reacts to form a compound which is difficult to decompose in the normal flame (air-acetylene); this causes low calcium results. The $Ca_3(PO_4)_2$ system will be used as an example. The approaches are:-

a) More insoluble compound formation: Lanthanum ions are added to the system. This metal forms a more insoluble compound with phosphate than does calcium, so the calcium ions are set free (1,54) to absorb radiation in the usual manner.

b) Ion exchange resins: The sample is passed through an anion exchange column, usually in the OH^- form, and the phosphate is replaced with OH^- (54).

c) Chelation: In this case the metal ion, Ca^{2+}, is chelated with ethylene diaminetetraacetic acid (EDTA) so making a complex with the cation. The ligand still protects the Ca^{2+} until the EDTA is burned away in the flame, and the Ca^{2+} can be reduced to the matal before it has a chance to react further with other anions, e.g. phosphate (53,54).

d) Alternatively, virtually all chemical interferences may be overcome by using a high-temperature flame e.g. nitrous oxide-acetylene flame (53,55).

FAAS is susceptible to chemical interferences; the use of the N_2O-C_2H_2 flame has helped reduce chemical interferences but has not eliminated them (54).

iv) Matrix effects

Matrix effects are due to chemical and physical differences between the sample and the standard (1).

Under this heading the interferences encountered include:-

a) Enhancement of sensitivity due to the presence of an organic solvent in the aqueous system.

b) Depression of sensitivity due to the sample having a greater viscosity than the standard solutions, and therefore not being nebulised at the same rate as the standards.

c) Depression of the results due to a high salt content.

These interferences can be readily overcome by using one of the following techniques:-

1. The method of standard additions.
2. Matching the matrix of the standards with that of the sample.
3. Solvent extraction to remove the cation to be determined from the interfering matrix (1,53).

v) Non-specific interferences

a) Light scattering

The enhancement of an analytical result at the µg/g or sub µg/g levels may be resultant because the solution contains a high concentration of dissolved salts. The effect is due to the presence of dried and semi-dried salt particles in the flame which scatter and absorb the incident radiation from the source. Since the intensity of the transmitted radiation will be decreased, there will be an increase in the absorption signal. This non-specific scatter effect is wavelength dependent and is more pronounced at shorter wavelengths. It is most significant below 250 nm (53).

Scattering has its origin in the induced secondary emissions of particles (whether single atoms or molecular aggregates) that lie in the path of radiation. Secondary radiation is scattered only if the particles:-

i) have dimensions approximately the order of magnitude or smaller than the incident wavelengths. Larger particles reflect the radiation. In the UV and visible regions of the spectrum, the scattering particles are those of colloidal size i.e. from 1 nm to 1 µm in greatest dimension;

ii) are randomly distributed in a medium of refractive index different from their own (55).

b) Molecular absorption

Although atomic absorption lines of each element are narrow and are easily distinguished from each other, molecular absorption is broadband and may take place over a wide range of wavelengths, e.g. 20 nm, frequently including the wavelengths of many atomic absorption lines (width < 1 nm). Broadband absorption may be caused by unburned

fuel in flames or unburned solvent, or fragments of molecules introduced from the sample. Consequently broadband absorption is common in flames (55).

The effect produced by non-specific interferences can be overcome by one of the following techniques:-

1. Solvent extraction to remove the element from the interfering matrix.
2. Repeating the determination at a nearby non-absorbing line and subtracting it from the signal obtained at the absorbing line (53).
3. By using a deuterium background corrector. The radiation from a deuterium lamp is measured at the same normal wavelength as the resonance line. The radiation from this lamp fills the spectral slit and therefore a waveband of approximately 0.1 nm or greater will reach the detector. Absorption by molecules in the flame is across the entire spectral slit width, providing a measurement of the molecular background absorption. Atomic absorption also takes place at this wavelength. However, the lines are very narrow (10^{-3} nm) and the total amount of energy absorbed is very small compared with the molecular energy absorbed. If an atomic line is completely absorbed from a waveband of 0.1 nm the total absorption is only 1%. This is a negligible amount and be ignored. Consequently, the absorption of the deuterium lamp is a measure of the background absorption (41).
4. Background correction using the Zeeman effect. Continuum source background correctors fail if background absorption is excessive, c. > 1 A unit, or if the background is structured

within the bandpass of the instrument. In these cases, the Zeeman effect can be used to obtain a superior correction (55).

In the Zeeman effect (56), the energy levels of a molecule are split under the influence of a varying strong magnetic field. If the atoms are not in a changing magnetic field they will absorb at a single wavelength (e.g. the resonance line). A strong magnetic field may then be switched on and the absorption line is split into fine structure with wavelengths greater than and less than the resonance line. If the magnetic field is strong enough the resonance line is eliminated entirely. Either the hollow cathode or the atomiser can be operated in the magnetic field (41,56).

Advantages of the Zeeman background corrector are:-

a) The Zeeman system is useful for analyses that are below the convenient detection limits of FAAS (57);

b) A single standard light source is used, thereby eliminating misalignment problems and extra source noise; intensity of the lamp does not change and no adjustment has to be made to the instrument;

c) Background correction is at the precise wavelength of the line. Background structure is usually not a problem;

d) Background absorbances as high as 2 can be tolerated in some systems;

e) Atoms outside the magnetic field give no response (41,55,58).

Disadvantages of the Zeeman system include:-

a) added cost and complexity;

b) shortened linear range;

c) currently Zeeman systems are available from only two manufacturers;

d) systematic errors can be encountered due to Zeeman shifts in the rotational spectra of diatomic molecules (55).

5. Smith-Hieftje background correction

The method was proposed by Siemer (59) for application in AAS. A single short duration high-current HCL power pulse (e.g. 170 μs, 280 mA) was used per measurement cycle. The radiation from the lamp was measured at the beginning and end of each current pulse and before self-reversal problems had time to develop fully. The difference between the absorbance signals observed during the two sampling periods was found to be unaffected by continuum absorption or line-scattering (60).

Potential advantages of this method include:-

a) avoidance of the beam misalignment problems prevalent in dual-source background correction methods (61);

b) the method is applicable to any atomiser (flame or electro-thermal) (60);

c) simplicity.

d) background correction at a wavelength very close to the absorption line (58).

The disadvantages include:-

a) reduced sensitivity;

b) possibility of reduced lamp life, unless applied to modified lamps;

c) commercially not available (58) except from one manufacturer.

vi) Ionisation interferences

To understand ionisation interferences, it is necessary to appreciate what is occurring in the flame during the aspiration of a sample. The flame is being used as a source of energy to convert elements in solution droplets created by the nebuliser into ground-state atoms.

$$MX \xrightarrow{E} MX \xrightarrow{E} M^\circ + X^\circ$$

solution salt particles ground-state atoms

Many determinations require the use of the nitrous oxide-acetylene flame and it is usually under these conditions that ionisation interferences occur. They arise from the energetic nature of the flame which gives ground-state atoms but also excites such atoms to some extent so that one or more electrons are lost and ionisation occurs.

$$M^\circ \xrightarrow{E} M^+ + e^-$$

The effect will obviously be greatest with elements having low ionisation potentials such as the alkali and alkaline earth metals, e.g. barium is approximately 80% ionised in the C_2H_2-N_2O flame. The ground state, therefore, becomes depopulated and the sensitivity will decrease.

A similar but opposite effect arises when an easily ionised element is being determined in the presence of another. There will be an enhancement of sensitivity compared with pure aqueous standards, which arises from the presence of excess free electrons which suppress further ionisation

$$M^+ + \underset{\text{excess}}{e^-} \longrightarrow M^\circ$$

This effectively increases the population of ground state atoms. In practical applications, some use may be made of this phenomenon. By adding an excess of a readily ionised salt to samples and standards, an increase in sensitivity may be achieved. Potassium chloride is usually chosen for this purpose owing to its high purity,low ionisation potential and lack of visible emission in the flame (53).

Table 2:4 lists the type of interference effects encountered, the problem caused and the techniques used to ameliorate the problem in FAAS.

Many reviews have been published on FAAS and its uses in the trace determination of metals in natural samples (62,63). There are many applications of FAAS in sewage and sludge (64), agriculture, pollution, food, petroleum and other areas (60).

Table 2:4. Selected Approach for Avoiding Interference Effects in FAAS

	Type of Interference Effects	Interference Problem	Solution
1	Spectral	Two metals, one effect on other due to close proximity of wavelengths	Choose an alternative absorption line
2	Emission	Emission and absorption at the same wavelength	Increase the source current, close down the slit
3	Chemical	Anything which prevents formation of ground state atom in flame	i) Add releasing agent such as $La(NO_3)_3$ ii) Use ion exchange resins iii) Chelate the metal ion with EDTA and leave the anion unassociated iv) Use high temperature flames such as $(N_2O-C_2H_2)$
4	Matrix	Samples and standard are different matrices	i) Use standard addition ii) Matching the matrix of the standards with that of the sample iii) Remove the metal ion to be determined from matrix by solvent extraction
5	Non-specific	Due to presence of dried particles in the flame which both scatter and absorb the incident radiation from the source	i) Use solvent extraction to remove metal ion interfering ii) Use deuterium background correction iii) Repeating the determination at a nearby non-absorbing line and subtracting it from the signal obtained iv) Using Zeeman effect if possible
6	Ionisation	The problem arises from the energetic nature of the flame in both ground state and ionic state	Adding ionised salt such as KCl to produce free electrons to react with ionised state and convert to free atoms

iii) Flameless Atomic Absorption Spectrometry

The development of electrothermal atomisers can be traced back to the work of King in 1905 and 1908. King was interested in the observation of emission spectra of elements. His aim was to obtain emission spectra (as nearly as possible), solely by the effect of heat, and he first used an arc-heated furnace. Then in 1959 L'vov reported the application of electrothermal furnace atomisers for quantitative atomic absorption analyses (65).

The method of electrothermal atomisation requires the deposition of the analyte solution on a metal or graphite surface whose temperature is gradually increased to evaporate the solvent and decompose the sample until it is possible to atomise it by rapidly raising the temperature of the surface to sufficiently high value. The method is more complicated to use but offers substantial advantages when compared to flame atomisation or to most other analytical methods. A marked improvement in sensitivity (compared to flame atomisation) is achieved and only small amounts of sample are required for a determination. The method only really gained popularity when Massmann introduced the electrical resistance heated tube atomiser in 1967. The system was compact and the tube was isolated from the atmosphere by a flow of an inert gas (argon or nitrogen) in and around the tube (39).

Various forms of electrically heated furnaces have been described in recent years; a fixed sample volume is introduced into the furnace and after thermal pretreatment is rapidly atomised. The result is a transient signal whose height or area is proportional to the quantity of element under study. It has been demonstrated that in a graphite furnace atom cell a substantially higher peak concentration of atoms may be expected compared with a flame. The gain results directly from avoidance of the dilution and expansion effects that occur in flame

cells. To assist the formation and maintenance of a dense free atom fraction of the element for AA analysis it is also an advantage that the chemical environment can be controlled by the use of an inert gas atmosphere (46).

Although graphite furnace atomic absorption spectrometry (GFAAS) has great advantages such as increased sensitivity and minute samples for determination of a trace amount of metals, it suffers from troublesome problems related to matrices and the nature of the graphite surface in the atomisation process. To overcome these problems many improvements in furnace design and performance have been developed; thus i) the graphite furnace was coated with a layer of pyrolytic graphite or metals, or ii) fitted out with a graphite platform and tantalum or tungsten liner, collar and wire (66).

When an analyte metal forms a stable carbide and has a low volatility, enhancements in the sensitivity were obtained by using a pyrolytically coated tube and a metal liner or boat. It was reported that the use of a nonpyrolytic graphite surface in place of a pyrolytic graphite surface enabled charring at higher temperature, resulted in interferences (67), and allowed an increased number of active sites being available for reduction by the graphite (68).

Many commercial electrothermal atomiser designs are available; including versions of the carbon furnace, cup, rod and the tantalum filament (55,69), while enclosed graphite tube furnaces (atomisers) are the most universal and popular versions used in commercial flameless spectrometers (47).

A small sample aliquot (1 to 50 ul often 10-30 ul) is introduced into a graphite tube which is aligned in the spectrophotometer optimal path and is enclosed in an inert gas atmosphere (47).

The furnace power supply and controller enables the basic steps normally considered to occur in the flame method of atomisation to be carried out in a sequential form, each stage governed by the particular phase of the controller programme. In its simplest form the programme will consist of four readily identifiable stages, namely "dry", "ash" ("char"), "atomise" and "clean-out". Each of these stages has to be carefully optimised to obtain the best results for any particular analysis (47,53). The graphite tube furnace is electrically heated up to 3000°C, the typical temperature values are:

i) "dry" - solvent evaporation 110-120°C.

ii) "ash" ("char") - of the residue 400°C.

iii) "atomise" - of the analyte < 3000°C.

iv) "clean-out" - for cleaning the furnace.

For atomisation the parameters of the heating programmes and the type and the flow rate of inert gas to be adopted to be determined for maximum AA signal in flameless atomisation. The resultant signal is a sharp narrow peak for volatile metals such as Cd and Pb, while a broad peak is obtained for involatile metals such as vanadium and molybdenum. The mean peak height and area signals are used in this technique; the peak height is the most widely accepted method for evaluating flameless signals (70,71), but when the peaks are broad the peak area sensitivity obtained may be better than the peak height result (72).

Very recently, a technique has been described for coating graphite tubes with pyrolytic materials which increases the sensitivity of the technique (71). The coated materials have low permeability to gases, high thermal conductivity, high sublimation point, high resistance and a small coefficient of thermal expansion (51).

The mechanisms of atom formation in GFAAS are either:

i) reduction of metal oxide by carbon with subsequent sublimation of the metal, or

ii) vaporisation of the oxide and thermal decomposition of oxide in the gas phase (65,73,74), as follows:

$$MO_{(s)} + C_{(s)} \xrightarrow{\text{Reduction}} M_{(g)} + CO_{(g)} \qquad (2.10)$$

$$MO_{(s)} \xrightarrow{\text{Dissociation}} MO_{(g)} \longrightarrow M_{(g)} + \tfrac{1}{2}O_2 \qquad (2.11)$$

While metal halide by vaporisation and thermal dissociation of vapour halide (72) as:-

$$MX_{(s)} \longrightarrow MX_{(g)} \longrightarrow M_{(g)} \qquad (2.12)$$

has been postulated.

Electrothermal AAS has tended to replace flame AAS due to its inherent high sensitivity (from 100 to 1000 times more) and low sample size (65). Table 2.5 shows the detection limits for FAAS and GFAAS, while Table 2.6 gives a comparison between flame and graphite furnace atomisers.

The detection limits are listed in Table 2.5; it shows different values for the same element. They are dependent on the procedure used to determine the detection limits. In many cases the detailed method of ascertaining the detection limit is not given but a common procedure is to use a value of two sigma above the background noise level for the system. The two sigma value represents a limiting situation and is often a very optimistic value. Therefore, some values are expressed in "ng" (ng/injection); others used "ng/ml". Other reasons for the differences may be due to the instrumentation used, for example some incorporate Zeeman correction and others do not.

Table 2:5. Comparison of Detection Limits of FAAS and GFAAS

Element	GFAAS Ref.(65) (ng/ml)	FAAS Ref.(65) (ng/ml)	GFAAS Ref.(75) (ng)	FAAS Ref.(75) (µg/ml)	FAAS Ref.(53) (ng/ml)
Cadmium	0.001	1.0	0.00008	1	2.0
Chromium	0.1	3.0	0.002	2.0	3.0
Copper	0.02	2.0	0.0006	1.0	4.0
Iron	0.03	10.0	0.01	4.0	3.0
Lead	0.02	20.0	0.004	10.0	10.0
Manganese	0.002	2.0	0.0002	0.8	3.0
Mercury	2.0(b)	500(a)	0.02	500	
Nickel	0.1	10.0	0.009	5.0	7.0
Selenium	3.0(c)	500(a)	0.009	100.0	160
Tellurium		300(a)	0.001 0.010(d)	50.0	70.0
Thallium	0.1	30.0	0.001	20.0	30.0
Zinc	0.0005	2.0	0.00003	1.0	2.6

(a) Ref. (54) (b) Ref. (76)
(c) Ref. (77) (d) Ref. (78)

Table 2:6. Comparison between FAAS and GFAAS (70)

	FAAS	GFAAS
1	Continuous atomisation	Discrete, transient atomisation
2	Requires 5 ml sample	Requires 10-20 ul
3	A small proportion of sample is measured	Total mass response
4	Low solid compatibility slurries may be atomised	Compatible with solid samples
5	Sample heated by gas from flame	Sample heated by graphite tube wall and gas
6	Temperature of flame is different according to gas and mixture	Temperature is programmed from ambient to 3000°C at variable heating rates and steps
7	Background interference low (in general)	Background interference high

However, GFAAS has disadvantages. The flame is more precise, faster, requires less skill and should always be used if sensitivity is adequate. Moreover, contamination can be a problem at these ultra-trace levels (46). The disadvantage of GFAAS is that the hot gases pass quite easily through the wall of the tube so that losses of atomic vapour can occur. To avoid this problem, the graphite tube may be impregnated with carbide forming elements such as Si, Ti, V and U. By firing at high temperatures, the metal forms the respective metal carbide layer which prevents the permeation of atomic vapour through the wall of the tube (70). Some workers (51,79,80) have tried to improve the production of free atoms in graphite tubes either by inserting metal foil liners inside the tube or using tubes made completely of high melting point metals such as tantalum, tungsten and molybdenum. Such systems should be especially useful when determining involatile metals, but unfortunately are not yet commercially available. Great skill and precise engineering is required for their manufacture and their non-availability is a limiting factor in the assessment of the performance of such tubes.

Although background effects are a major source of error in GFAAS, there are other interference effects which can cause problems. They are physical, chemical and spectral. Table 2.7 illustrates the mechanisms by which they are produced (65,70,72,74).

Background correction has always been a rather controversial topic since the inception of electrothermal atomisation. There now exists a variety of high-performance systems designed to cope with the problems of matrix-background absorption. A comparison of the Zeeman effect with other methods of background correction was made by Slavin and Carnrick (60).

A variation in the wavelength modulation technique is the use of

Table 2:7. Selected Approach for Avoiding Interference Effects in GFAAS (65,70,72,74)

	Type of Interference Effect	Interference Problem	Solution
1	Physical Causes, such as volatility, viscosity or surface tension It is less important than with FAAS	i) Different response from the same injected volume; irreproducible and low precision ii) The spread of the sample solution within graphite tube means different response	i) Match physical properties of sample to that of the standard ii) Dilute viscous samples with a suitable solvent iii) Correct injection technique, use an automatic dispenser
2	Spectral	i) Large concentration of matrix vaporised during atomisation stage and can cause scattering of the incident light beam ii) Molecular species which vaporise during the atomisation stage can cause molecular absorption by their band structure	i) Use a long ashing period to eliminate matrix vaporisation during atomisation stage ii) Use background correction technique to eliminate molecular absorption
3	Chemical Resulting either from a matrix component which can chemically combine with the metal, altering the ground state atom population with the absorption or from reactions taking place in the graphite tube prior to, and during, the atomisation stage of a furnace cycle. This type of interference effects originate from:	Changes in the sensitivity of the method and high error in the accuracy of measurement	

Table 2:7 continued

Type of Interference Effect	Interference Problem	Solution
i) Pyrolysis loss	The element may be present in the sample in volatile form at pyrolysis temperature employed	Add matrix modifier reagent to decrease the volatility of normally volatile compound
ii) Condensation	When the atomised element leaves the hot surface of an atomiser, it is transported into a much cooler region where condensation of the atomic species can occur	Use platform technique
iii) Memory effect	Incomplete atomisation of an element causes an enhancement in subsequent analytical determination thus V, Mo, W form stable refractory oxides	Use higher atomisation temperatures or long atomisation time
iv) Carbide formation	Formation of stable carbide which prevents formation of the atom population	Use pyrolytically coated tube
v) Nitride formation	When N_2 used as an inert gas, some metal may form stable nitrides and so reduce the sensitivity	Use argon as inert gas

a sectored-wheel square-wave system (81), which measures background at two wavelengths adjacent to the analyte wavelength rather than from a wavelength scan (60).

The technique of "matrix modification" has been successfully employed in many cases to overcome chemical interference effects (74). Ediger (83) was the first to propose the technique, aimed at either decreasing the relative volatility of the analyte or increasing the volatility of the matrix or both. Two classic examples are:

i) the Ni addition to stabilise Se even up to 1100° to 1200°C (as thermally stable nickel selenide), and

ii) the addition of NH_4NO_3 (or HNO_3) so as to convert the NaCl matrix (i.e. sea water) into more volatile salts: $NaNO_3$ and NH_4Cl (or HCl).

Matrix modification is now an indispensable part of GFAAS procedures for volatile elements such as As, Bi, Cd, Hg, Sb, Se, Sn, Te, etc., and is very useful with moderately volatile analytes as well (Cu, Mn, Pb, etc.) (47,84). Table 2.8 lists the chemical modifiers which have been proposed for specific elements (in GFAAS).

The influence of valency state on the atomisation of Se, when stabilised with nickel, is important. Without modifier, Se(VI) is effectively retained on the atomiser up to 1000°C but Se(IV) may be lost at temperatures above 500°C. However, Se(IV) is stabilised in the presence of nickel up to 1000°C (60).

Table 2:8. Chemical Modifiers for Specific Elements in GFAAS

Analyte	Modifier	Effect	Reference
Selenium	Ni (in 20 fold excess)	Forms stable selenide which atomises at higher temperature	(51,77,83,85)
	$Ni + Mg(NO_3)_2$		(78)
	$1\% HNO_3 + 0.05\% K_2Cr_2O_7$	Extends the maximum permissible ashing temperature	(85)
Tellurium	Ni (excess)	Forms stable telluride	(51,78)
Mercury	$(NH_4)_2S$ in excess	Permits a higher ashing temperature and stabilises the signal	(74)
	$K_2Cr_2O_7 + HNO_3$	Extends the maximum permissible ashing temperature	(51,85)
Cadmium	H_3PO_4 (excess)	Conversion to less volatile phosphate which atomises at a higher temperature	(51,74)
Ag, Pb, Zn	H_3PO_4 (excess)	Conversion to less volatile phosphate which atomises at a higher temperature	(51,74)
Thallium	1% v/v H_2SO_4	Increases ashing temperature and reduces $HClO_4$ interferences	(78,82)

iv) Cold-Vapour Atomic Absorption Spectroscopy (CVAAS)

For CVAAS the method developed by Jones and Nickless (86) was used, which was based on the principle first described by Poluektov et al. (87) and taken up later by Hatch and Ott (88). The aeration cell was a slightly modified version of that described by Simpson and Nickless in 1977 (89), which utilised similar principles to that reported by Hawley and Ingle (90). The apparatus is shown schematically in Figure 2:3 and photographically in Figure 2:4.

The unique properties of mercury, namely:-

i) Hg^{2+} ions may be easily reduced to the element (Hg°), and

ii) the high vapour pressure of elemental mercury as a monoatomic vapour at room temperature,

have been utilised for the trace determination of mercury in what are termed "cold-vapour techniques". The principle of this general method involves the conversion of mercury in a matrix to solution as Hg^{2+}, usually by wet acid oxidising conditions, and then reduction of Hg^{2+} to elemental mercury by a reducing agent such as Sn^{2+}, followed by flushing of the Hg° vapour with a purge gas such as N_2 into an absorption cell placed in the radiation path of a mercury hollow cathode lamp (91,92).

The main difference between the Jones-Nickless system and that of Hatch-Ott was that the former was an open ended system and the latter a closed one (93). The open ended system is called the "exhaust" method where the mercury vapour from the aeration vessel is passed through the absorption cell and then to exhaust (94). A transient signal is thus produced (92).

The dead volume of a CVAAS system should be kept low and the absorption cell should be rational in shape and dimensions. The optimum shape of the absorption cell may cause marked improvement in

FIGURE 2.3 SCHEMATIC OF MERCURY COLD-VAPOUR ANALYSER

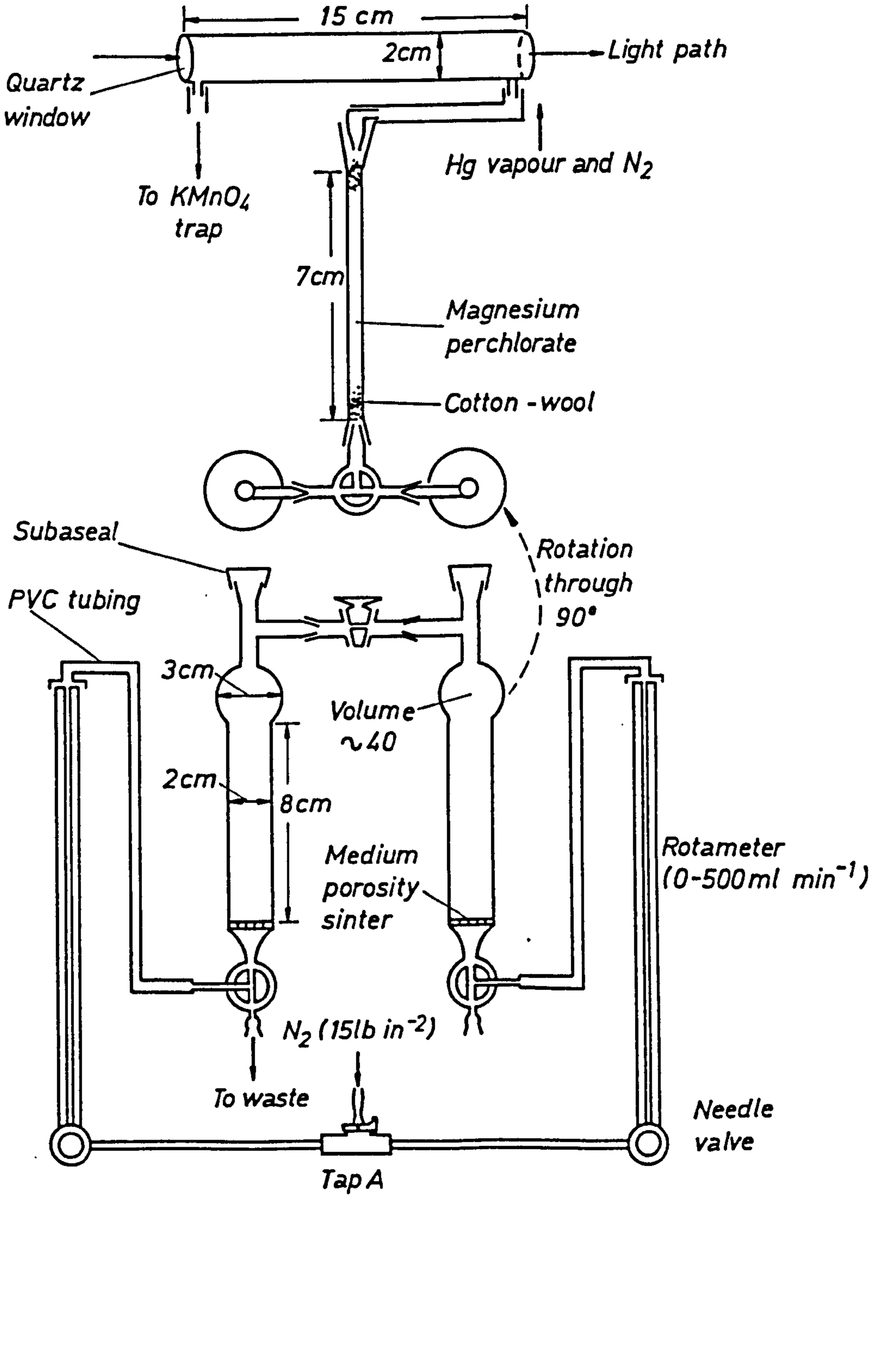
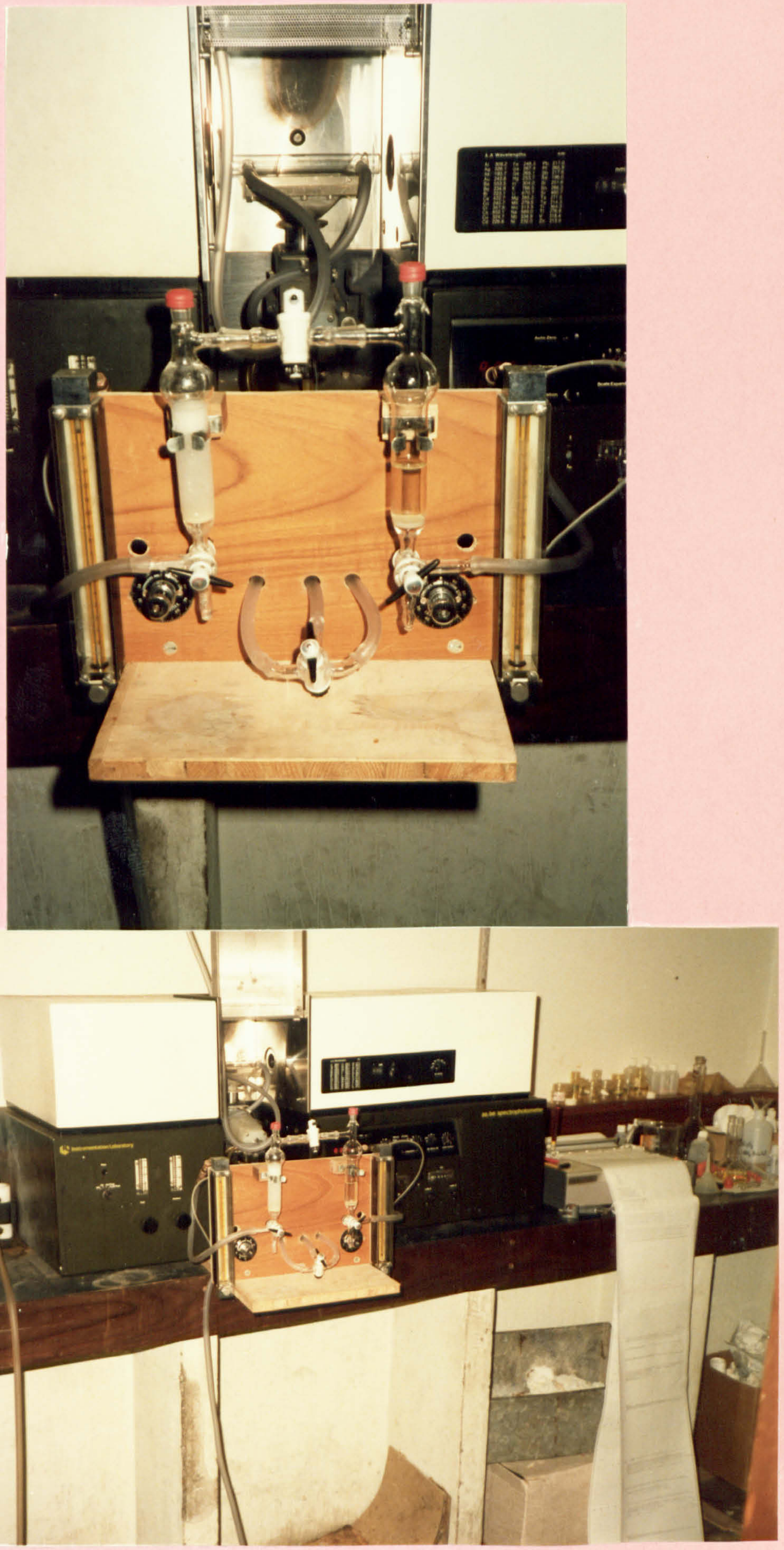


FIGURE 2.4 MERCURY COLD-VAPOUR ANALYSER



precision, sensitivity and detection limits (92).

In the cold vapour procedure, the excess of oxidant remaining after digestion is destroyed by addition of a reducing agent such as hydroxylammonium sulphate or ascorbic acid. The stannous sulphate or chloride which reduces the mercury to Hg° is added immediately before the aeration. A number of techniques and modifications have been described which reduce interference and increase sensitivity (95).

A common interference is water vapour, which is carried into the spectrophotometric cell from the reduction vessel and tends to condense on the cell walls and windows. Water has been removed by passing the gas stream through a drying tube usually containing magnesium perchlorate $Mg(ClO_4)_2$. This drier may need frequent changing; moreover, it is now realised that $Mg(ClO_4)_2$ drier, especially when getting damp, may be responsible for lower sensitivity, memory effects (due to trapping of some Hg), tailed peaks, prolonged measurements and gradual sensitivity drift (92,95,96).

Although the cold-vapour method is usually used for the determination of total mercury, selective reduction methods have been developed which can determine organic mercury from the difference between the signals obtained for total mercury and the inorganic form of the element (91,97). Inorganic mercury is determined by complexing the mercury with cysteine in an acidic solution followed by the addition of $SnCl_2$ and NaOH. Addition of cadmium or copper salts will also release the organically bound mercury (91).

Magos (98) digested biological samples with acid cysteine. Subsequently, reduction with $SnCl_2$ released only inorganic mercury but reduction with alkaline $SnCl_2$-$CdCl_2$ released all mercury.

Mercury levels in environmental and biomaterials range over

several orders of magnitude: from subnanogram to above micrograms per gram. Of all the analytical methods for mercury, the cold-vapour technique of AAS is the best routine method: it is easily accessible, sufficiently sensitive, reproducible, relatively simple, fast (about two minutes for each sample), has a low detection limit (about 0.1 ng/ml) and is inexpensive (89,91,92).

2:2:3 Electroanalytical Techniques

The everyday application of electrochemistry in analytical laboratories has increased tremendously in the past 15 years. The increase is largely due to the availability of instrumentation that has made it possible to perform voltammetry, particularly the powerful pulse voltammetric techniques, conveniently and inexpensively. The first commercial and easy to use pulse polarograph, for instance, became available in the early 1960s (99).

Electrochemical methods of analysis have the advantage that the species of the particular metal being analysed can be characterised. The methods will distinguish, for example, between the metals present in an ionic state and those that are complexed. Hence the methods are of particular value when analysing water samples where the form or species of the metals is of concern (1).

Electrochemical techniques depend upon the exploitation of the current-voltage relationships to determine the concentration of electro-active species in solution. Advances in modern electronics which allow the measurement of current and voltage at the micro and nano level have led to significant advances in sensitivity of these techniques. Determination of most components in solution that can be oxidised or reduced at an electrode is now possible. .Many metal ions are reduced at some form of mercury cathode to form an amalgam with the mercury (70).

i) Polarography

The term "polarography" refers specifically to a technique which is a subclass of voltammetry in which the indicator electrode is a liquid-metal (normally mercury) electrode whose surface is continuously or periodically renewed such that long-term accumulation of the products of electrolysis at the electrode-solution interface is prevented. A mercury electrode, called a dropping mercury electrode (DME), is used as the working electrode (99,100).

The working electrode (cathode) consists of easily polarised droplets of mercury emerging regularly (at a rate of one drop every 3 to 5 seconds, the drop time should not exceed 7-8 seconds) from the end of a fine bore (0.05 to 0.08 mm i.d.) capillary tube, and continuously exposing a fresh mercury surface to the electrolyte. Thus the DME has proved to be the most essential component of all polarographic experiments (101,102), despite studies on other micro electrodes e.g. tungsten or platinum.

The characteristic common to all modern polarographic instrumentation is "potentiostatic" control of the working electrode potential. The potentiostat controls the potential at the working electrode-solution interface and in the two-electrode situation (where only working and reference electrodes are used) can yield data which are considerably in error where the solution resistance is too high and the resultant voltage drops between the electrodes. Eliminating the error due to the solution resistance is thus very desirable and if a suitable system was available would be applicable to a much wider range of electrochemical systems. A potentiostat usually accomplishes this end by making use of a three-electrode system. Here, a reference electrode of constant potential (inserted in the system and positioned as closely as possible to the working electrode) is connected to

the instrument through a circuit which draws essentially no current from it. There is thus no current flow between the tip of the reference electrode (or its connecting bridge) and the instrument, and thus no voltage drop (103).

The DME is useful over the range +0.3 V to -2.8 V versus the saturated calomel electrode (SCE). At potentials more positive than 0.3 V, mercury dissolves and gives an anodic wave. At potentials more negative than -1.2 V, visible hydrogen evolution occurs in 1M HCl solutions, and at -2 V the usual supporting electrolysis of alkali salts begins to discharge (10).

The technique consists of applying a gradually (c. 1.0 V/min) increasing potential difference across the electrodes immersed in the solution under analysis, and measuring small currents of the order of microamperes produced by the reduction of ions at the dropping mercury cathode, or oxidation if the electrode is operating anodically. Since the solution is not stirred, the currents are due to:-

i) the migration of ions at working electrode (DME) in the electrical gradient set up, and

ii) the diffusion of ions in the concentration gradient produced by the removal of ions from solution in the proximity of the DME (101).

Only the latter current is required, since the magnitude is dependent on the concentration of the reducible substance and it is most fortunate, therefore, that the migration current can be readily suppressed by the addition of an excess of an indifferent salt to the sample solution. The solution of this indifferent salt is known as a "supporting electrolyte" or "indifferent electrolyte" (101).

The effect of migration is usually eliminated by adding a 50- or 100-fold excess of the inert "supporting electrolyte". The ions of this electrolyte migrate to relieve the electric fields but do not undergo an electrochemical reaction at the electrode. A potassium salt is often employed (10).

The current plateau corresponds to the condition where metal ions are reduced as fast as they reach the electrode by natural diffusion - a condition of complete concentration polarisation. Reducible and oxidisable species which show such polarographic characteristics are often referred to as depolarisers. The magnitude of the diffusion-controlled currents at DME are direct functions of depolariser concentration and in this fact lies the quantitative analytical significance of polarography. All quantitative analysis by the technique is based on the direct proportionality between the limiting diffusion controlled current and depolariser concentration (104) as expressed in the Ilkovic equation:-

$$i_d = 607\ n\ D^{\frac{1}{2}}\ m^{\frac{2}{3}}\ t^{1/6}\ C \qquad (2.13)$$

where i_d is the average diffusion current (μA)

n is the number of moles of electrons involved in the reaction equation (this number is also equal to the valence charge of the metal)

D is the diffusion coefficient of the reducible ion in solution (cm^2/s)

m is the mass of mercury flowing (mg/s)

t is the drop time (seconds)

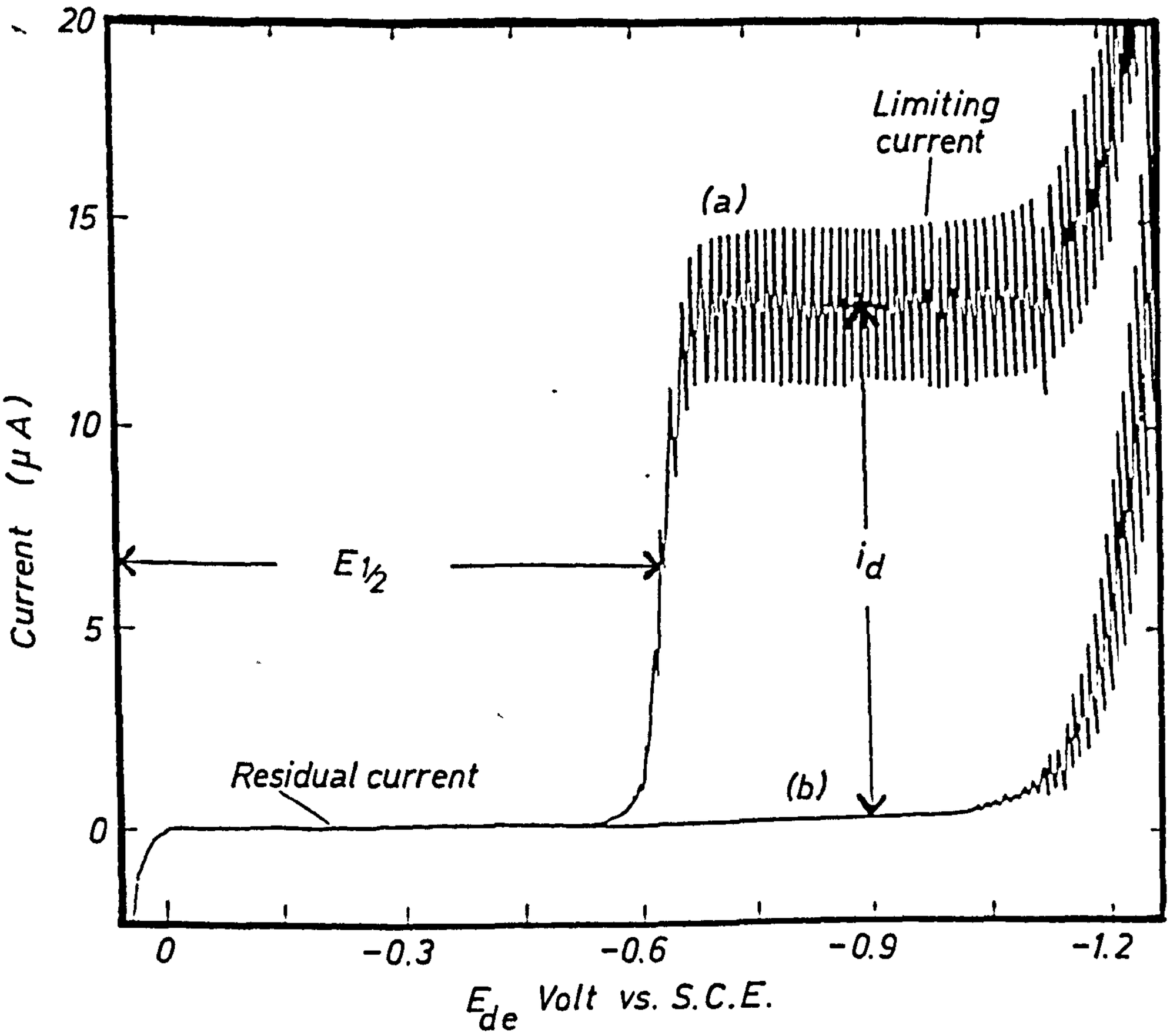
C is the concentration of the reactant (mM/L)

Thus the diffusion current is directly proportional to concentration (10,104,105). The diffusion current at DME is affected by both the parameters of the DME and the properties of electroactive species in solution as shown by the Ilkovic equation (70). For analytical applications of polarography, the original empirical Ilkovic equation (2.13) is adequate and much more convenient; its errors tend to cancel out in practical use (10).

Polarography is mainly performed at the DME, and when the input voltage used is DC voltage the technique is called direct current polarography (DCP). The DCP current response is represented in Figure 2:5 which is a direct reflection of current variation during the lifetime of each mercury drop (99). DCP involves measurement of the diffusion current when an applied potential is changed by typically 100 mV/min in a linear but negative direction from -0.1 to -1.80 volts w.r.t. Ag/AgCl. Due to the double layer formation at the DME-solution interface by electrostatic attraction of cation and anion respectively, a significant background current is observed in the current-applied potential and the phenomenon effectively limits the sensitivity of DCP (70) which has a detection limit of around 1 ppm (106).

Although of limited analytical utility, DC and sampled DCP are still useful to distinguish between oxidation and reduction processes (106).

FIGURE 2.5 POLAROGRAMS FOR (a) Cd^{2+} ION, IN DILUTE HCl, AND (b) DILUTE HCl ALONE



(a) Single electroactive species and supporting electrolyte

(b) Deoxygenated supporting electrolyte alone

$E_{1/2}$ Half - wave potential

i_d Diffusion current

Pulse Polarography

Pulse polarography takes advantage of the fact that, following a sudden change in applied potential, the capacitative current surge decays much more rapidly than does the faradic current. In this technique a small amplitude voltage pulse, in addition to the linearly increasing DC ramp of about 1 mV/sec normally used for DC polarography, is applied to the polarographic cell. As each mercury drop forms it is allowed to grow for a period of time, perhaps 1.9 sec at the DC ramp potential, after which a sudden voltage pulse of perhaps 50 msec duration is applied. The pulse is synchronised with the maximum growth of mercury drop, when a DME is used. The current is measured 40 msec after the application of the pulse, to allow time for the charging current to decay to a very low value (10).

Pulse polarography takes two forms:-

a) normal pulse polarography (NPP),

b) differential pulse polarography (DPP) (105).

The techniques of normal pulse and differential pulse polarography have largely displaced DCP for analytical purposes. The pulse waveforms are designed to enhance the faradic current relative to the charging current, leading to significantly improved detection limits. With the pulse techniques the drop time must be controlled, i.e. the mercury drop is dislodged by mechanically tapping the capillary. The drop knocker must be incorporated into the electrode system (106).

a) Normal pulse polarography (NPP)

In the technique of NPP, the potential of the drop is held at some nominal initial value E_I during most of the drop to a new potential E_{pulse} and holds it there. The duration of this pulse is usually some 50 to 100 msec, and the current is measured during the last few msec of the pulse. In the pulse mode of the PAR Model 174A, for example, the pulse is 57 msec long, and the current is measured (averaged) over the final 16.7 msec of the pulse. The pulses are of slowing increasing height, such that E_{pulse} changes slowly just as the potential ramp does in classical polarography (107).

b) Differential pulse polarography (DPP)

DPP is the most widely used form of pulse polarography. In DPP, a slow continuous DC scan is used as in classical polarography. On this slow voltage scan is superimposed a series of voltage pulses, themselves of constant magnitude rather than of increasing magnitude as in NPP (107). For DPP uniform square voltage pulses are imposed upon the linear voltage ramp of classical polarography. This approach yields the current response. The purpose of the pulse technique is to minimise the amount of capacitative charging current in the current measurement (105). The pulses (magnitude 5-250 mV) are added once during each drop lifetime and last about 60 msec as in normal pulse. The current is measured twice - once before applying the pulse and once during the last 17 msec of the pulse. The first current value is instrumentally subtracted from the second current value (106). The difference between these two current values is amplified and fed to a recorder producing peak output (70). The sensitivity of DPP lies between that of DCP and NPP but is often preferred because DPP discriminates more effectively against charging current as shown in Table 2:9 (99).

Table 2:9. Typical Sensitivity and Detection Limits for DCP, NPP and DPP

Technique	Sensitivity μA/mM	Detection Limits (M)
DCP	5	10^{-5}
NPP	30	10^{-6}
DPP	20	10^{-7}

Polarography is a highly sensitive technique (detection limit from 10^{-5} to 10^{-7}M) detecting and determining electroactive substances. Unfortunately, oxygen is capable of dissolving in aqueous solution to the extent of forming millimolar solutions at room temperature and pressure, and this dissolved oxygen is in itself electroactive. Complications in the polarograms obtained are introduced both by the voltammetric behaviour of oxygen and from the associated chemical reactions which take place. Oxygen is reduced at the DME in two stages (108). The first stage involves the reduction of oxygen to hydrogen peroxide and/or hydroxide ion; the second stage involves the reduction of oxygen to hydroxide ion or water.

The most convenient method of removing oxygen is to deaerate the analyte solution with pre-purified nitrogen gas, using an oxygen scrubbing system to remove the last traces of oxygen (55,108).

Vanadous chloride solution can be used for oxygen scrubbing, as shown in Figure 2.6 (108).

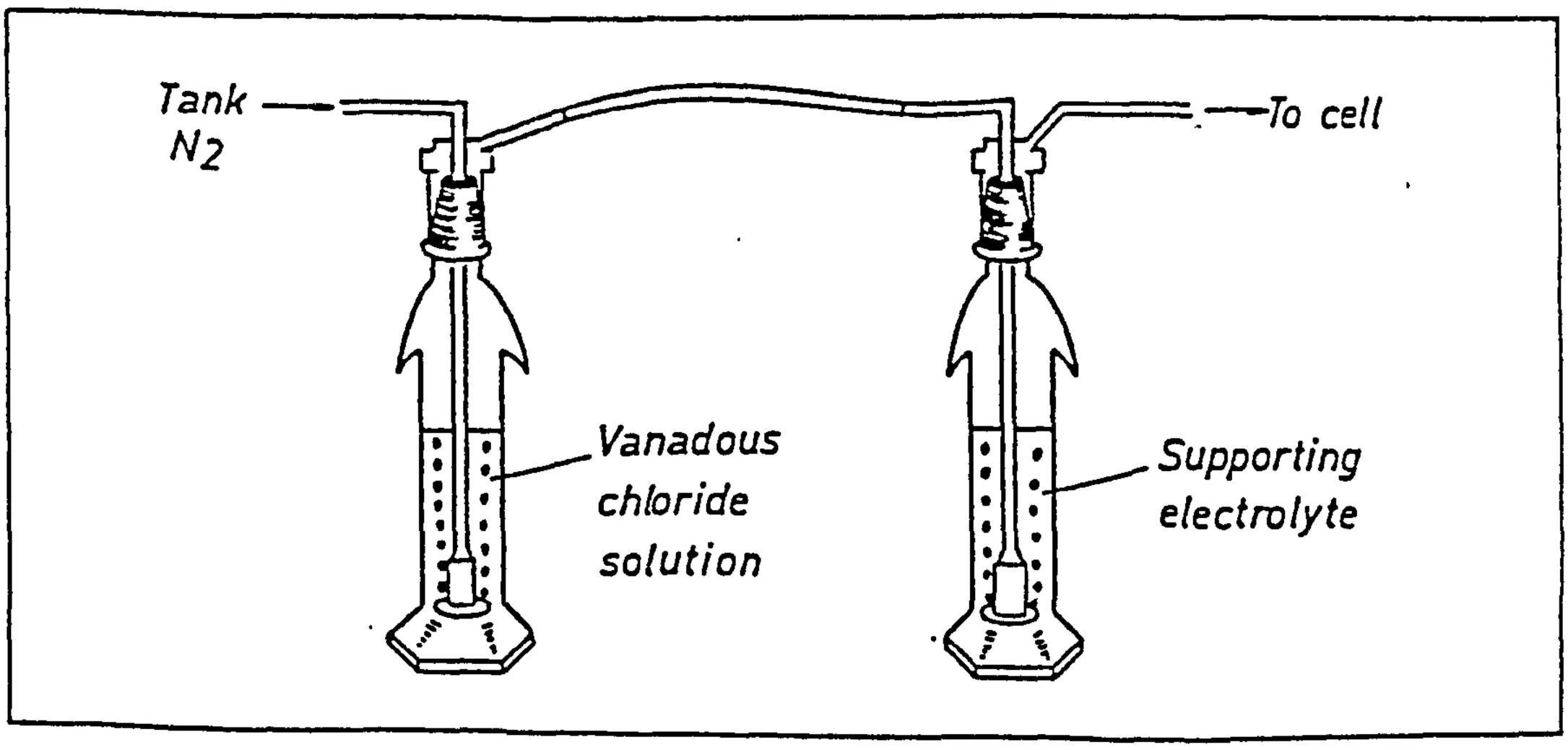
Applications

Polarographic techniques are applicable to trace analysis, being particularly useful for the determination of inorganic constituents of

water. Not only is the sensitivity of polarography competitive with other techniques, but its selectivity permits the determination of many constituents without prior chemical separations. Moreover, the range of concentrations which can be determined makes possible the analysis of all types of water ranging from highly contaminated industrial effluents and mineral springs to water prepared for laboratory use by distillation or ion exchange(55).

Determinations of inorganic or organic species that are either molecular or ionic can be performed if they undergo oxidation or reduction at a mercury electrode in the region of potential bounded at the positive limit by the potential of oxidation of mercury in the medium employed, and at the negative limit by the potential at which the supporting electrolyte or the solvent is reduced. Non-aqueous solvents can be used for organic substances that are insoluble in water, the only limitation being that if the resistance of the medium is high, the polarographic wave may be severely distorted (10).

FIGURE 2.6 OXYGEN SCRUBBING SYSTEM



In fact, differential pulse polarography can be used to determine antibiotics and many other materials at concentrations well below the ppm level (103).

ii) Voltammetry

The technique of stripping voltammetry has been used in trace analysis with relative ease and success in a variety of analytical applications. Metals, non-metals (such as selenium), ions and organics can be determined in a wide range of sample matrices with minimal sample preparation. This electrochemical technique is routinely capable of identifying and quantifying trace components (109) and its ability to determine simultaneously several elements at concentration levels of ng/ml or less in solution with acceptable precision and accuracy makes the technique a very attractive one. It is also possible to automate the determination by means of relatively inexpensive instrumentation (44,110).

Stripping voltammetry is very similar to polarography, with a small, but significant, change in procedure. Stripping voltammetry is a two-step technique in which the first step consists of the electrolytic deposition of a chemical species onto an inert electrode surface

at a constant potential. This preconcentration step can involve either an anodic or cathodic process, according to the character of the stripping process (reduction or oxidation, respectively) (109,111). An example of an anodic process is the deposition of a selenide onto a hanging mercury drop electrode (HMDE) in the form of an insoluble film of mercuric selenide (112):

$$Se(IV) + 6e + 2H^{+} \longrightarrow H_2Se \qquad (2.14)$$

$$H_2Se + Hg \longrightarrow HgSe + 2H^{+} + 2e^{-} \qquad (2.15)$$

However, the most common use of stripping voltammetry involves a cathodic process in which a metal ionic species is reduced from the solution onto a mercury electrode, resulting in the formation of an amalgam. The second step consists of the application of a voltage scan to the electrode which causes an electrolytic dissolution, or stripping, of the various species in the amalgam or film back into solution at characteristic potentials (109).

1. The Deposition Step

The extremely low detection limit of stripping analysis is attributable to the preconcentration that takes place during the deposition step. The deposition step is usually carried out employing a controlled-potential electrolysis for a definite time and under reproducible hydrodynamic (mass-transport) conditions in the solution.

(a) In the anodic variant of stripping analysis, the metal ions of interest are reduced at the controlled negative potential. At the mercury working electrode used for measurement of amalgam-forming metal the electrode reaction is (113):-

$$M^{n+} + n\,e^{-} + Hg \xrightarrow{Red} M(Hg) \qquad (2.16)$$

At solid electrodes, used for measurement of ions with positive redox potential, the corresponding electrochemical reaction is:

$$M^{n+} + n\ e^- \xrightarrow{Red} M \qquad (2.17)$$

resulting in a metallic film on the surface. Obviously the reducible forms of the element, which form such deposits, will be determined, in addition to the accumulation of metal ions by their reduction into or onto the working electrode.

(b) The cathodic variant of stripping analysis utilises the deposition of various organic and inorganic species as sparingly soluble compounds on the electrode surface.

In all cases, the deposition potential imposed on the working electrode is chosen according to the species to be determined and is maintained for a deposition period depending on their concentrations (113).

2. The Stripping (Oxidation) Step

Usually the stripping step consists of scanning the potential anodically (toward more positive potentials). When the potential reaches the standard potential of a metal-metal ion couple, that particular metal is reoxidised back into solution and a current is produced:-

$$M(Hg) \longrightarrow M^{n+} + n\ e^- \qquad (2.18)$$

The resultant voltammogram (current-potential plot) recorded during this step provides the analytical information of interest. The stripping current, because of the oxidation of the metal, is proportional to the concentration of that metal in or on the electrode and, therefore, to its concentration in the sample solution. Peak

potential serves to identify the metals on the sample (109,111,113). Whereas the deposition step of the analysis is usually the same for the different stripping techniques, the primary differences are associated with the approach used during the stripping step (113).

Electrodes for Stripping Voltammetry

Unlike polarography, the DME is not used in stripping voltammetry. The electrode must be stationary. The ideal working electrode should have a reproducible surface, a reproducible area, and a low residual current (109). Solid electrodes of gold, silver, platinum, carbon and bismuth have been used successfully. The determination of those metals with oxidation potentials anodic of that of mercury (e.g. Au, Hg and Ag) requires the use of such solid electrodes. Some of the noble metals have a mutual tendency for the formation of intermetallic compounds (e.g. AuHg). To avoid this a carbon electrode is often used (110). Although solid electrodes give a sensitive response, they generally can be used for the determination of only one species. When a solid electrode is employed for the determination of several species, it is almost impossible to obtain the required homogeneity of the deposited materials prior to the stripping step (109).

The most popular working electrodes for stripping voltammetry are a) the hanging mercury drop electrode (HMDE); and b) the thin film mercury electrode (TFME) (102,109,114).

a) Hanging mercury drop electrode

The HMDE is the best working electrode for stripping voltammetry because of its extremely reproducible surface. The hanging mercury drop is formed at the tip of a microsyringe or capillary, with a

micrometer to control the drop size (109,112,114). A simple HMDE was developed by Gerischer in the 1950s. Using his approach 1 or 2 mercury drops (falling) from the classical DME are collected and transferred to a small metal contact sealed in glass or in plastic material. Platinum or gold-plated platinum wires are used (102). The design has now been replaced by the Kemula-type HMDE, with a glass capillary (115). The Kemula-type HMDE allows easy and reproducible renewal of the mercury drop and use of relatively long deposition periods. In this electrode, mercury is displaced from a reservoir through a capillary (0.15-0.50 mm i.d.) by a screw-driven plunger. Reproducible drops are formed at the tip of the capillary by adjustment of a calibrated micrometer (113).

The surface of the mercury drop is perfectly smooth and is constantly renewed by the dislodgement process. With solid electrodes, frequent polishing or cleaning is necessary in order to regenerate the original surface. Because the size of the mercury drop is very small, only an extremely low portion of the sample actually reacts during an analysis, i.e. the amount reacting is insignificant, and voltammetry is, therefore, considered a non-destructive technique (55,116).

The use of mercury is nearly an ideal choice for the operation of the working electrode for several reasons:

i) the surface area of such an electrode is highly uniform and reproducible (if the mercury is clean) and has low background current (113);

ii) mercury electrodes are used most frequently because they fulfill the sensitivity and reproducibility requirements and the element has a wide cathodic potential range (113);

iii) mercury behaves as an inert electrode in most aqueous solutions;

iv) it can be used for cathodic stripping voltammetry e.g. for determination of selenium by the formation of an insoluble film of the salt on the electrode surface (3,117);

v) the high hydrogen overvoltage of mercury allows the analyte to reach more negative potentials than any other metal electrode before the reduction of hydrogen ions commences (55,116).

The disadvantages of the HMDE are:

1) it is difficult to maintain a stable drop on the end of the capillary; the mercury drop can fall off during the experiment and so the run must be aborted (109);

2) mercury is a toxic material and the oxidation of mercury occurs at +0.4V, preventing the determination of materials that are oxidised at more positive potentials (such as Au, Ag) (116);

3) interferences are caused by intermetallic compound formation inside the mercury drop. When metals such as Cu and Zn are present in solution at high concentrations, they can affect the size or position of the peak current and overlapping stripping peaks have occurred (114,118).

In order to avoid some problems encountered with the HMDE, the TFME has been used in stripping voltammetry.

b) Thin film mercury electrode (TFME)

A TFME is prepared by deposition of a film of mercury onto a glassy carbon electrode, although other electrode materials may be used (109). Gold is not a suitable solid electrode substrate, because gold amalgam is formed, but platinum may be used (119). Glassy carbon is an especially good support for the mercury film electrode (120,121) because it does not interact with mercury. The most suitable forms are glassy carbon or the wax-impregnated graphite electrode polished flat, since a smoother and less porous surface is better for deposition (119).

The TFME consists of a very thin (1 to 100 μm) layer of mercury covering an inert support electrode, so that the electrode exposes only mercury to the electrolyte (113,119). The TFME can be prepared by

placing the glassy carbon electrode in a well-stirred solution of $Hg(NO_3)_2$ made slightly acidic with nitric acid at -0.4 V vs SCE for 5 min. Once the TFME is generated, it must be protected from oxygen to prevent oxidation of the film, principally because the layer of deposited mercury is extremely thin (109).

The TFME can also be prepared "in situ" by adding 2-5 ppm mercuric nitrate directly to the sample solution and electrodepositing mercury and trace metals simultaneously. The trace metals are then anodically stripped from the mercury film. After the analysis the mercury film can be completely removed from the electrode by simply wiping with a tissue (121).

The TFME is generally used only for anodic stripping voltammetry (ASV). Such an electrode is most useful where maximum sensitivity is required. The TFME exhibits high sensitivity because only an extremely small amount of mercury is incorporated into the film, resulting in the formation of a very concentrated amalgam during the deposition step (109). The plating efficiency and sensitivity are enhanced because the electrode can be used easily with vigorous convection conditions (high rates of rotation or stirring). In addition to having high sensitivity, mercury films give superior selectivity as diffusion from the bulk of the film to the surface is very fast (113). The use of the TFME should be limited to analyte concentrations less than 10^{-7}M (109). Because the same electrode surface is used for repetitive analyses, the condition of the surface is a major consideration. Steps must be taken to ensure that the surface of the TFME is as reproducible as possible prior to each analysis. Failure to guarantee a consistent surface will inevitably give rise to irreproducible results. This problem, of course, is not a consideration with the HMDE since a new mercury drop is used for each determination. The TFME cannot be considered

appropriate for routine analytical purposes (109).

The major problems with TFME (which is deposited on rotating glassy carbon electrodes) are change in the surface of the solid substrate and the deposition film thickness. These changes result in the irreproducible and often multiple or split stripping peaks (122,123).

Another approach to eliminate errors caused by intermetallic compound formation has been the preferential formation of another intermetallic compound (110,113,124). This approach is based on the addition of a "third" element that forms a more stable intermetallic compound with one component of the interfering binary system. For example, the Cu-Zn problem is usually circumvented by adding an excess of Ga^{3+} to the sample solution. Because the Cu-Ga intermetallic compound has a larger formation constant than the Cu-Zn compound, the gallium preferentially combines with copper so that zinc may be determined without interference. Copper then can be determined in a separate experiment in which only copper is plated (113,124).

Differential Pulse Stripping Voltammetry

Perhaps the most widely used stripping mode is differential pulse voltammetry, designed to compensate for the charging background current. In the differential pulse stripping mode, pulses of equal amplitude are superimposed on an anodic potential scan. The pulses have an amplitude of 25 or 50 mV, a duration of about 50 msec, and a repetition rate of 0.5-5 sec. Usually, the basic potential scan rate is slow, e.g. 2-10 mV/sec, so that the ramp potential does not change significantly during the pulse life. The currents are sampled twice: prior to the pulse application and just before the pulse termination. The first

current is subtracted instrumentally from the second one, and the current difference is plotted versus potential. A peak-shaped response is obtained, with the peak potential corresponding to the maximum rate of metal oxidation for a given potential change (113).

In differential pulse stripping voltammetry, all the considerations of the DC stripping voltammetry case apply. However, the far greater sensitivity and signal processing capabilities of the pulse-modulated detection technique permit significantly higher instrument sensitivities to be used and thus allow either much shorter deposition times or much lower instrument gains. Under these circumstances, deposition times are kept to a few minutes so that electrode instabilities are less of a problem, and diffusion into the body of the electrode is less important (103).

By use of the pulse stripping technique on both hanging drop and film electrodes, sensitivities similar to those obtained by DC stripping voltammetry can be obtained with much shorter experiment times (103).

Owing to the introduction of relatively inexpensive commercial instruments, differential pulse stripping voltammetry is widely used for extensive analytical applications, especially when measurements of metals at the parts per billion level (ng/g) and below are concerned (113).

There are two types of stripping voltammetry:

a) Anodic stripping voltammetry (ASV), and

b) Cathodic stripping voltammetry (CSV).

Although very similar in concept, they are used to determine completely different types of materials (109).

a) Anodic Stripping Voltammetry (ASV)

ASV is used to determine the concentration of trace metals. ASV consists of a deposition potential that is more negative than the half-wave potential of the metals to be determined, and an anodic (positive-going) scan to oxidise the reduced metal back into solution. During deposition, an amalgam is formed by the elemental metal and the mercury on the electrode. ASV can only be used to determine those metals that exhibit appreciable solubility in mercury such as antimony, arsenic, bismuth, cadmium, copper, gallium, germanium, gold,[a] indium, lead, mercury,[a] selenium,[a] silver,[a] thallium, tin and zinc. ([a] Must be determined on a solid electrode, such as glassy carbon or gold.) (109, 117, 125).

The high sensitivity of ASV has attracted a great deal of attention to the potential of the metal for determination of metal ions at the 10^{-9}M level (126).

b) Cathodic Stripping Voltammetry (CSV)

CSV is used to determine a wide range of organic and inorganic compounds that form insoluble salts with mercury (the electrode material) (123,127). In CSV, the mercury working electrode is not inert, but takes an active part in the formation of the deposit (see equation 2.15). The application of a relatively positive potential to a mercury electrode in the presence of such a material will result in the formation of an insoluble film on the surface of the mercury electrode. Stripping in CSV consists of a cathodic (negative-going) scan to reduce the deposited salt back into solution (109).

Low concentrations of halide ions, selenide ions, thiocyanate ions, sulphide ions, and oxyanions, such as $[MoO_4]^{2-}$, $[CrO_4]^{2-}$, $[WO_4]^{2-}$ and

$[VO_3]^{2-}$, can be determined by CSV. These determinations are based on the reactions of the anion of interest with the electrogenerated Hg_2^{2+} ion to form a partially insoluble mercurous film that can be preconcentrated on the mercury electrode surface (113).

Because CSV involves the formation of a film on the surface of an electrode and not a homogeneous amalgam as in ASV, it is not unusual for calibration curves in a CSV analytical procedure to display non-linearity at higher concentrations. Even though the curves may be nonlinear, they are generally quite reproducible and can be used with confidence (109).

Much attention has been paid to the determination of selenium by CSV; this is because of the difficulties associated with trace measurements of selenium by other techniques (3,112,113,117). Solutions of selenium (VI) (electroinactive) are not reduced on a mercury electrode, but Se(IV) is reduced to selenide when in acidic solution (113).

The CSV method has recently been used for the determination of aluminium in natural samples with a detection limit of around 10^{-9}M (128).

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CHAPTER 3

1,2-DIAMINOBENZENE DERIVATIVES AS REAGENTS FOR DETERMINATION OF SELENIUM (BY GLC +ECD)

CONTENTS

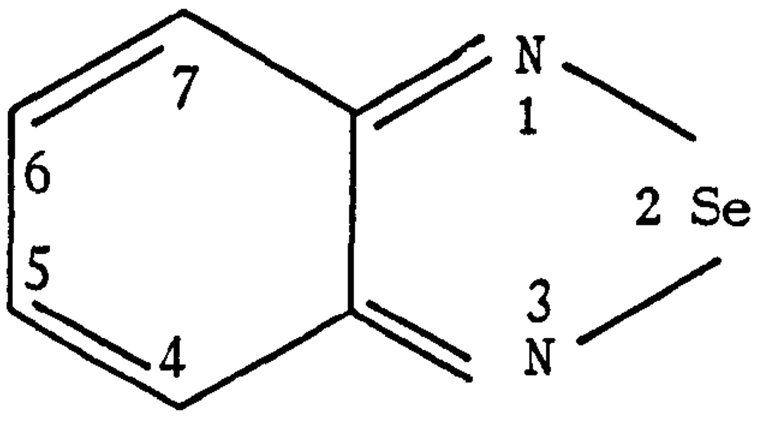
3:1 INTRODUCTION

Estimation of Se by gas-liquid chromatography (GLC) is almost always based on the measurement of the amount of piazselenol formed during the reaction of Se(IV) with a chosen o-phenylenediamine (o-PDA), in acidic solution (usually HCl). Piazselenols are easily extracted with toluene, whence they can be subsequently determined by chromatographic methods. In GLC, piazselenols are usually estimated using an electron capture detection (E C D) system due to its extremely high sensitivity and selectivity. Apart from the superior sensitivity and selectivity, the GLC method allow for the elimination of interferences from the matrix (1).

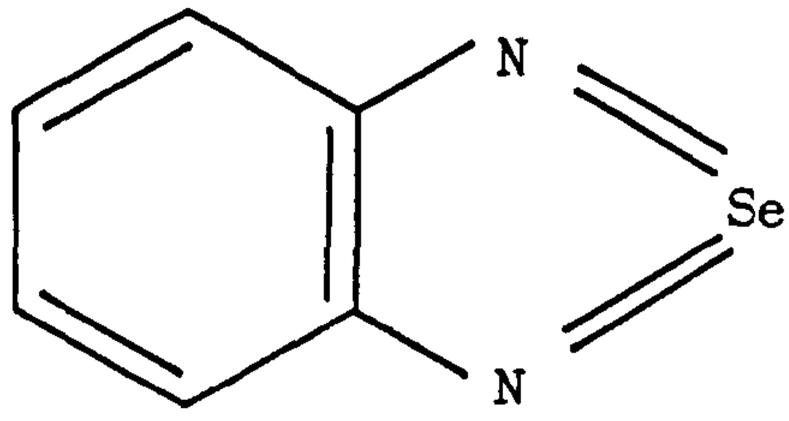
The parent piazselenol was first synthesised in 1889 by Hinsberg (2) who demonstrated that only Se(IV) reacted with o-PDA to form a piazselenol (selenium complex).

The parent piazselenol is a stable compound (as are the derivatives of analytical interest), and its sulphur and oxygen analogues are well known (3).

From various approaches (including X-ray crystallography (4), infra-red (5,6) and nuclear magnetic resonance (7) spectroscopy), piazselenol is best represented by an ortho-quinonoid structure (I) consistent with the considerably greater single-bond character of the N-Se bond (1.83 Å) than either the C_4-C_5 bond (1.30 $\pm$ 0.04 Å) or the C_5-C_6 bond (1.42 $\pm$ 0.04 Å). Indeed, it has been concluded (6) that piazselenol has greater ortho-quinonoid character than its sulphur or oxygen analogues and is comparable with that found in other benzo-heterocycles (3,5).



(I)



(II)

Nakashima and Toei (1968) first investigated the GLC method for estimation of Se using 4-chloro-o-PDA and reported a sensitivity of 4×10^{-8} g (8). Shimoish (1977), looking for increased sensitivity (in the determination of Se) studied 13 derivatives of 1,2-diamino-benzene, and reported the sensitivity depended upon the substituents present and was in the order H < Cl < Br < NO_2 for the compounds studied. 4,6-Dibromopiazselenol had the highest sensitivity and was capable of detecting a level of 1 ng Se (9).

In the present work the gas chromatographic properties of 10 piazselenols were studied in order to obtain a more sensitive reagent, of which four are new reagents, namely:-

a) 4-Bromo-6-trifluoromethylpiazselenol

b) 4-Bromo-6-fluoropiazselenol

c) 4-Chloro-6-trifluoromethylpiazselenol

d) 4-Chloro-5-fluoropiazselenol.

3:2 EXPERIMENTAL AND RESULTS

3:2:1 Experimental Preparation

i) Reagents and Glassware

a) Reagents

AnalaR hydrochloric, hydrobromic, nitric, perchloric, sulphuric acids, magnesium nitrate hexahydrate, bromine, metallic selenium, sodium selenate and urea; reagent-grade selenium dioxide; and selenious acid standard solution "Spectrosol" (for atomic spectroscopy) were obtained from BDH, Poole, U.K. 4-Chloro-1,2-phenylenediamine and 4-nitro-1,2-diaminobenzene were obtained from Koch-Light (Colnbrook, Bucks., U.K.). 1,2-Phenylenediamine dihydrochloride, 2-nitroanaline and N-bromosuccinimide were obtained from Aldrich (Gillingham, U.K.). 4-Fluoro-2-nitroaniline and 3,4-diaminobenzotrifluoride were obtained from Fluorochem (Glossop, Derbyshire, U.K.). 4-Fluoro-5-chloro-1,2-phenylenediamine, 3-bromo-5-trifluoromethyl-1,2-diaminobenzene, 3-chloro-5-trifluoromethyl-1,2-diaminobenzene, 1,2-dinitro-3,4,5,6-tetrachloro-benzene were obtained from Maybridge (Trevillett, Tintagel, Cornwall, U.K.). Chromosorb W AWDMCS was obtained from PhaseSep (Queensferry, Clwyd, U.K.). OV-17 was obtained from Jones Chromatography (Llanbradach, Glamorgan, U.K.). Toluene was double distilled.

Standard solution:

i) Se(IV): Stock solution 1000 ppm = 1000 µg/ml was prepared by:

1) 0.5 g of metallic selenium was dissolved in 10 ml concentrated nitric acid and diluted to 500 ml with double distilled water (DDW).

2) alternatively 0.7026 g of selenium dioxide was dissolved in 500 ml DDW.

3) using selenious acid standard solution.

ii) Se(VI): A 1000 µg/ml Se(VI) stock solution was prepared by dissolving 2.3372 g of $Na_2SeO_4.10H_2O$ (sodium selenate) in 2M HNO_3.

Working solutions of Se(IV) and Se(VI) were made by dilution of the stock solution with 1M HCl and 2M HNO_3 respectively (10).

b) Glassware

Glassware was washed with "Teepol" detergent, rinsed with tap water and distilled water and placed in 40% v/v nitric acid (reagent-grade) for at least 48 hours. The glassware was rinsed with distilled water, then DDW and oven dried.

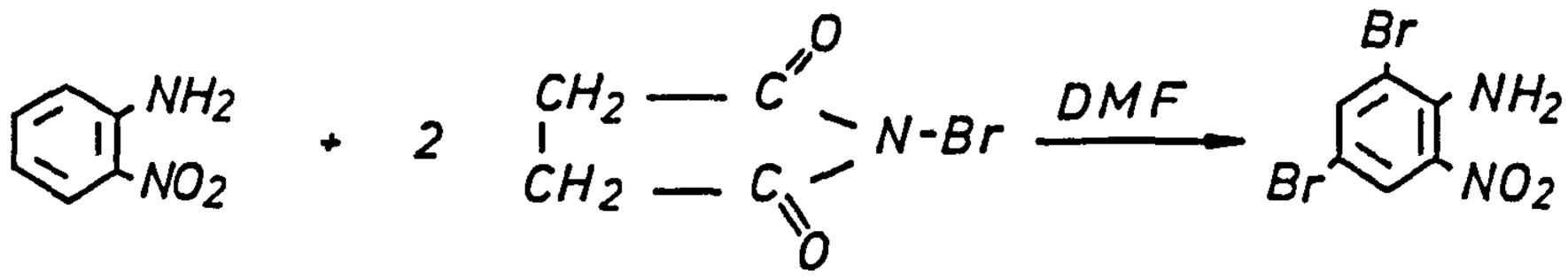
ii) Synthesis of Diamines

a) 1,2-Diamino-3,5-dibromobenzene dihydrochloride

Shimoishi (9) reported that 1,2-diamino-3,5-dibromobenzene was synthesised from 2-nitroaniline by simple bromination with bromine, followed by reduction with tin or zinc in hydrochloric acid.

Mitchell et al. (11) used N-bromosuccinimide-dimethylformamide (NBS)-(DMF) as the reagent for bromination of aromatic compounds.

The following procedure was the most convenient:

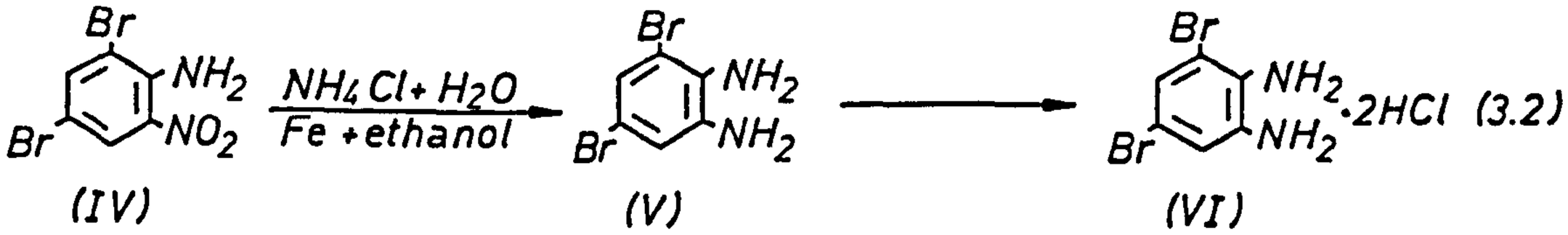


(3.1)

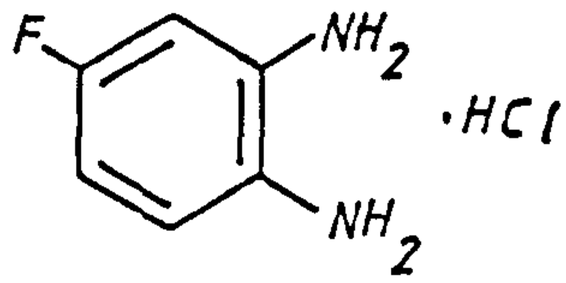
(III) *(IV)*

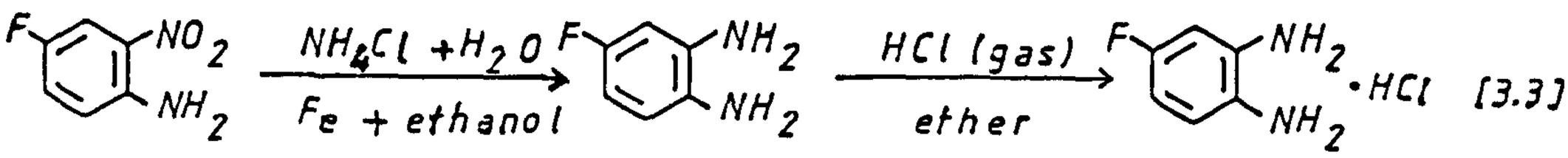
2-Nitroaniline (50 g = 0.362M) dissolved in 150 ml of dimethylformamide was mixed with N-bromosuccinimide (130 g = 0.730M) dissolved in a similar volume of dimethylformamide, by adding the latter dropwise over a period of about 10 minutes. The resultant mixture was refluxed and stirred for an overnight period (> 12 hours). After allowing to

cool, the reflux mixture was poured into one litre of distilled water. The slurry was stirred and filtered by suction to dryness (overnight). The yield (compound (IV)) was 100 g (93%) as yellow crystalline solid.



The reduction scheme is described in equation (3.2). Compound (IV) (20.72 g = 0.07M) was reduced by reaction of 30 g of iron (reduced by hydrogen), together with 20 g of ammonium chloride and 150 ml ethanol and approximately 100 ml water. The mixture was refluxed for two hours, using a water bath. The hot mixture was filtered, the filtrate maintained at room temperature and the precipitate washed with diethyl ether. The ethanol was removed by evaporation using a rotatory evaporator coupled to a water pump and a warm water bath. The resultant residue was then extracted with 400 ml of diethylether. The ether layer was separated off and made acid by passage of hydrogen chloride gas through the solution to precipitate the hydrochloride [compound (VI)]. The yield was 19 g (80%) as pale pink crystalline solids with m.p. 235-237°C (decomposed).

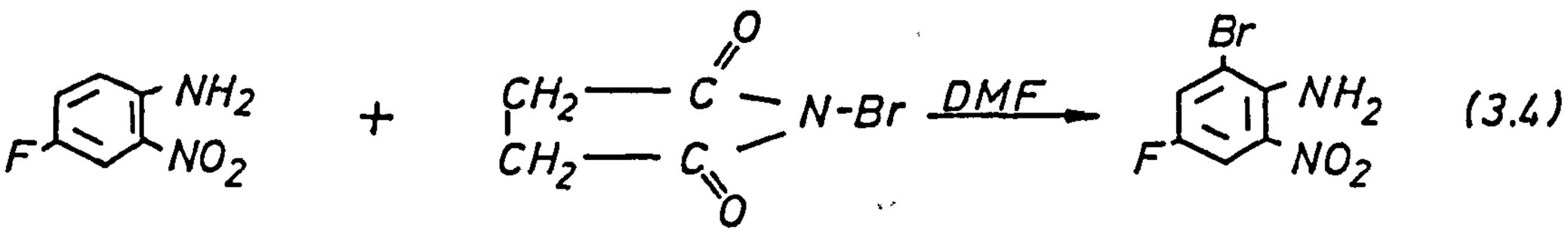
b) 1,2-Diamino-4-fluorobenzene hydrochloride 



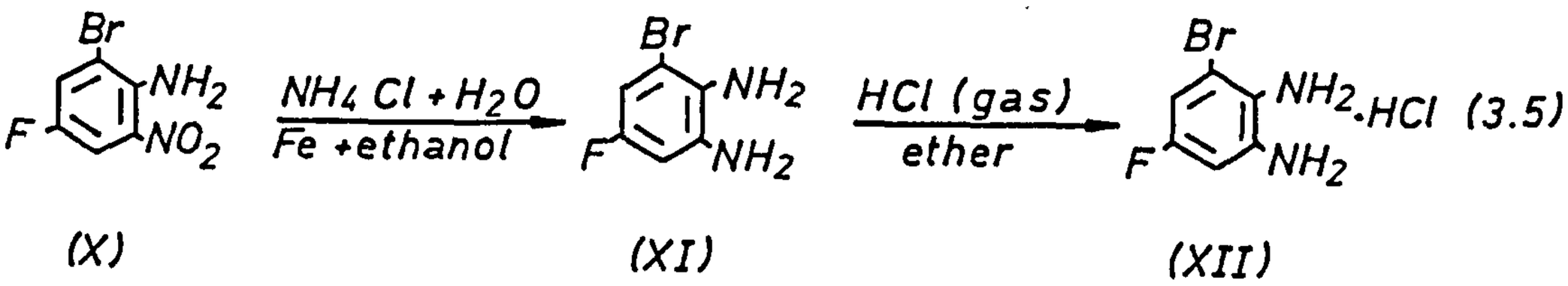
(VII) (VIII) (IX) [3.3]

4-Fluoro-2-nitroaniline (VII; 12.50 g = 0.08M) was reduced as described in equation (3.3) by 16.5 g of iron metal (reduced by hydrogen), together with 11 g of ammonium chloride, 100 ml ethanol and 75 ml water. The mixture was refluxed for two hours, using a water bath. The hot mixture was filtered, the filtrate maintained at room temperature and the precipitate washed with diethyl ether. The ethanol was removed by evaporation using a rotary evaporator coupled to a water pump and warm water bath. The resultant residue (crude VIII) was then extracted with 300 ml of diethyl ether. The ether layer was separated off and made acid by passage of hydrogen chloride gas through the solution to precipitate the hydrochloride (Compound IX). The yield was 13.10 g (82%), as a pale grey crystalline solid.

c) 1,2-Diamino-3-bromo-5-fluorobenzene hydrochloride



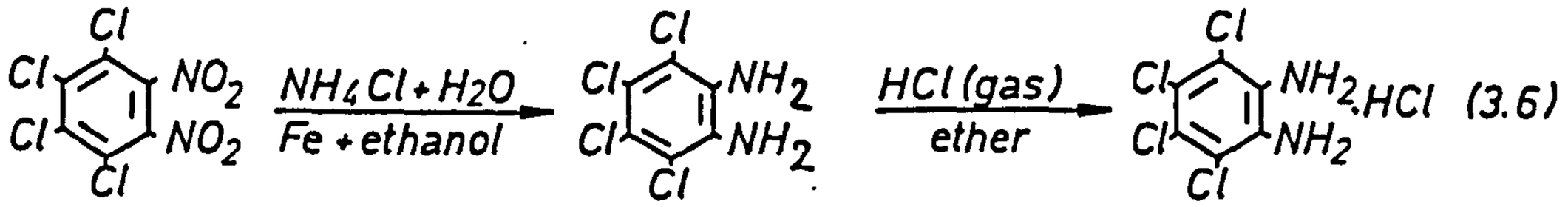
(VII) (X) [3.4]



(X) (XI) (XII) [3.5]

Compound (XII) was synthesised from 4-fluoro-2-nitroaniline (10 g = 0.064M) (VII) by simple bromination with N-bromosuccinimide (11.41 g = 0.064M) and 50 ml of dimethylformamide (N-BS)-(DMF), followed by reduction with an iron metal (reduced by hydrogen) and ammonium chloride. The resultant crude (XI) was dissolved in diethyl ether and hydrogen chloride gas passed through the solution to convert to the hydrochloride salt as described in Equations (3.4) and (3.5). The yield (10.06 g = 66%) was a pale brown crystalline solid with m.p. 210-211°C (decomposed).

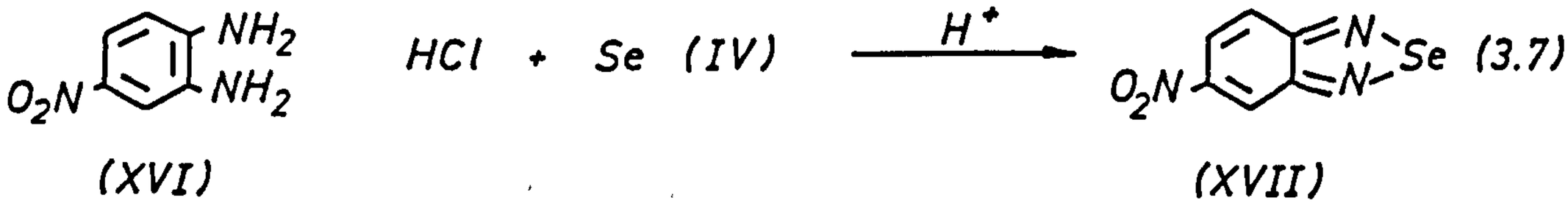
d) 1,2-Diamino-3,4,5,6-tetrachlorobenzene hydrochloride



(XIII) *(XIV)* *(XV)*

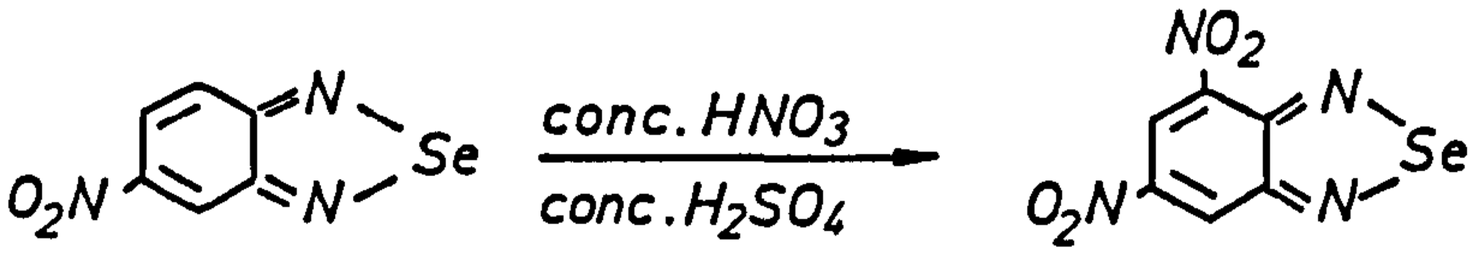
Compound (XV) was synthesised by the reduction of compound (XIII) (10.71 g = 0.035M) with 30 g of iron metal (reduced by hydrogen) and 22 g of ammonium chloride, 100 ml ethanol and 75 ml water. The mixture was refluxed for two hours, using a water bath. The hot mixture was filtered, the filtrate maintained at room temperature and the precipitate washed with diethyl ether. The ethanol was removed by evaporation using a rotary evaporator coupled to a water pump and warm water bath. The resultant residue, crude (XIV), was then extracted with about 300 ml of diethyl ether. The ether layer was separated off and made acid by passage of hydrogen chloride gas through the solution to precipitate the hydrochloride salt (compound (XV)). The yield was 7.1 g (63.6%) as a white crystalline solid with m.p. 212-214°C (decomposed).

e) 1,2-Diamino-3,5-dinitrobenzene



(XVI) (XVII) (3.7)

Compound (XVI) (20.85 g = 0.11M) was dissolved in 0.2M HCl and selenium dioxide (12.21 g = 0.11M) was dissolved in 0.2M HCl. The two acidic solutions were mixed together and the mixture allowed to stand at room temperature for two hours. The solid piazselenol compound (XVII) was filtered off through a glass-fibre filter paper. The yield (compound (XVII)) was 23 g (92%) as a pale yellow crystalline with m.p. 222-223°C.



(XVII) (XVIII) (3.8)

(XVIII) (XIX) (3.9)

5-Nitropiazselenol (compound (XVII)) (20 g = 0.088M) was dissolved in concentrated sulphuric acid (160 ml) and fuming nitric acid (d. 1.5; 120 ml) added. The solution was heated slowly to 90°C and after 15 min, cooled and poured onto crushed ice. The precipitate was filtered off, washed thoroughly with water, dried and recrystallised from acetic acid (12) to give pale yellow needles of 4,6-dinitropiazselenol (compound (XVIII)) (17.3 g = 72%), m.p. 210-212°C.

Compound (XVIII) (4.1 g = 0.015M) and 55% hydriodic acid (d 1.7; 35 ml) was mixed and heated at 50°C for two hours under refluxing conditions. The cooled mixture was treated with sodium hydrogen sulphite to remove iodine and made alkaline with 30% w/v sodium hydroxide solution and extracted with diethyl ether. The crude product was recrystallised from acetic acid (12) to give brick red needles of 3,5-dinitro-o-phenylenediamine (compound (XIX)) (1.8 g, 60%) with m.p. 216-218°C.

iii) Purification of the Ligands (diamines)

All the diamines which were commercially available or those from synthetic work had to be purified by recrystallisation, and at the same time converted from the free diamines to hydrochloride form (salts), prior to use in the piazselenol reaction. The following procedure was commonly used on all ligands:-

Reagent (diamine) was dissolved in 2M HCl and activated charcoal added and the solution warmed. The hot solutions were filtered and an equal volume of concentrated hydrochloric acid added to the filtrate. The solution was then cooled in an ice/salt bath. The crystals of hydrochloride were collected on a glass-fibre filter-paper supported on a glass sinter, washed with concentrated hydrochloric acid, dried by suction, and placed in a vacuum desiccator for drying (13,14).

Ligand solutions

The requisite diamine hydrochloride was dissolved in 10% (v/v) hydrochloric acid (except 3,5-dibromo-o-phenylenediamine which was

dissolved in concentrated hydrochloric acid) at a concentration of between 0.1 to 0.5% (w/v). The resulting solutions were stored in a refrigerator. New solutions were prepared every two weeks or when needed.

iv) Preparation of Piazselenol Standards

For the synthesis of the piazselenol standards, equimolar amounts of 0.04M diamine hydrochloride salt and selenium (IV) (as selenium dioxide) were mixed together in 0.2M HCl, as in equation (3.7). The mixture was allowed to stand at room temperature for two hours. The solid piazselenols were filtered off through a glass-fibre filter-paper and the damp solids recrystallised from 95% ethanol-acetone mixture (13,14). Melting-point data (measured in air) are listed in Table 3.1.

3.2.2 Analytical Results for Ligands and Piazselenols

Table 3.1 shows the melting-point data for 12 piazselenols, these results are very close to those already reported in the literature. Microanalysis for ligands (diamines) are listed in Table 3.2, while Table 3.3 shows the results for the microanalysis of the corresponding piazselenols.

Mass spectra of ligands were used to confirm the exact molecular weight of these ligands (diamine derivatives) as illustrated in Figures 3.1a-3.11a. Mass spectra of piazselenols are shown in Figures 3.1b-3.11b.

Table 3:1. Melting Points of Piazselenols

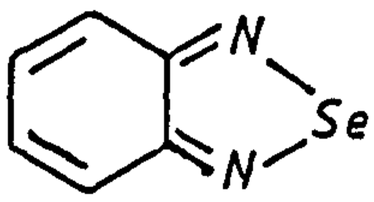
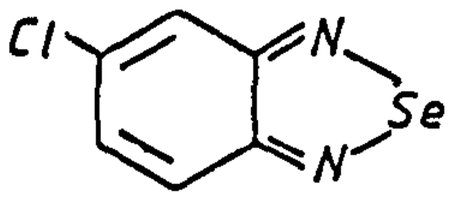
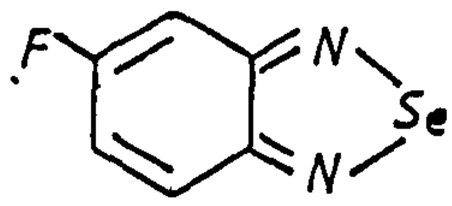
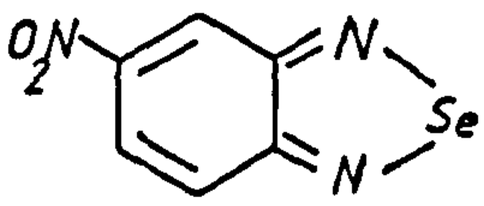
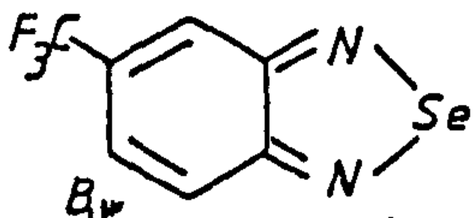
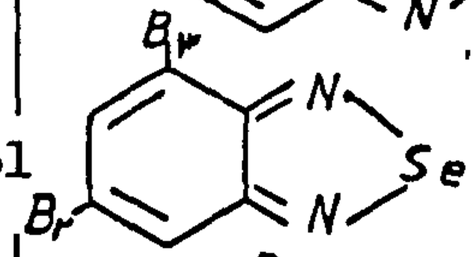
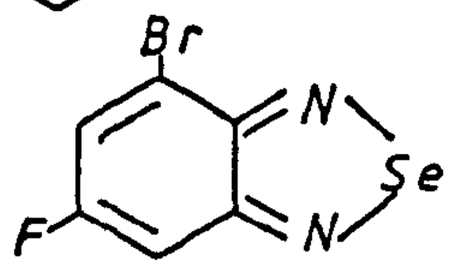
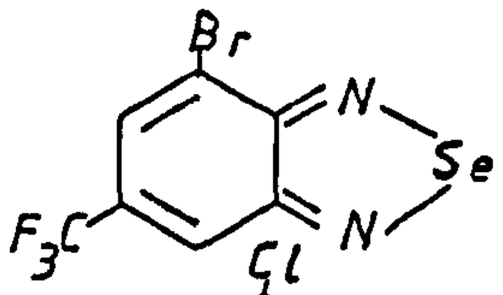
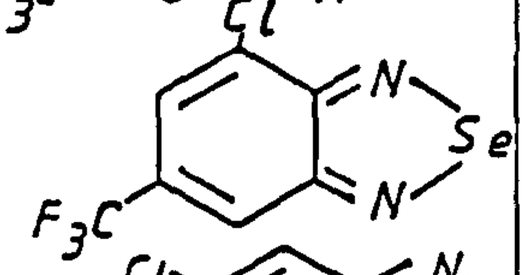
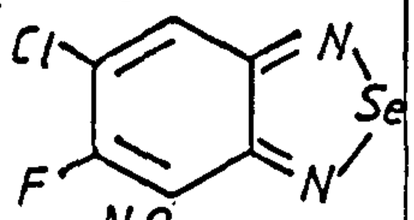
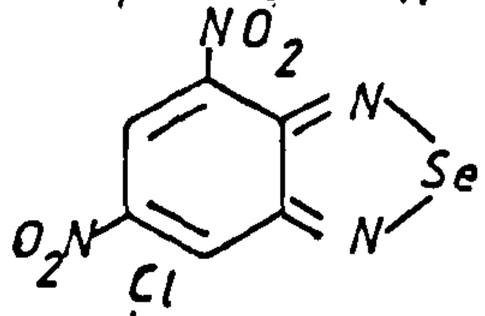
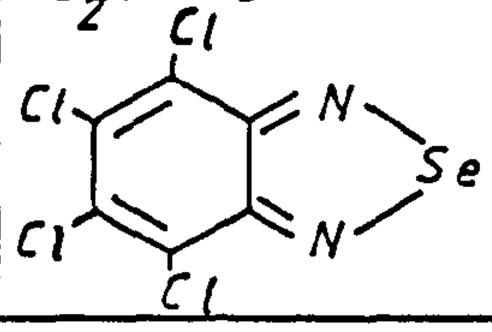
Name	Structural Formula	Melting Point °C		Reference
		Found	Reported	
Piazselenol		74	73-74	(9,15,16)
5-Chloropiazselenol		117-118	118-119	(16,17)
5-Fluoropiazselenol		104-106	104	(15)
5-Nitropiazselenol		222-223	222-224	(9,15,17)
5-Trifluoromethyl-piazselenol		90-91	91	(15)
4,6-Dibromopiazselenol		215-217	217-218	(9)
4-Bromo-6-fluoro-piazselenol		155-156		
4-Bromo-6-trifluoro-methylpiazselenol		160-161		
4-Chloro-6-trifluoro-methylpiazselenol		167-168		
5-Chloro-6-fluoro-piazselenol		130-131		
4,6-Dinitropiaz-selenol		210-212	211-213	(12)
4,5,6,7-Tetrachloro-piazselenol		198-200		

Table 3:2. Microanalysis of the Diamine HCl Derivatives

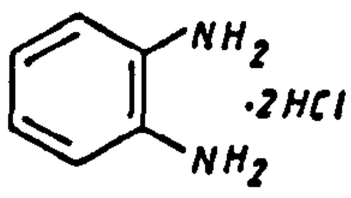
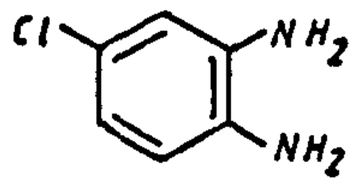
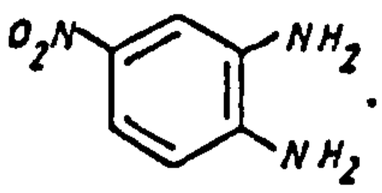
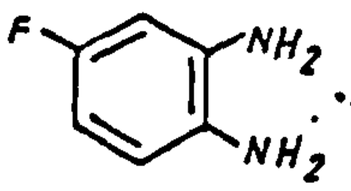
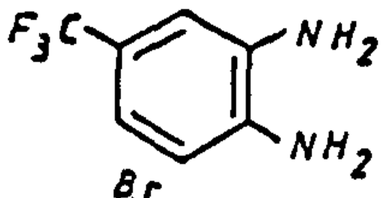
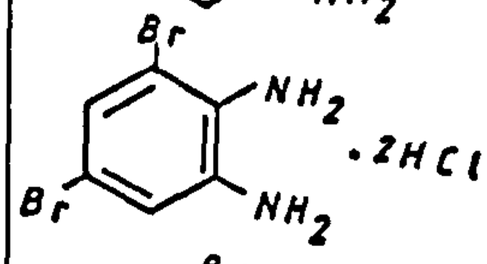
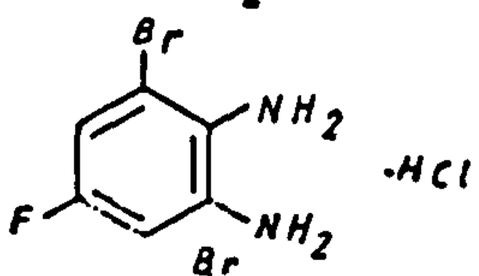
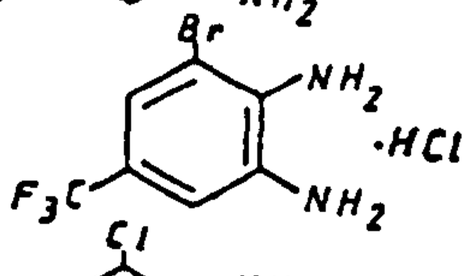
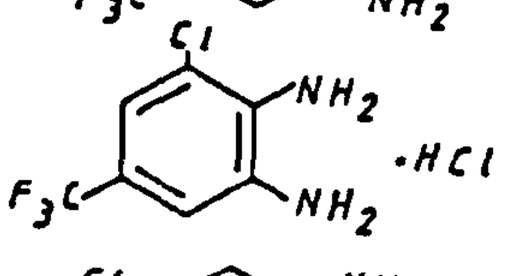
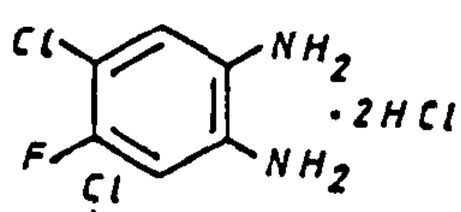
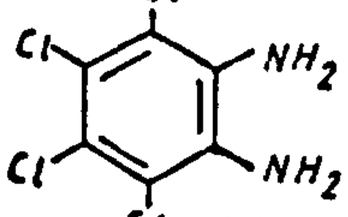
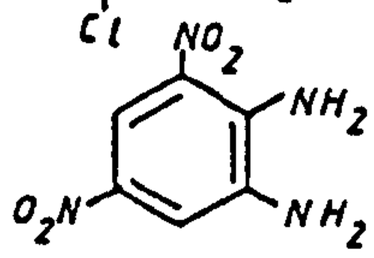
Structural Formula	Molecular Weight	% C	% N	% H	% Br	% Cl	% F	% O	
	181.064	39.43	15.26	5.68		39.50			Found
		39.80	15.47	5.57		39.16			Requires
	215.51	33.39	13.31	4.40		48.82			Found
		33.44	13.00	4.21		49.35			Requires
	189.54	38.01	21.71	4.27		18.91			Found
		37.98	22.17	4.25		18.70		16.88	Requires
	199.055	35.70	14.50	4.59		35.47	9.37		Found
		36.20	14.07	4.56		35.62	9.54		Requires
	249.06	33.90	11.73	3.49		26.79	22.58		Found
		33.76	11.25	3.64		28.47	22.88		Requires
	338.86	21.96	8.00	2.15	46.86	19.72			Found
		21.27	8.27	2.38	47.16	20.92			Requires
	241.43	29.73	11.53	2.92	33.41	14.84	7.66		Found
		29.92	11.60	2.92	33.10	14.68	7.87		Requires
	291.43	28.70	9.38	2.44	27.42	12.72	19.53		Found
		28.83	9.61	2.42	27.42	12.16	19.56		Requires
	246.98	33.60	11.00	2.73		28.49	22.94		Found
		34.01	11.34	2.86		28.71	23.08		Requires
	233.44	31.29	11.53	3.50		45.38	9.53		Found
		30.85	12.00	3.45		45.56	8.14		Requires
	318.78	22.80	8.71	1.74		60.79			Found
		22.59	8.79	1.89		66.73			Requires
	198.14	36.46	28.08	2.85					Found
		36.36	28.21	3.05				32.15	Requires

Table 3:3. Microanalysis of Piazselenols

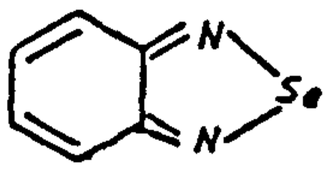
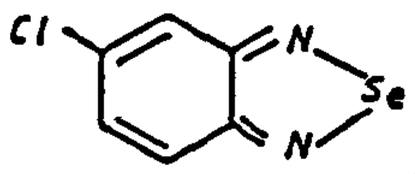
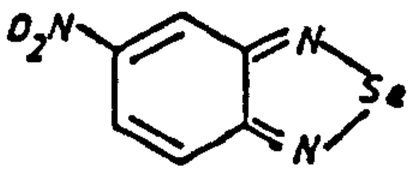
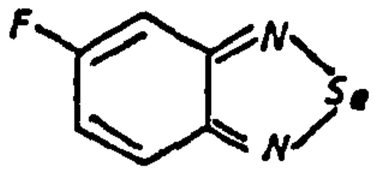
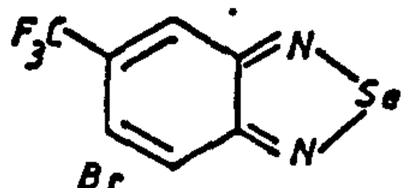
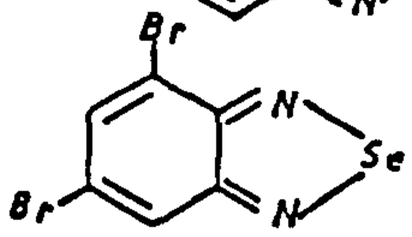
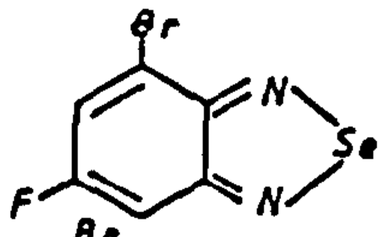
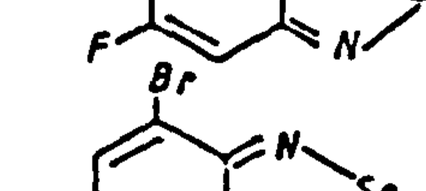
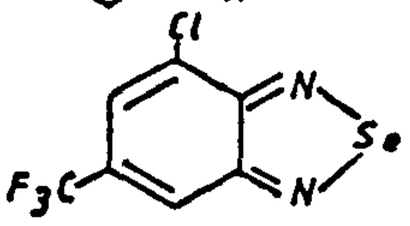
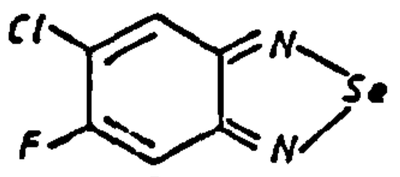
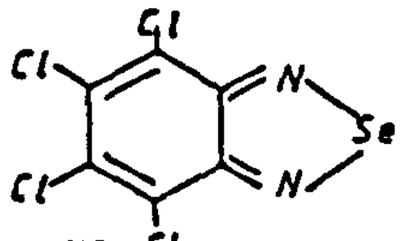
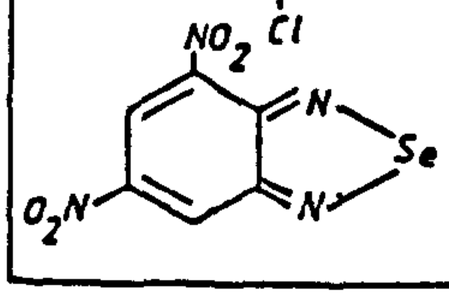
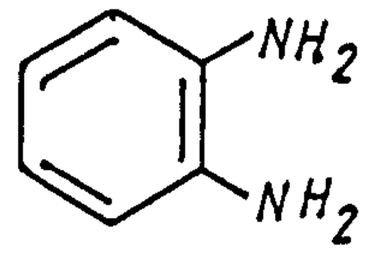
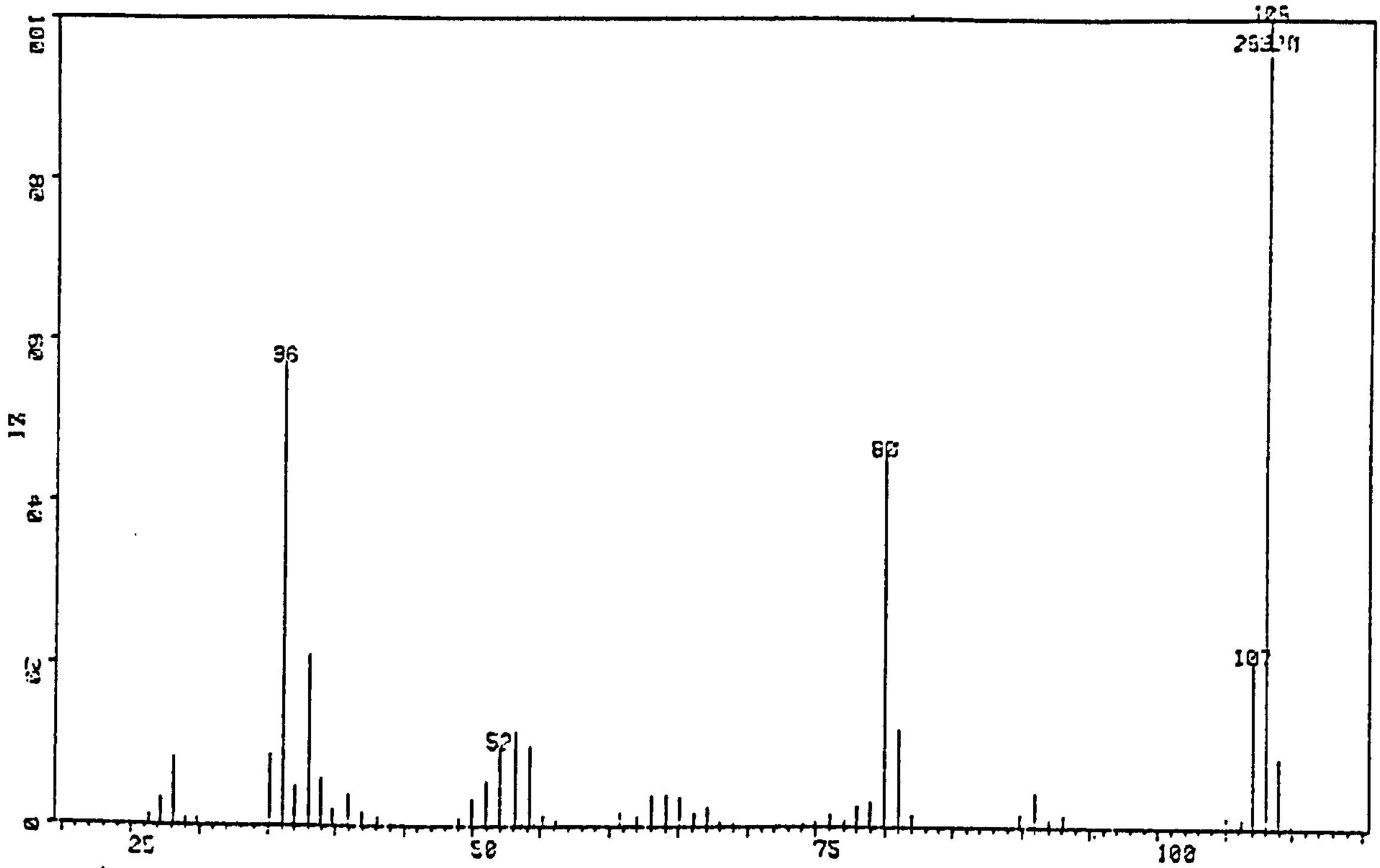
Piazselenol	Molecular Weight	% C	% N	% H	% Br	% Cl	% F	% O	% Se	
	183.07	38.94	15.10	2.22						Found
		39.36	15.30	2.20					43.13	Requires
	217.52	33.65	13.18	1.28		16.99				Found
		33.13	12.88	1.39		16.30			36.30	Requires
	228.07	31.56	18.52	1.165						Found
		31.60	18.42	1.325				14.03	34.62	Requires
	201.06	35.09	13.67	1.39			9.03			Found
		35.81	13.93	1.504			9.54		39.27	Requires
	251.07	33.64	11.18	1.22			22.58			Found
		33.49	11.16	1.20			22.70		31.45	Requires
	340.86	21.20	8.20	0.61	44.84					Found
		21.14	8.22	0.593	46.88				23.16	Requires
	279.90	26.01	10.25	0.65	28.45		6.83			Found
		25.73	10.01	0.72	28.55		6.79		28.20	Requires
	329.90	25.78	8.77	0.68	22.40		19.29			Found
		25.46	8.49	0.611	24.22		17.28		23.93	Requires
	285.44	29.37	9.85	0.72		12.40	19.74			Found
		29.43	9.81	0.706		12.42	19.97		27.66	Requires
	235.44	30.43	11.90	0.70		15.76	10.52			Found
		30.58	11.90	0.856		15.06	8.07		33.53	Requires
	320.79	21.48	8.86			42.38				Found
		22.45	8.73			44.21			24.61	Requires
	273.06	26.22	20.25	0.64						Found
		26.39	20.51	0.74				23.43	28.91	Requires

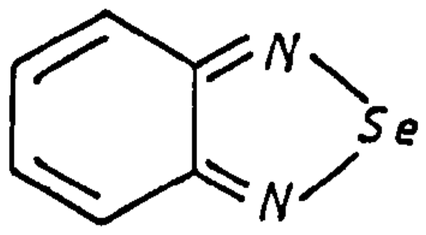
FIGURE 3.1 MASS SPECTRA OF:

(a) 1,2 - DIAMINOBENZENE





(b) PIAZSELENOL



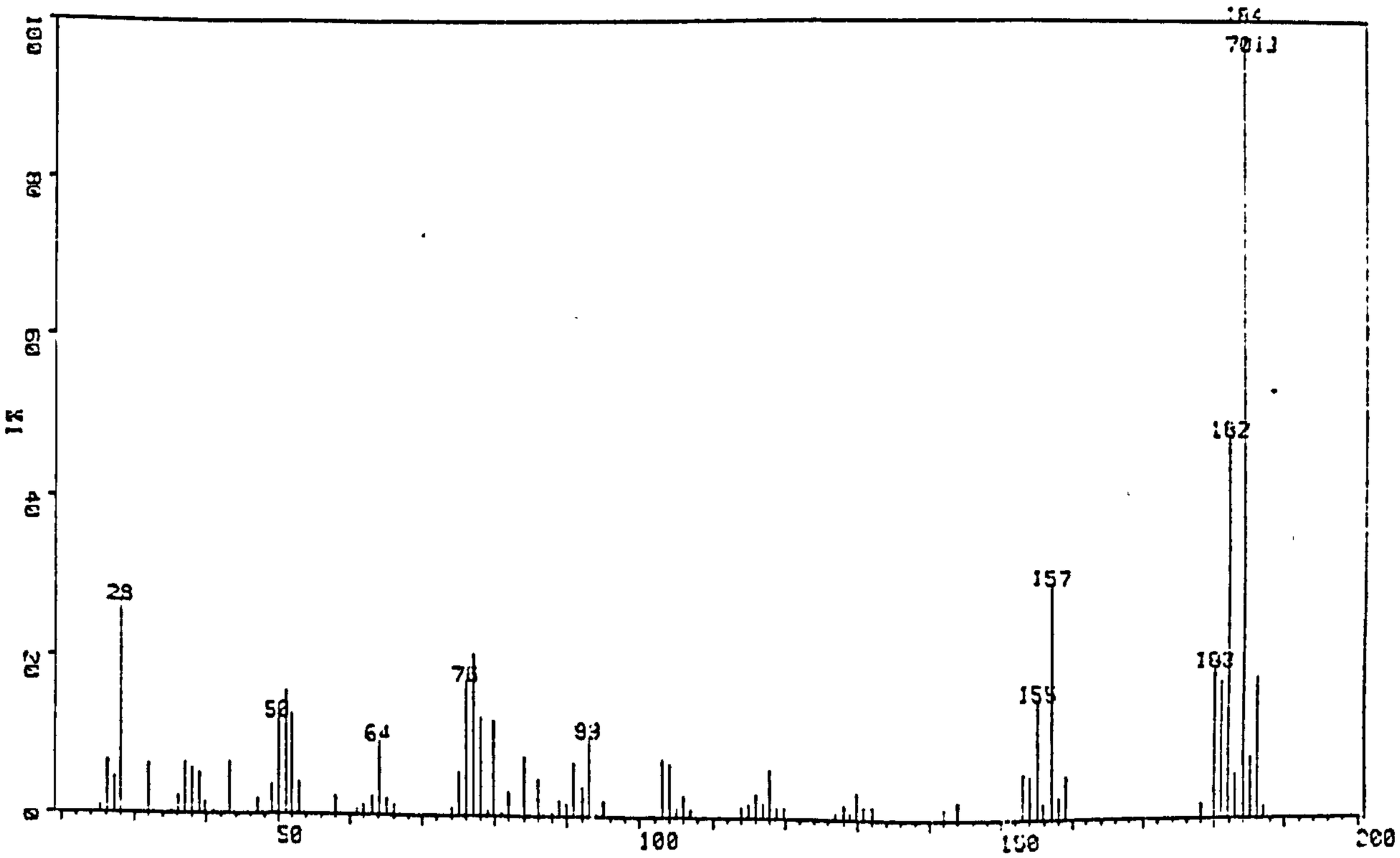
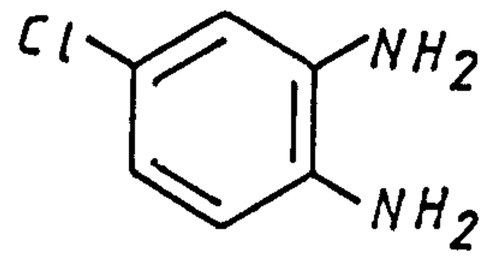
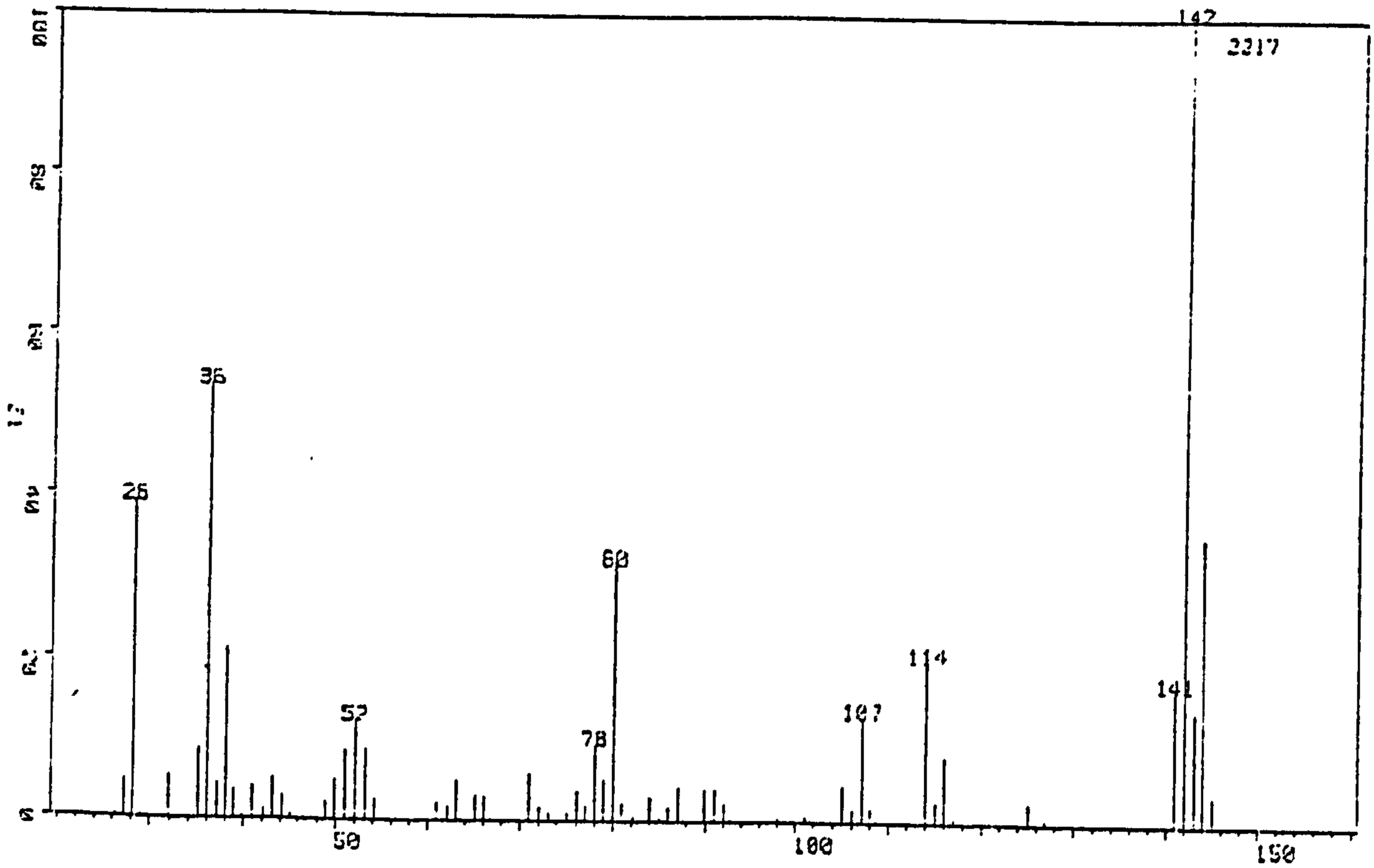


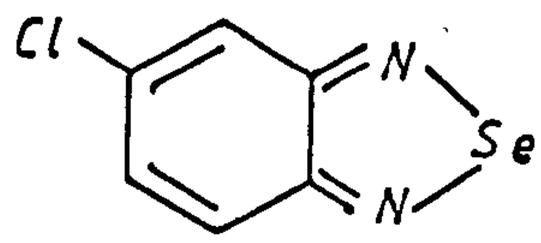
FIGURE 3.2 MASS SPECTRA OF:

(a) 4-CHLORO-1,2-DIAMINOBENZENE





(b) 5-CHLOROPIAZSELENOL



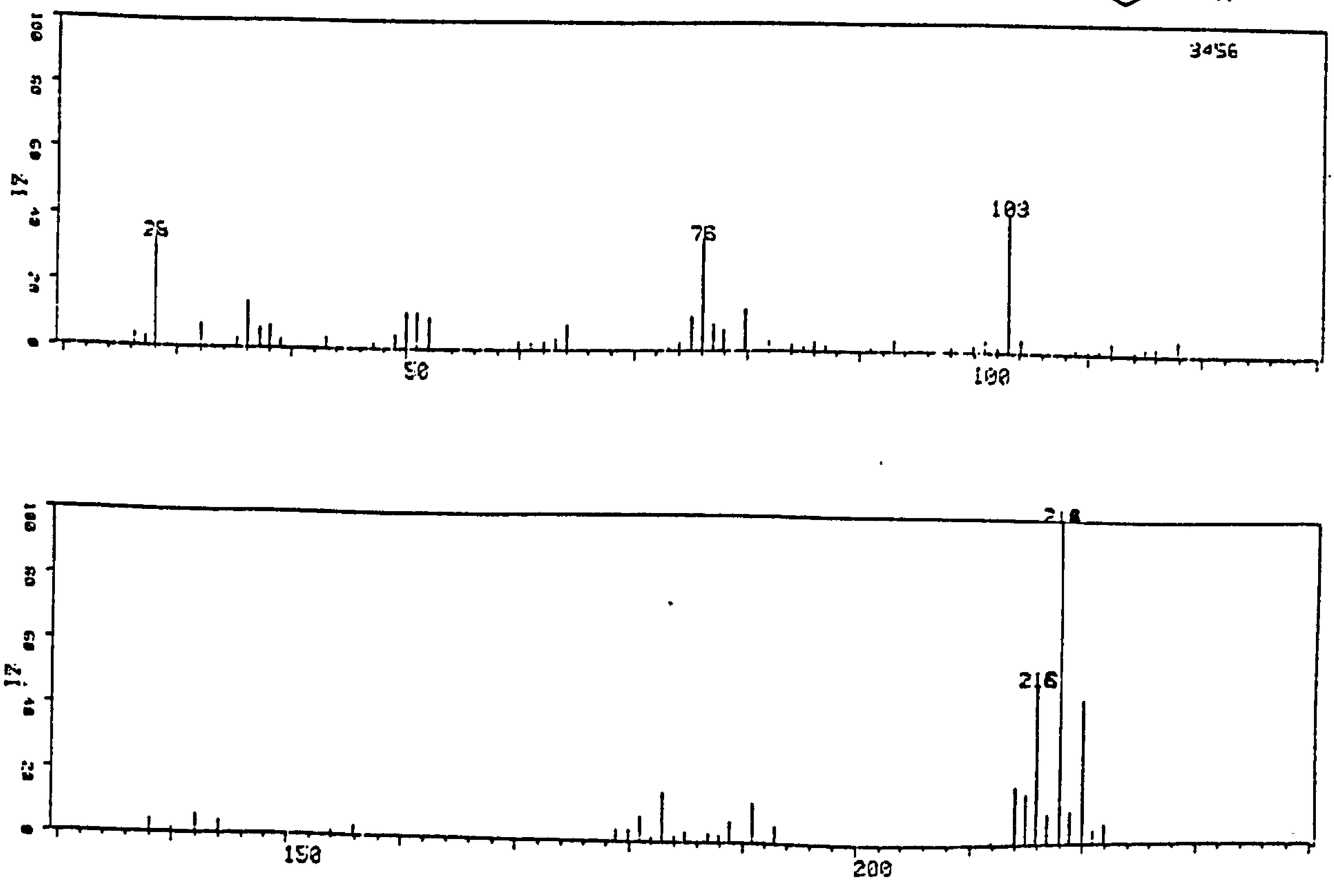
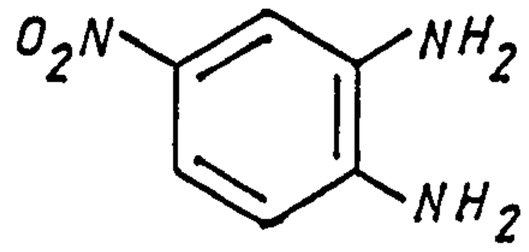
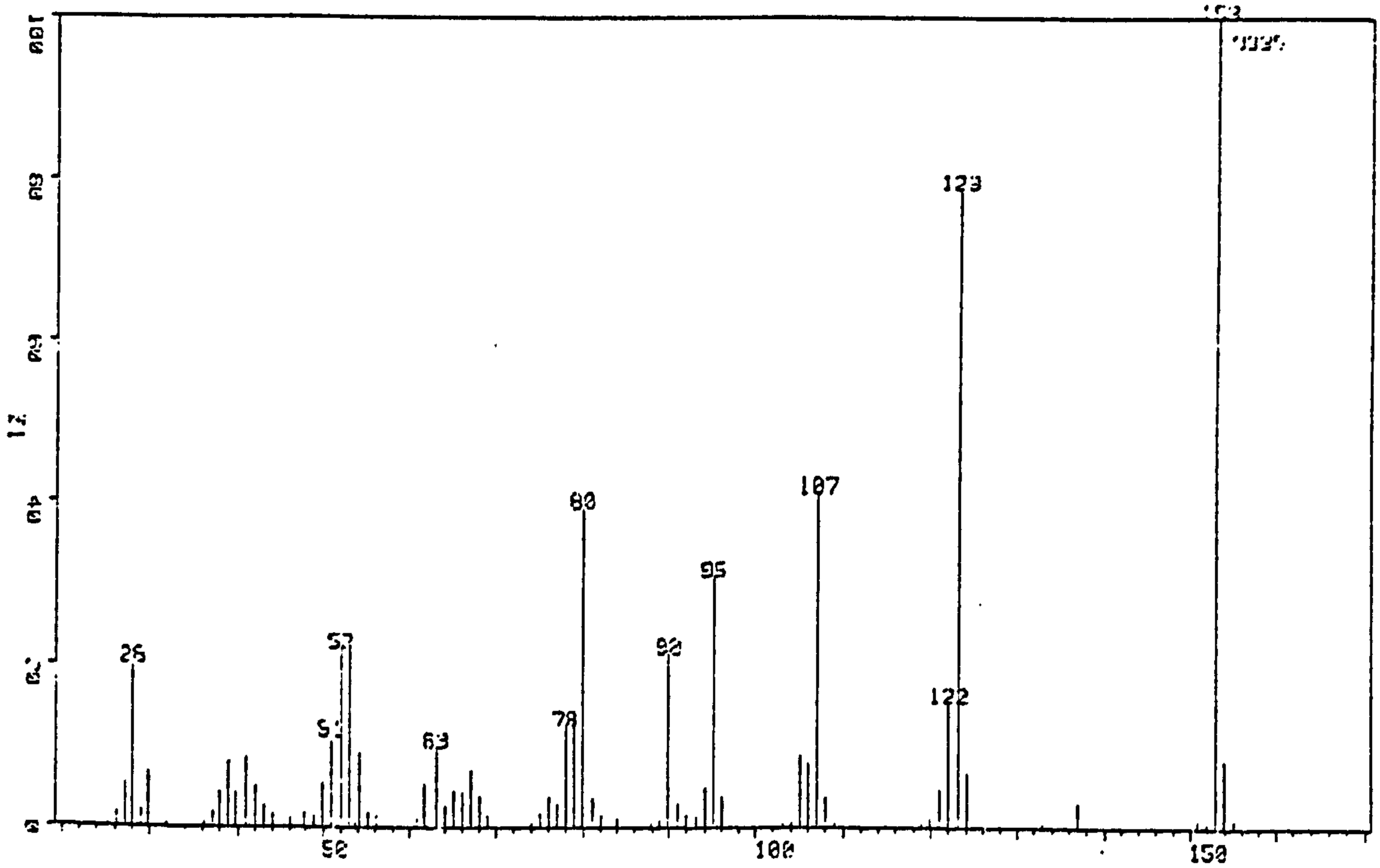


FIGURE 3.3 MASS SPECTRA OF:

(a) 4-NITRO-1,2-DIAMINOBENZENE

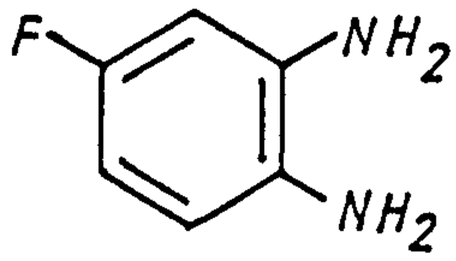


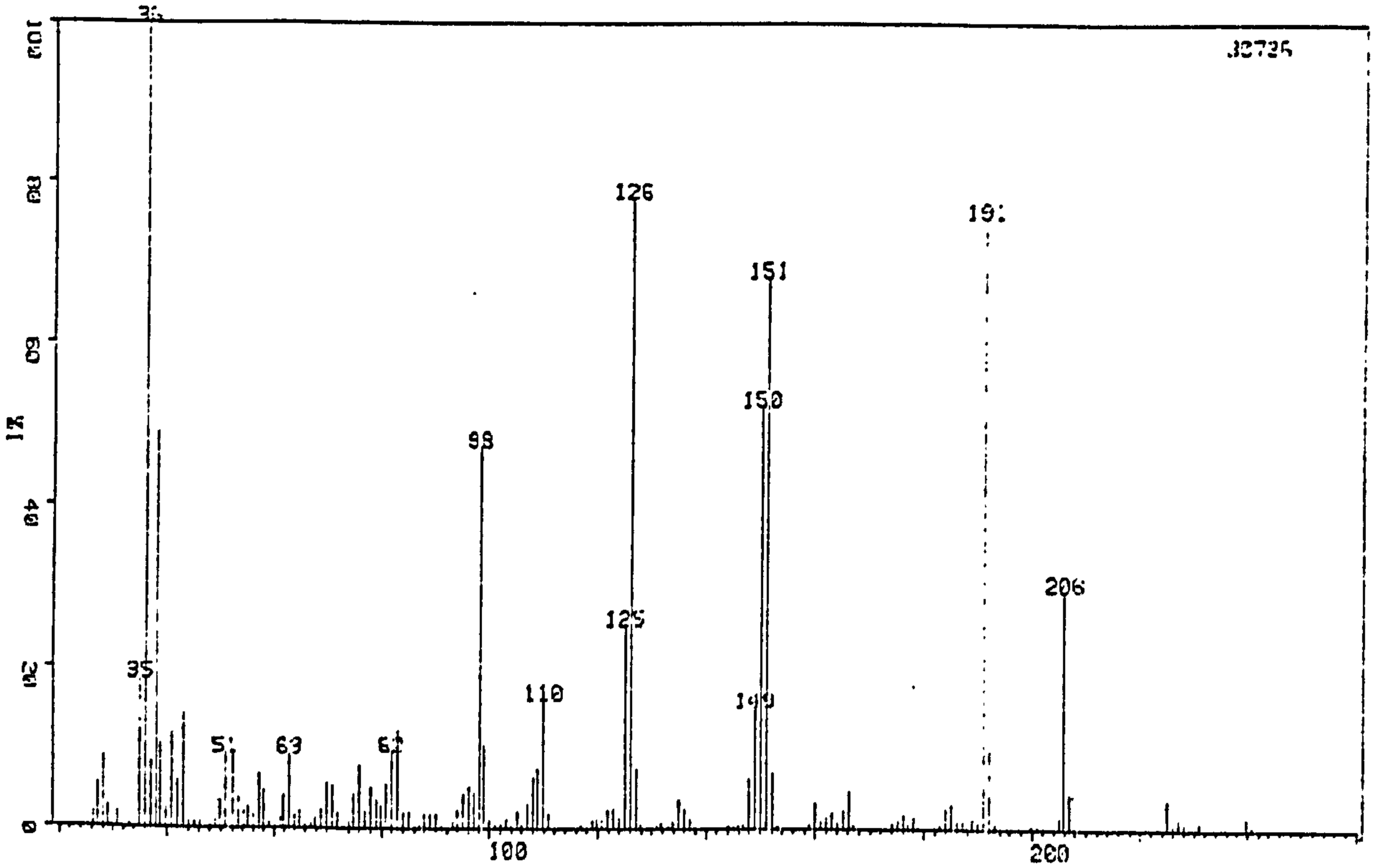


(b) 5-NITROPIAZSELENOL

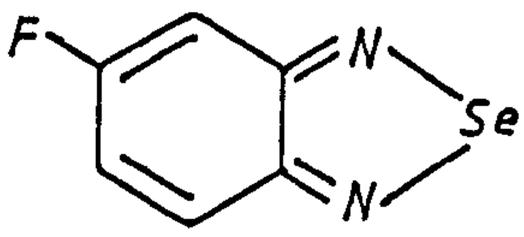
FIGURE 3.4 MASS SPECTRA OF:

(a) 4-FLUORO-1,2-DIAMINOBENZENE





(b) 5-FLUOROPIAZSELENOL



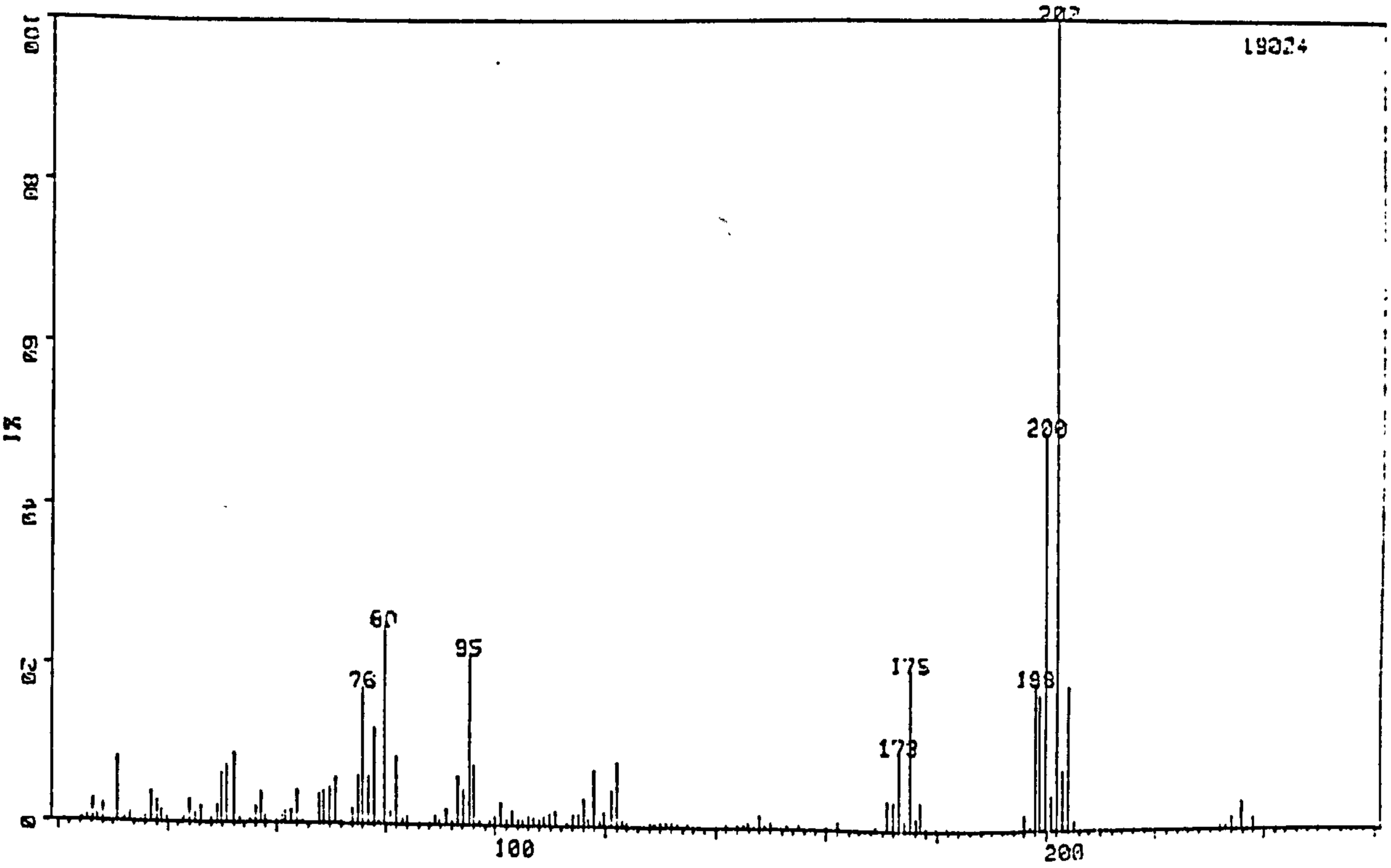
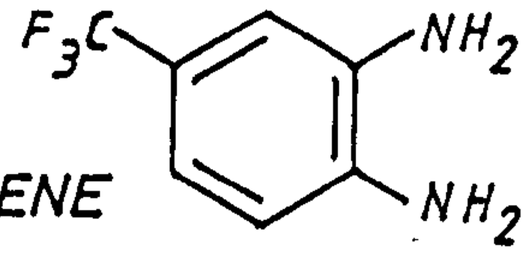
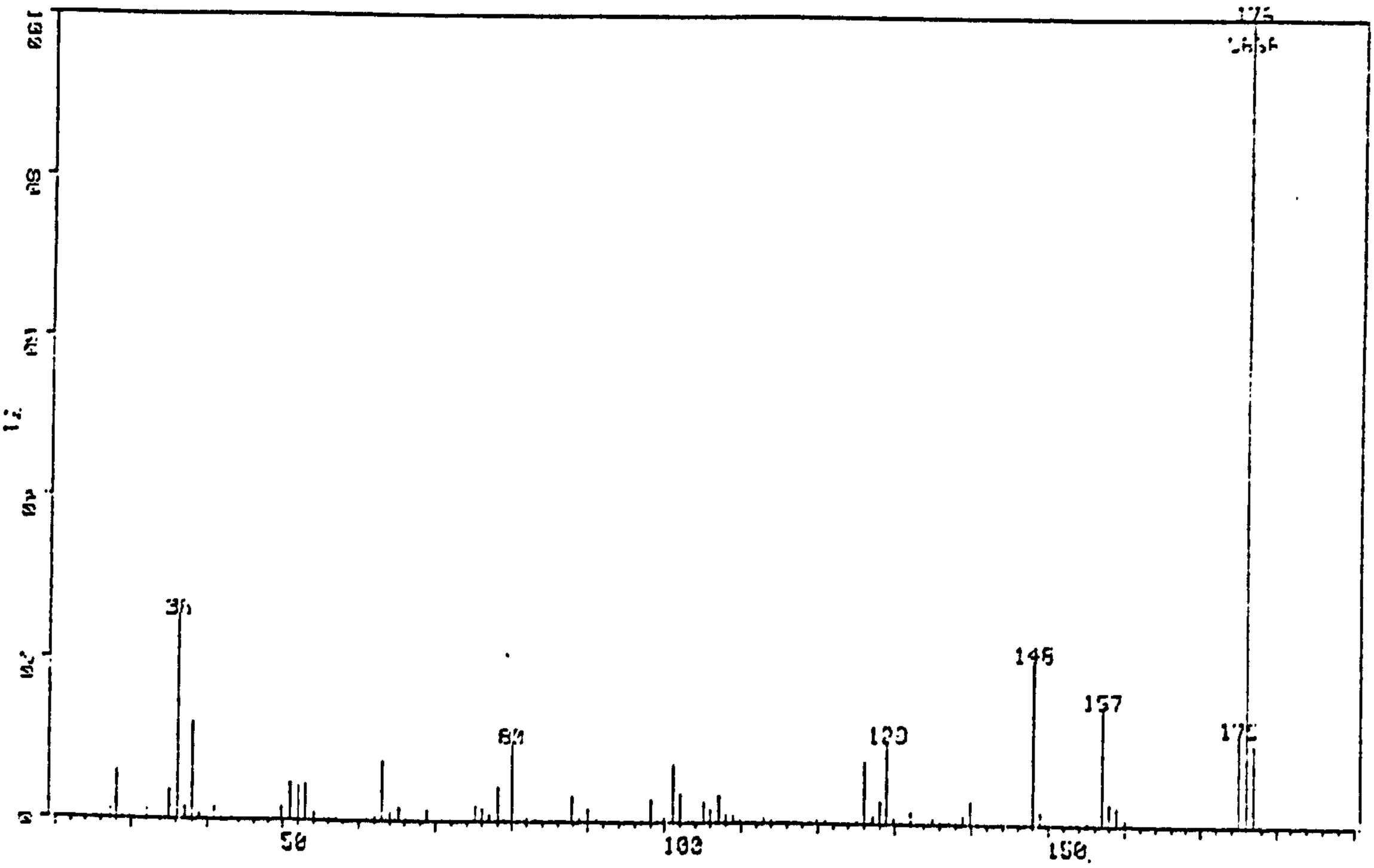


FIGURE 3.5 MASS SPECTRA OF:

(a) 4 - TRIFLUOROMETHYL - 1,2-DIAMINOBENZENE





(b) 5 -TRIFLUOROMETHYLPIAZSELENOL

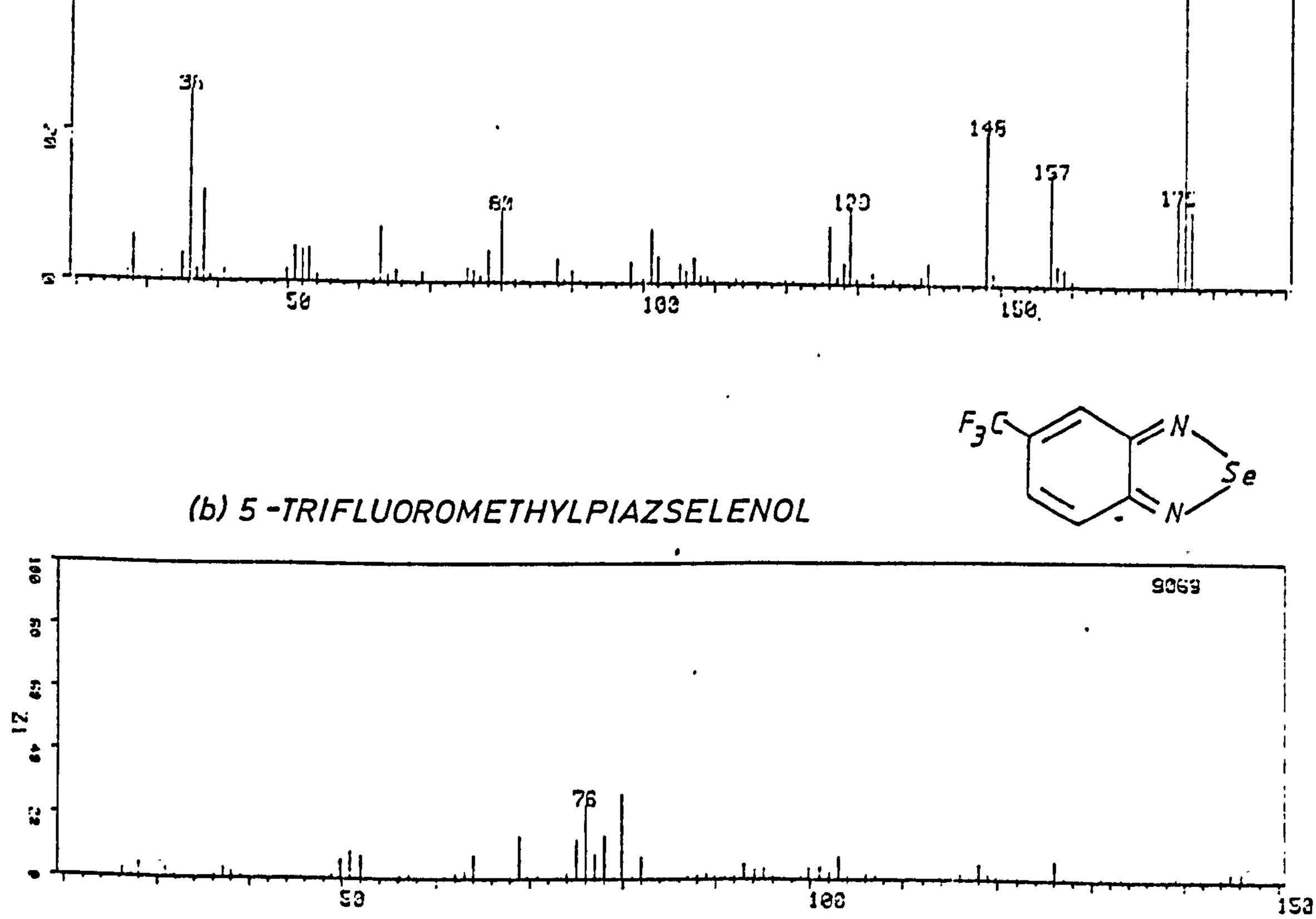
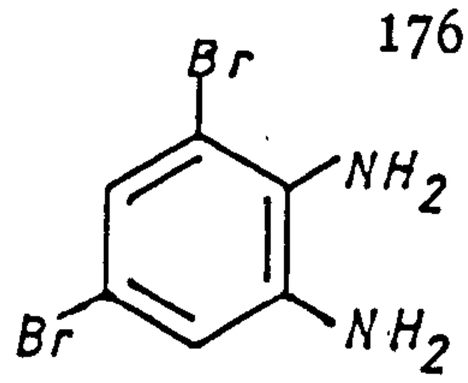
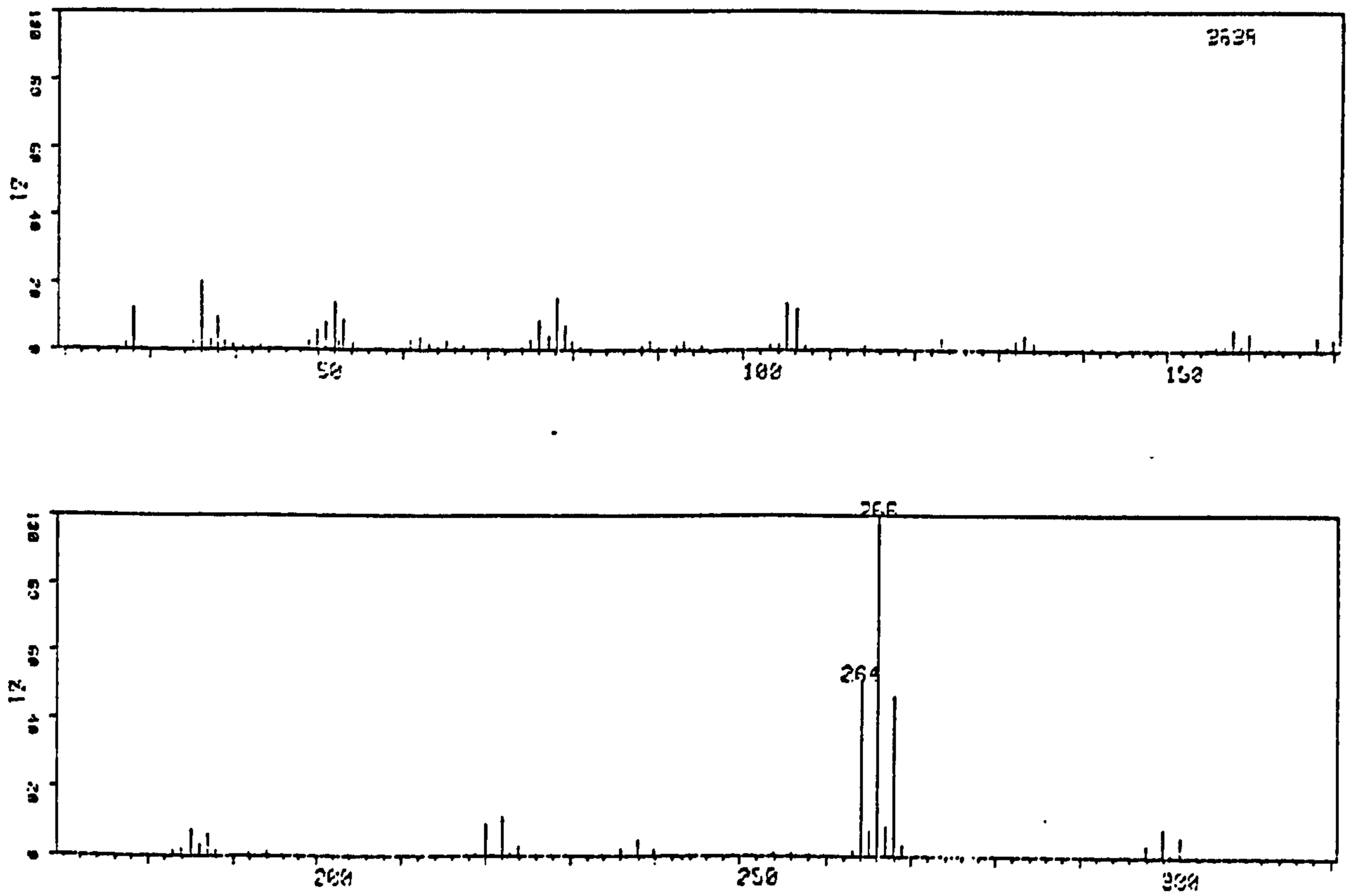


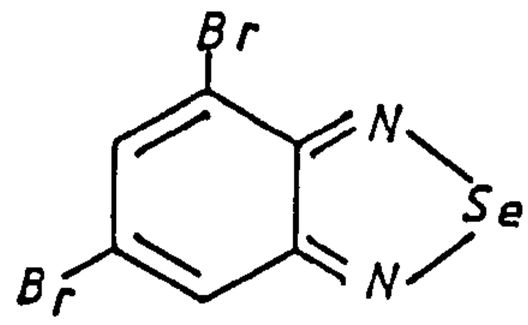
FIGURE 3.6 MASS SPECTRA OF:

(a) 3,5 - DIBROMO-1,2 -DIAMINOBENZENE





(b) 4,6-DIBROMOPIAZSELENOL



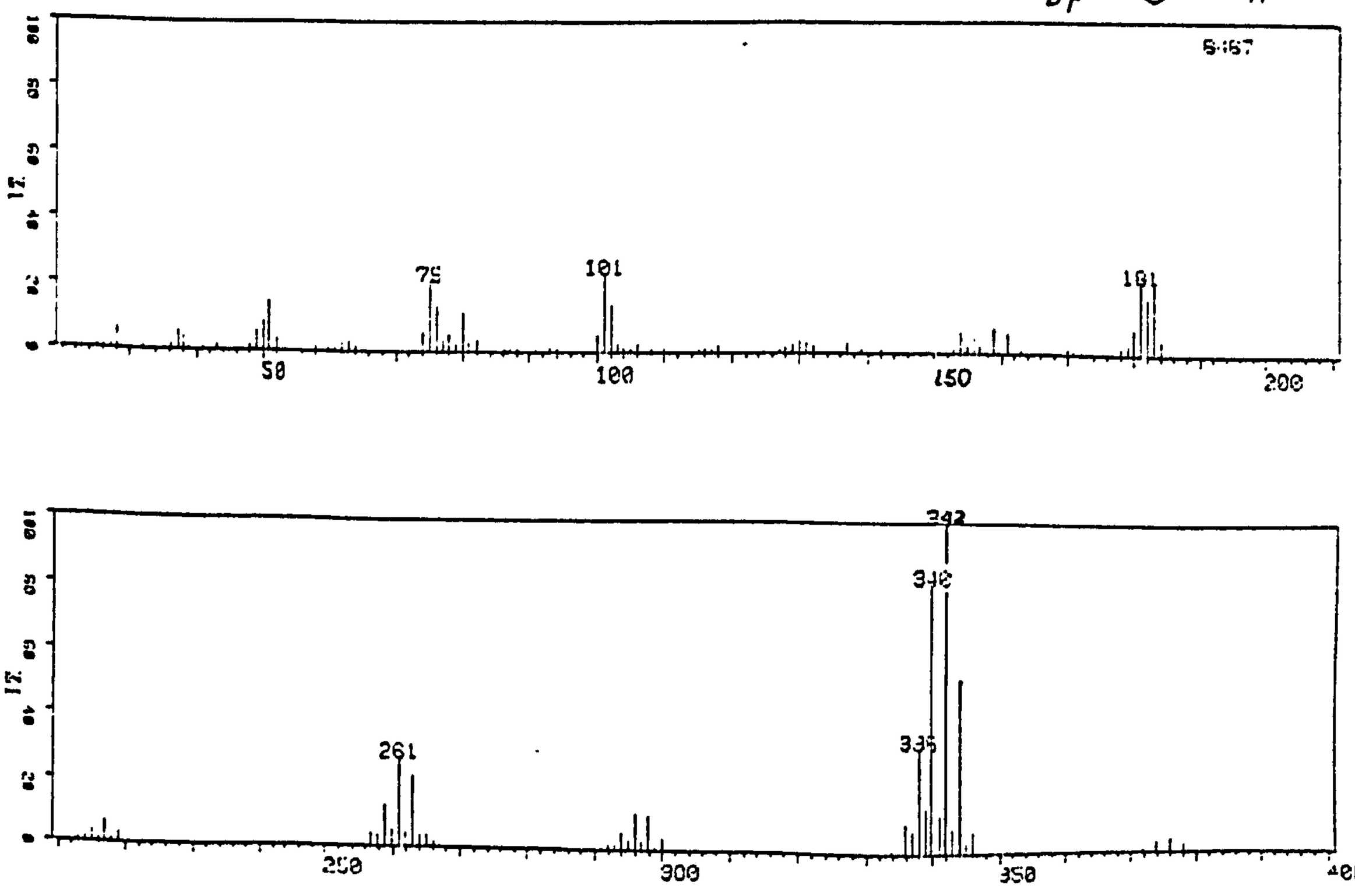
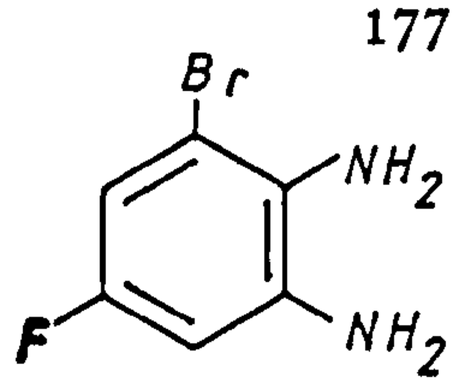
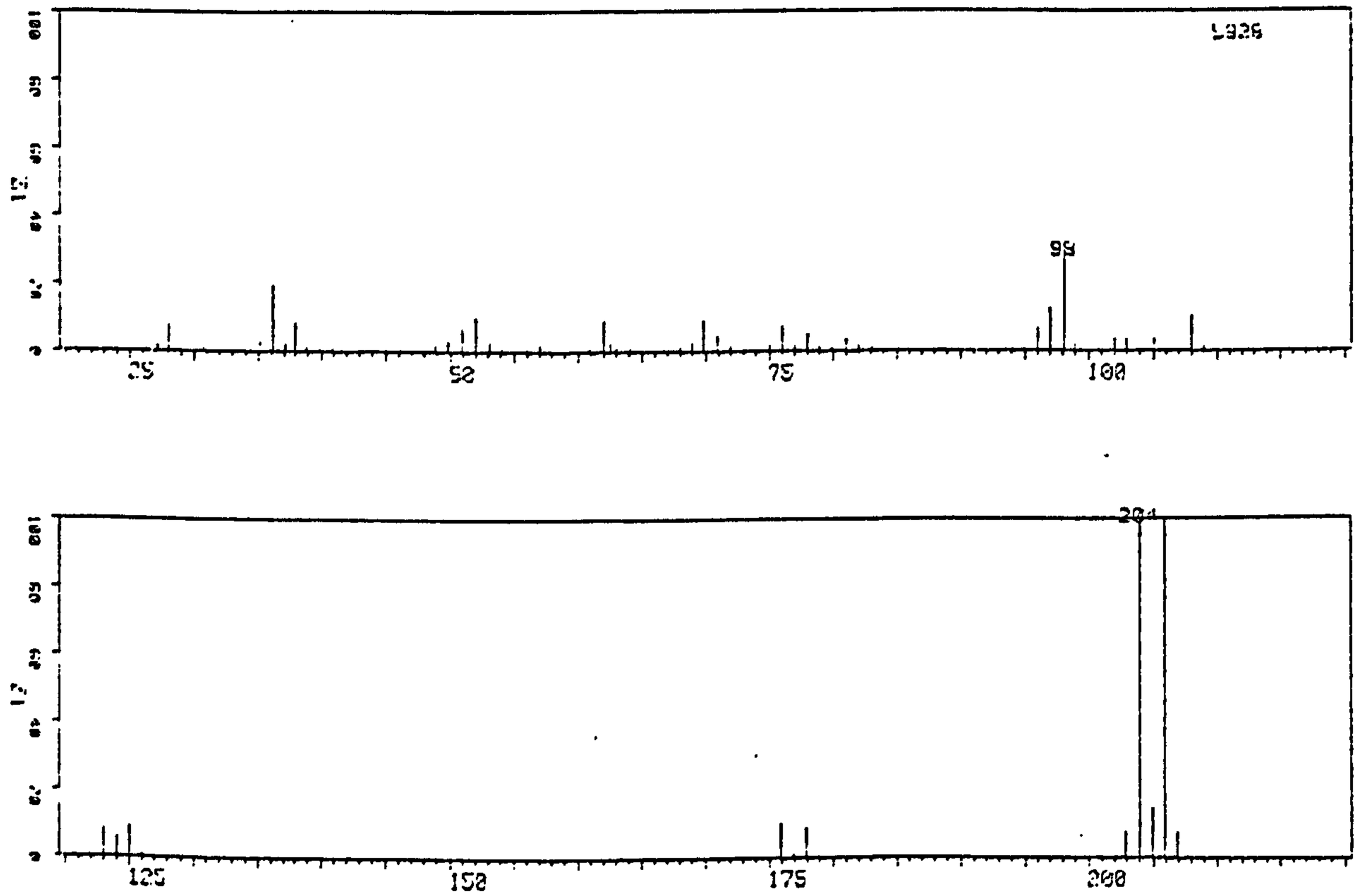


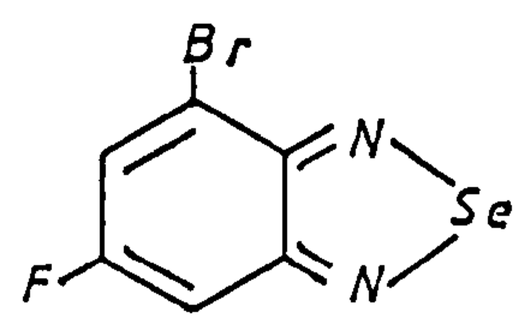
FIGURE 3.7 MASS SPECTRA OF:

(a) 3 -BROMO-5-FLUORO-1,2-DIAMINOBENZENE





(b) 4 -BROMO-6-FLUOROPIAZSELENOL



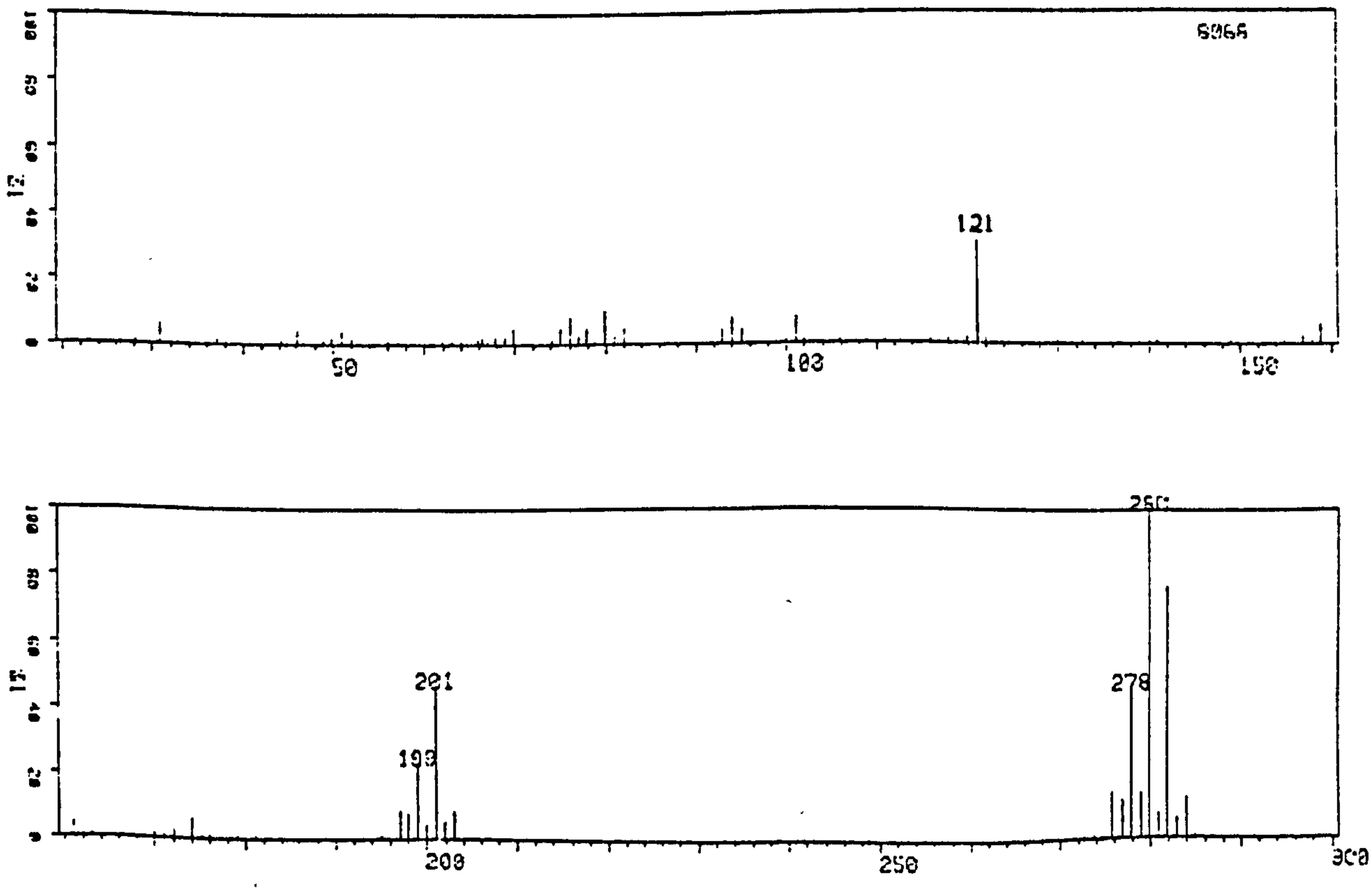
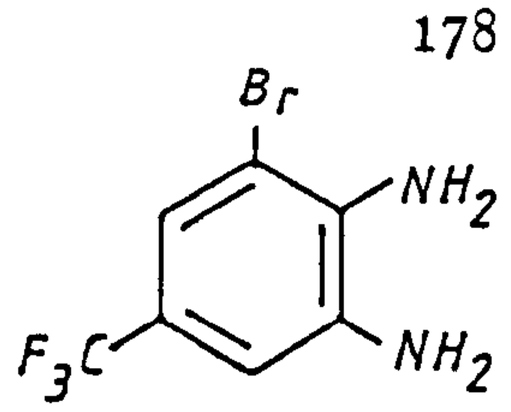
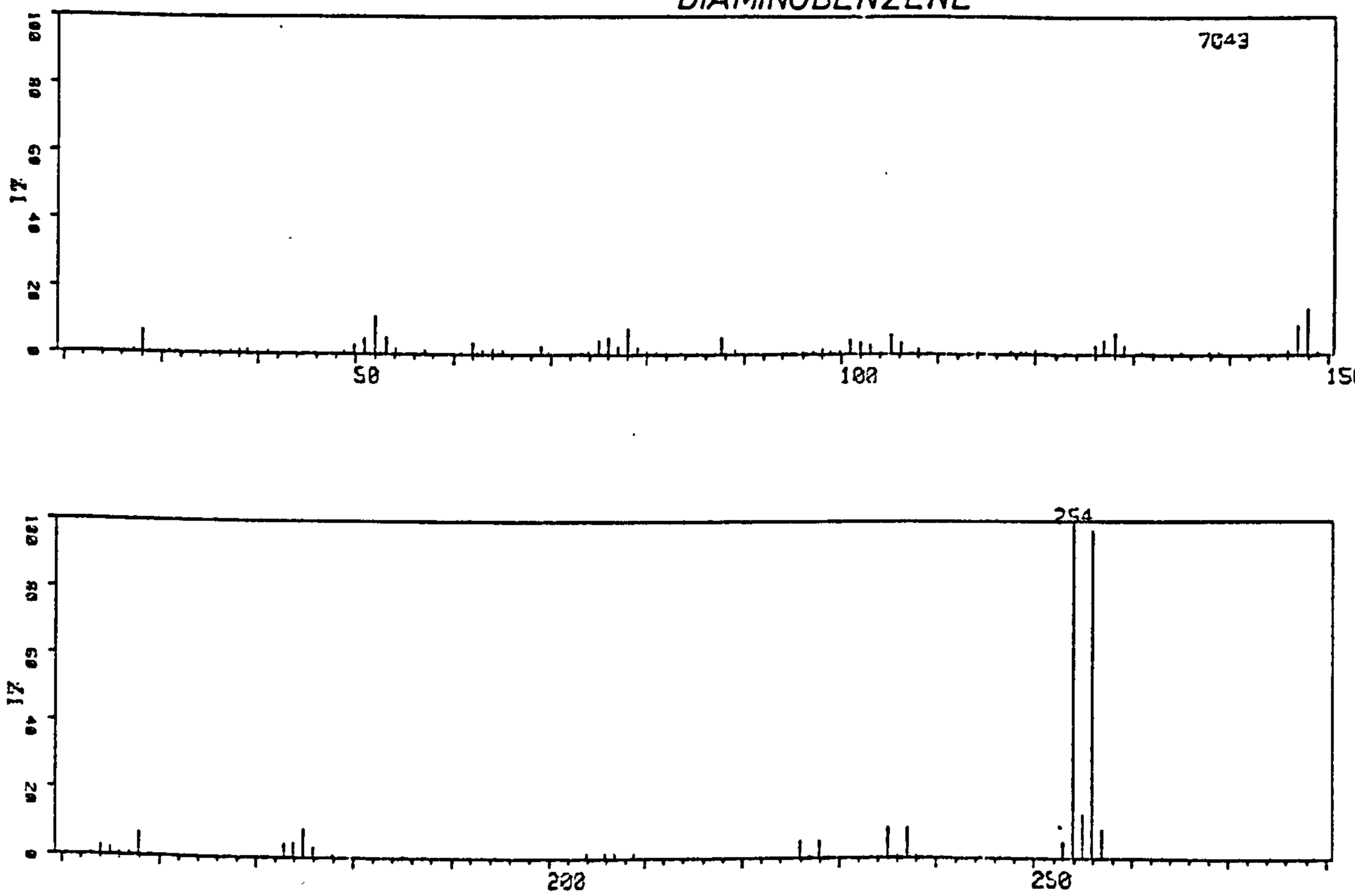


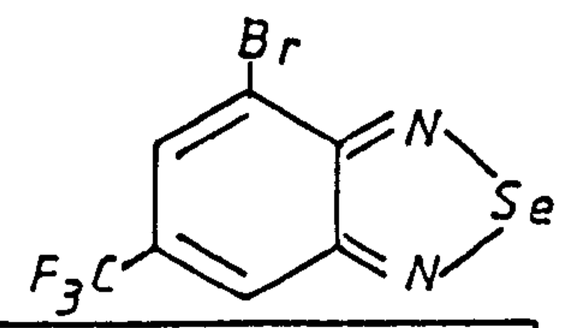
FIGURE 3.8 MASS SPECTRA OF:

(a) 3-BROMO-5-TRIFLUOROMETHYL-1,2-DIAMINOBENZENE





(b) 4-BROMO-6-TRIFLUOROMETHLPIAZSELENOL



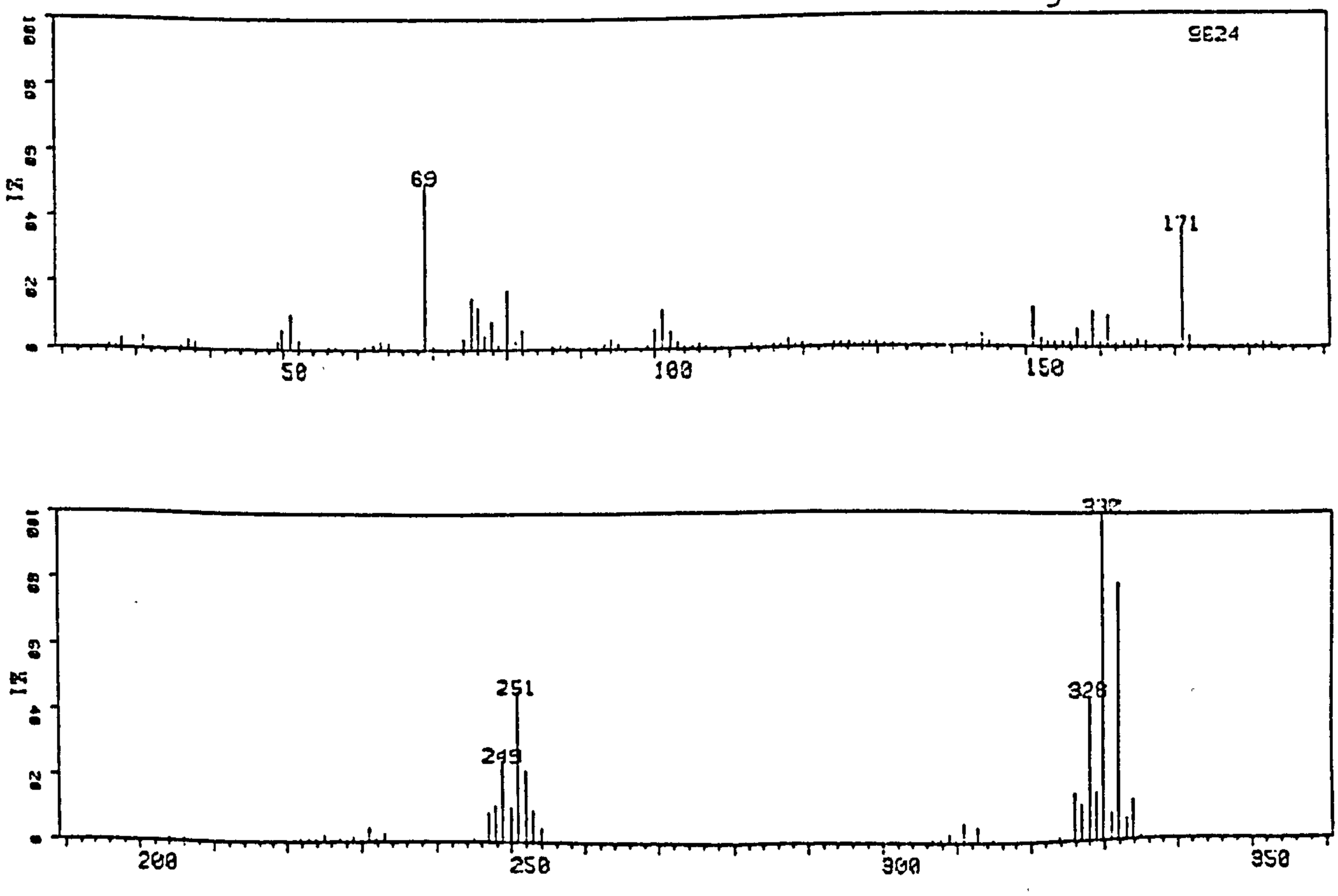
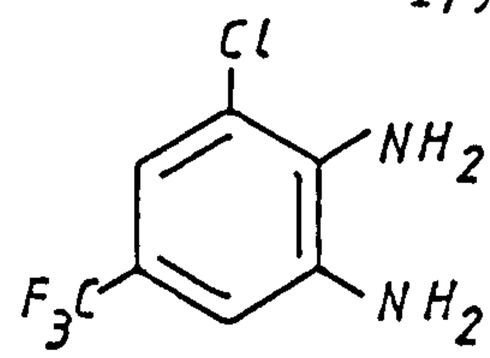
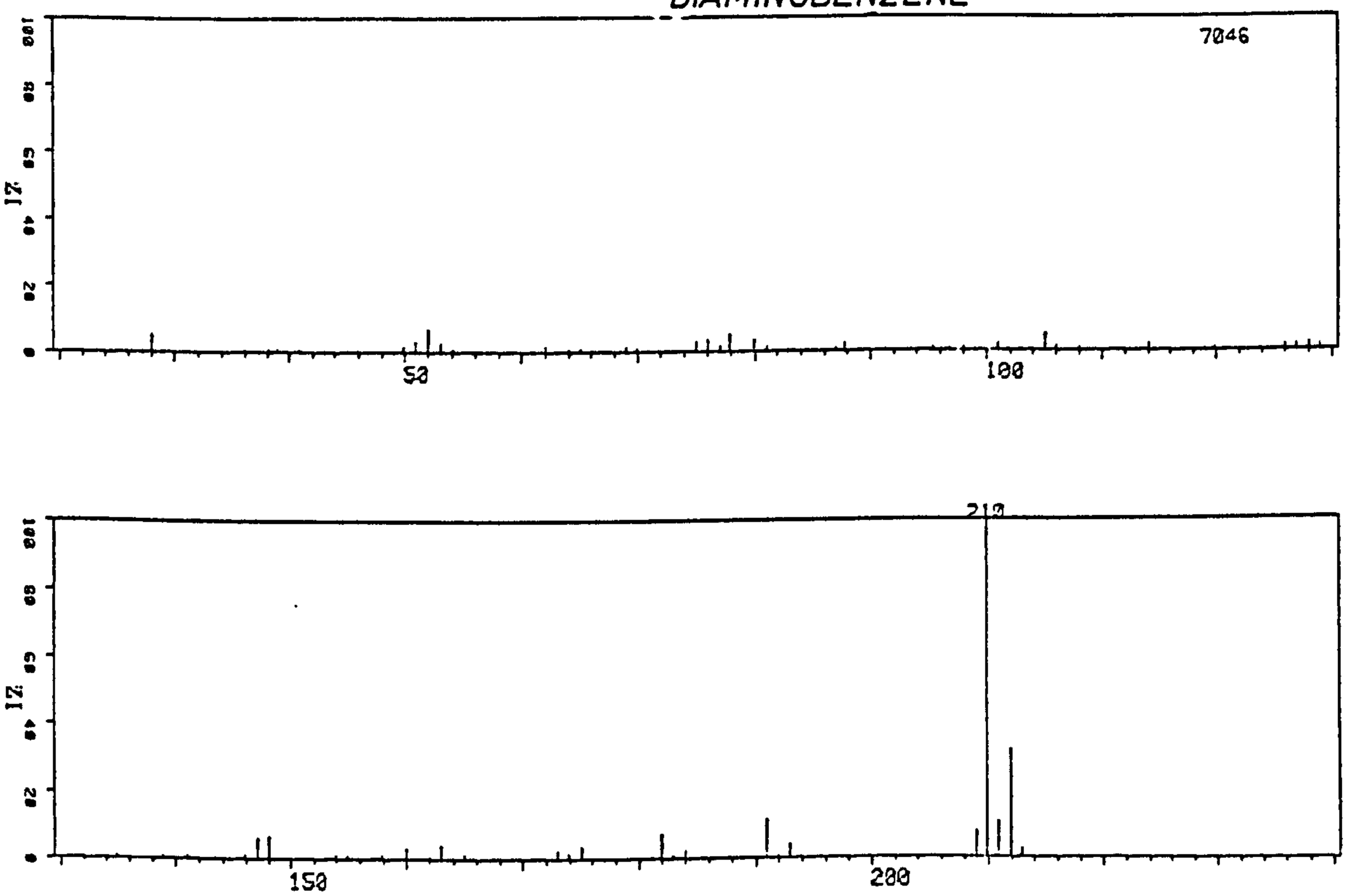


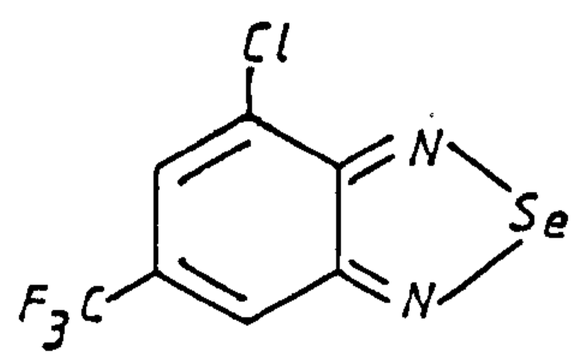
FIGURE 3.9 MASS SPECTRA OF:

(a) 3-CHLORO-5-TRIFLUOROMETHYL-1,2-DIAMINOBENZENE





(b) 4-CHLORO-6-TRIFLUOROMETHYLPIAZSELENOL



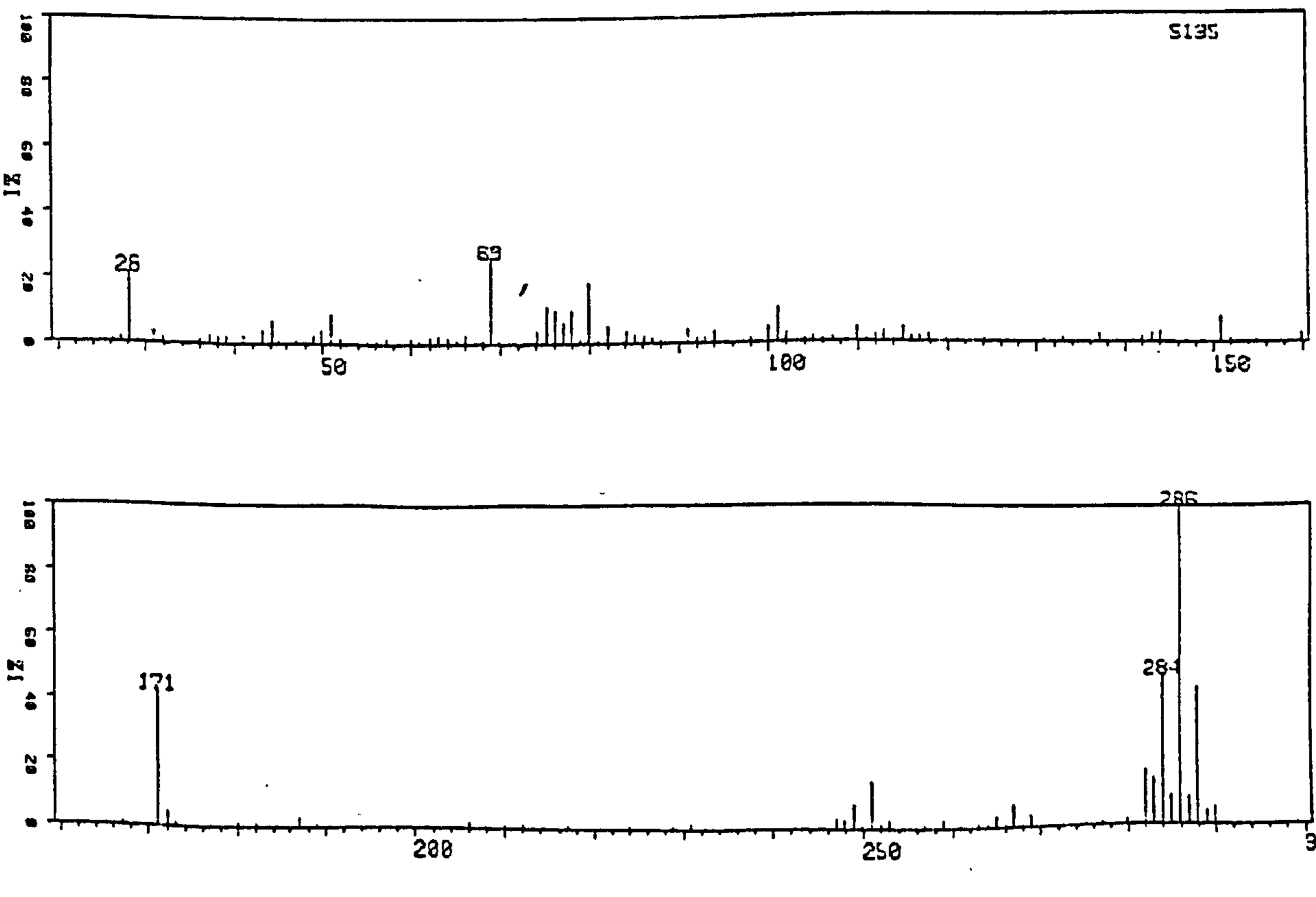
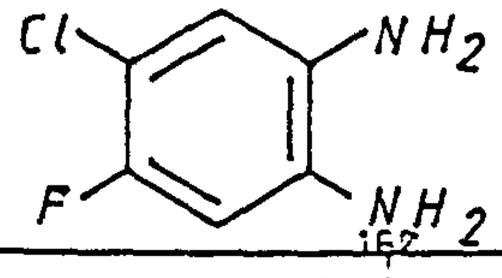
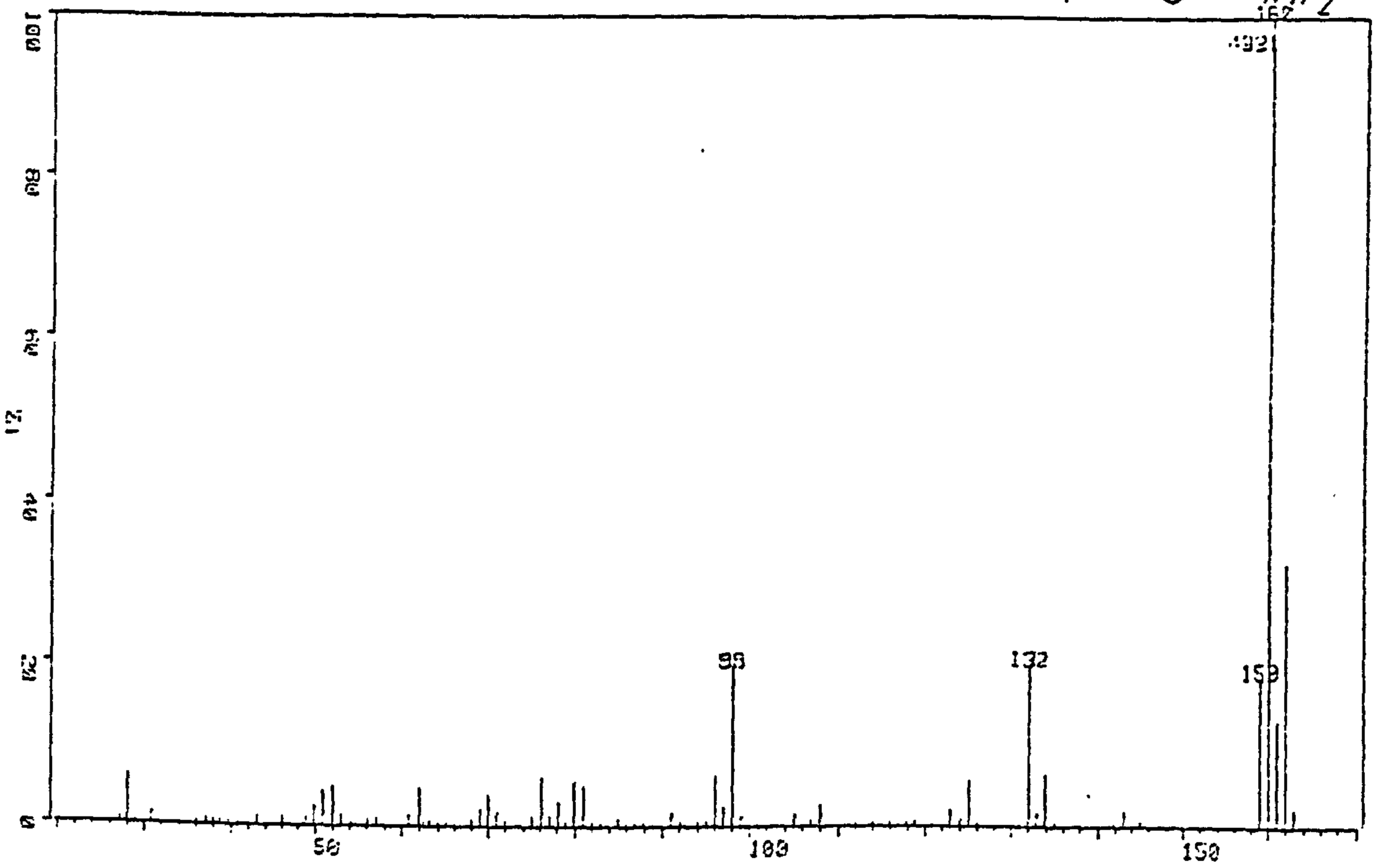


FIGURE 3.10 MASS SPECTRA OF:

(a) 4-CHLORO-5-FLUORO-1,2-DIAMINOBENZENE





(b) 5-CHLORO-6-FLUOROPIAZSELENOL

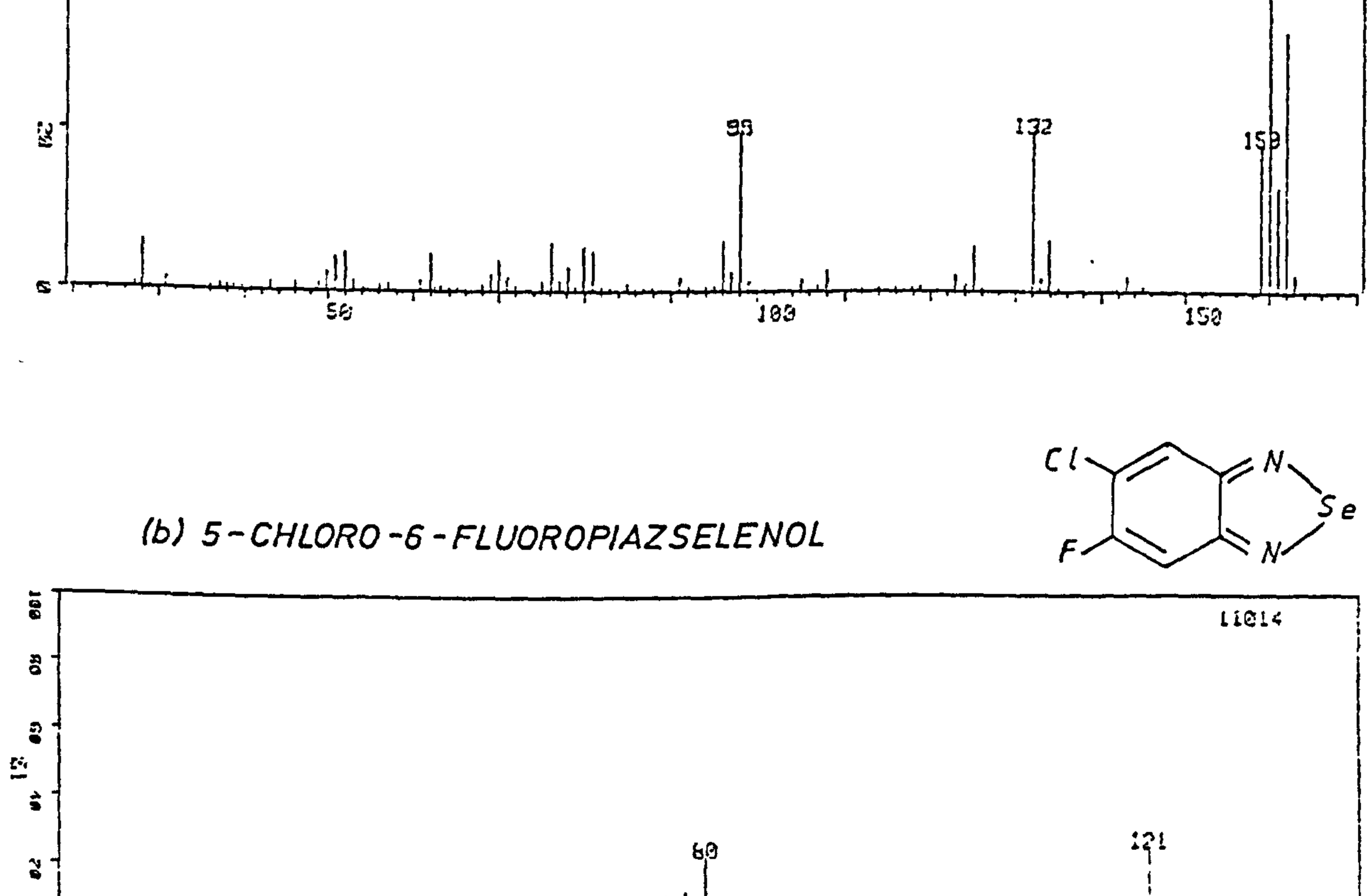
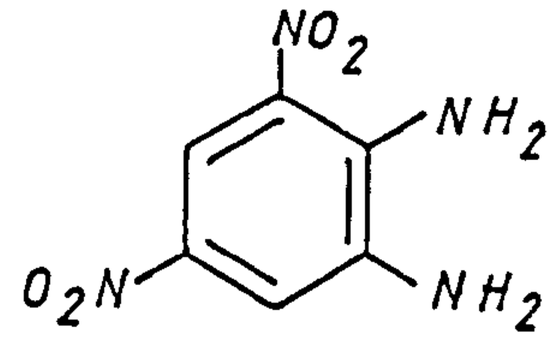
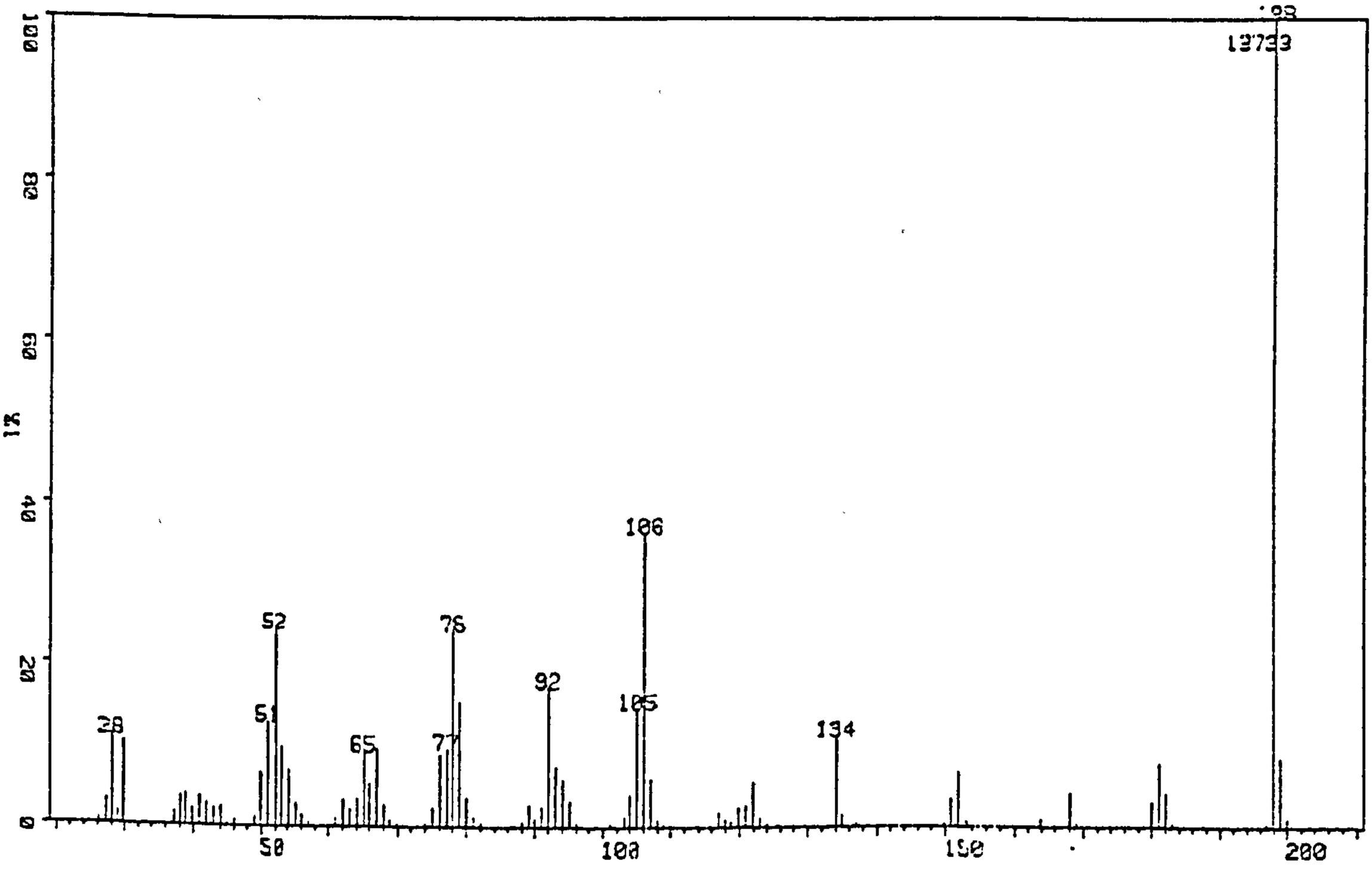


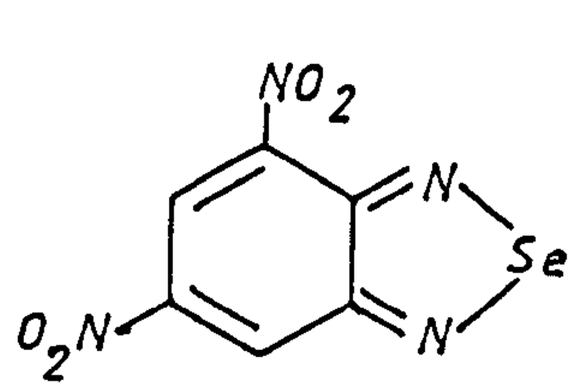
FIGURE 3.11 MASS SPECTRA OF:

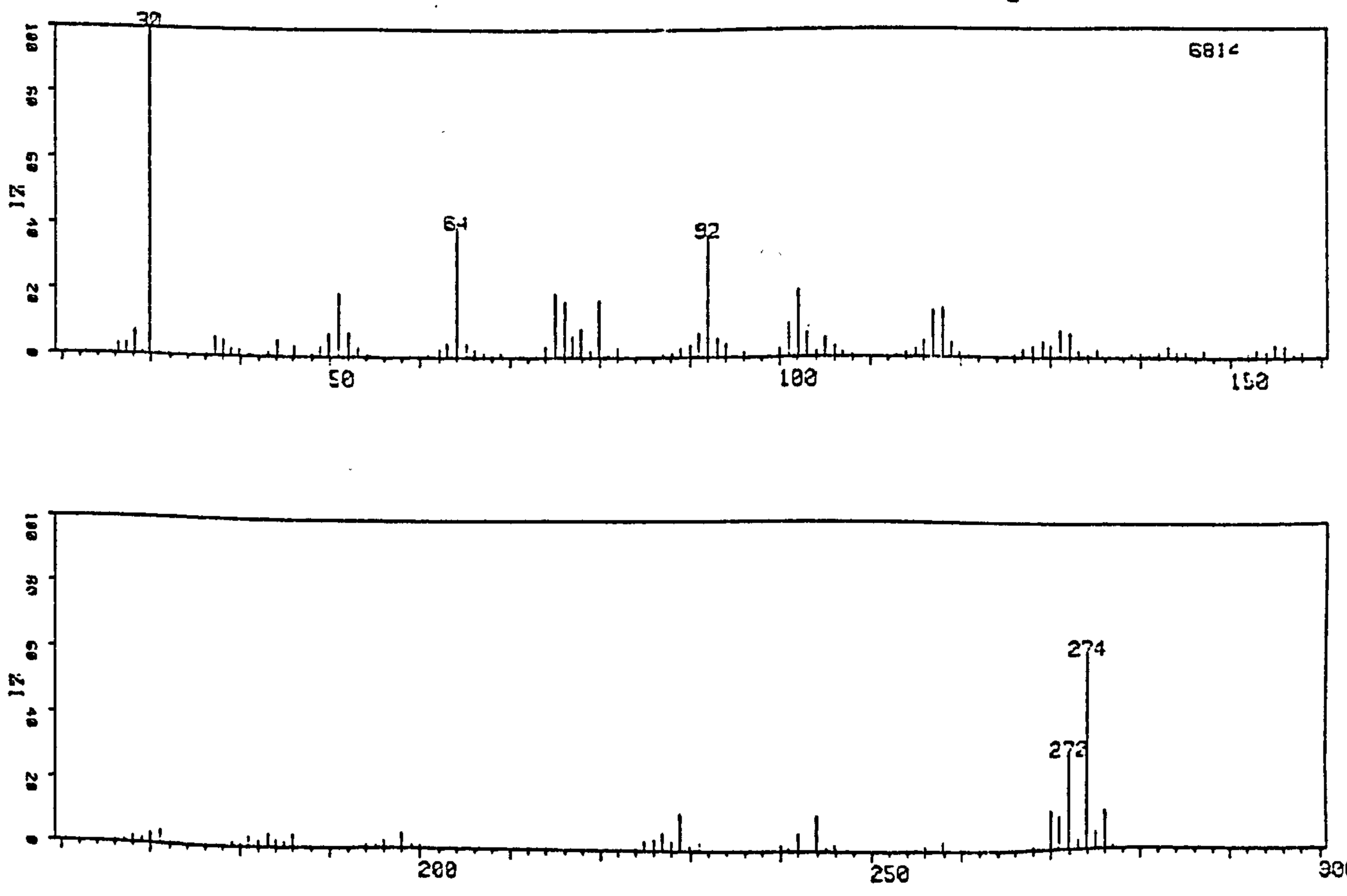
(a) 3,5 -DINITRO-1,2 - DIAMINOBENZENE





(b) 4,6 - DINITROPIAZSELENOL





3:2:3 Piazselenols and GLC

i) Instrumentation

Gas liquid chromatography (GLC) was carried out with a Pye-Unicam Series 104 chromatograph using an electron capture detector (ECD) Ni^{63} in the pulse mode (150 μsec), a detector oven controller, and a column oven controller. Extracts were separated on a 150 cm x 0.3 cm i.d. x 0.6 cm o.d. Pyrex glass column [the column packing prepared by coating Chromosorb W-AW-DMCS (80-100 mesh) with 5% (w/w) OV-17 using chloroform as a solvent and rotatory evaporation]. Packing of the column was assisted by agitation with a mechanical vibrator and suction with a vacuum line (14).

The column was conditioned at 220°C for at least three days (more than 72 hours) before use. Recordings were made on a Smith's Servoscribe potentiometric recorder RE 541.20 using the 2, 5, 10 mV ranges where appropriate.

Typical conditions used were:

Column temperature	=	200°C
Detector oven temperature	=	300°C
Flow rates white spot nitrogen	=	30 ml/min for 150 cm column
	=	purge 10 ml/min
Detector pulse mode	=	150 μ second
Attenuator (amplifier)	=	1×10^2, 2×10^2, 5×10^2
Recorder range	=	2 mV, 5 mV calibration
Chart speed (recorder)	=	2 mm/min, 5 mm/min.

ii) "Clean-up" methods

Since the retention time of free o-diamine and the corresponding piazselenol is closer than is desirable for an analytical procedure (15), two methods of removing excess reagent were examined.

A measured amount (2 ml) of a solution of the purified diamine reagent [0.5% (w/v) in 2M hydrochloric acid (except for 3,5-dibromo-o-phenylenediamine which dissolved in concentrated HCl acid)] was added to a solution (100 ml) containing 10-100 ng of selenium (IV) (depending on the sensitivity of the diamine). After standing for more than two hours at room temperature, the mixture was shaken with 10 ml toluene. The toluene phase was then washed three times with:

a) 3 ml of perchloric acid (72%, 1:1 by volume with water),

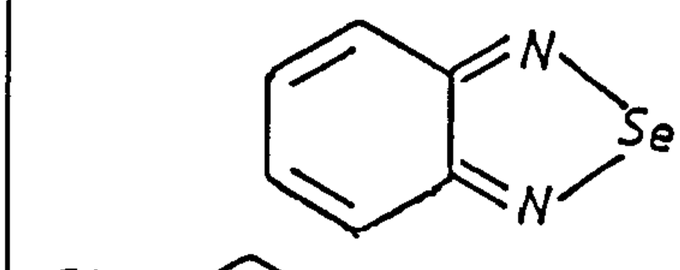
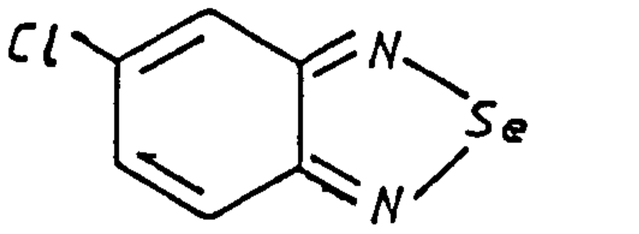
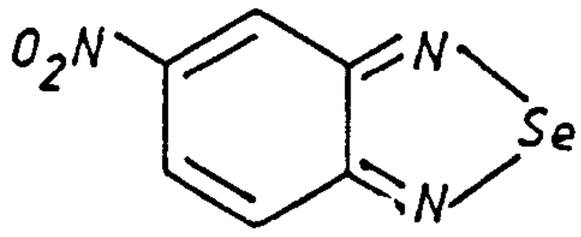
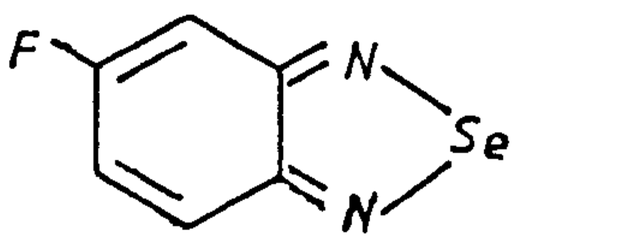
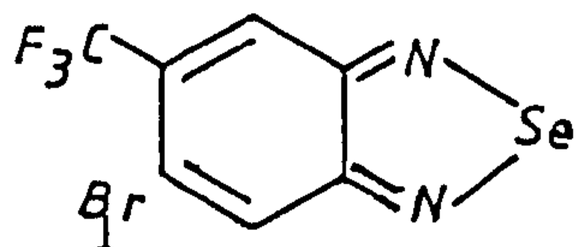
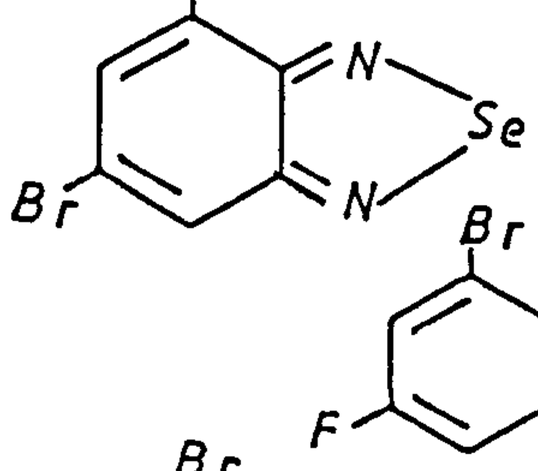
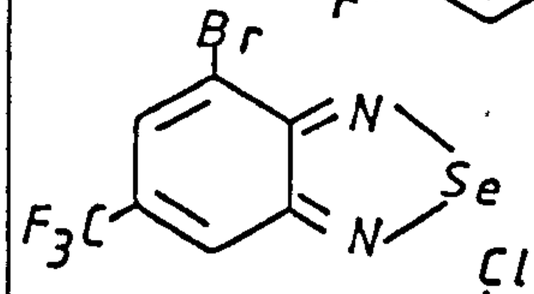
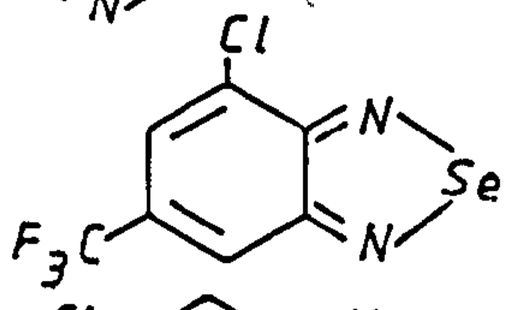
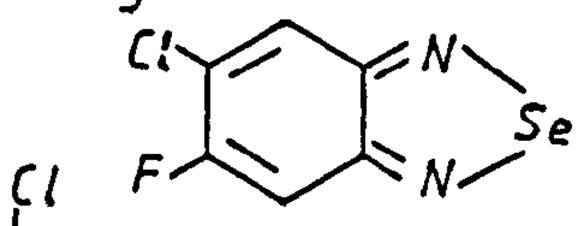
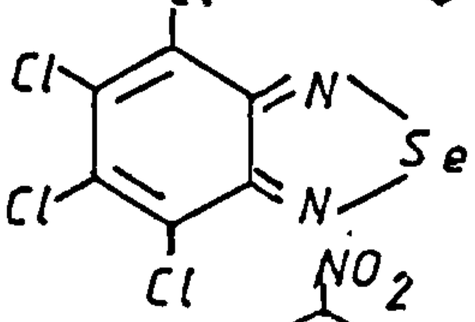
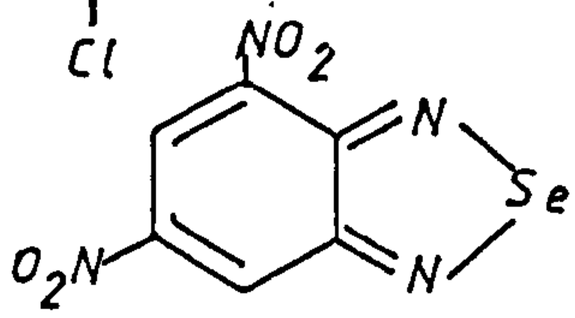
or b) 3 ml of 9M hydrochloric acid,

and examined by GLC + ECD (2 µl injection volumes).

iii) Retention time

Retention times under isothermal conditions of 12 piazselenols were measured using a column temperature of 200°C, plus the calculation of detection limits for each piazselenol using the resultant peak heights for a series of standard solutions of suitable concentrations. Table 3:4 lists the retention times and the relative sensitivity for the respective piazselenols related to the parent (unsubstituted) piazselenol, in terms of peak height response ratios.

Table 3:4. Properties of Piazselenol Derivatives

Piazselenol	M.P. (°C)	Retention Time (Minutes)	Relative Sensitivity
	74	1.0	1
	117–118	2.25	5.25
	222–223	7.4–7.5	9.4
	104–105	1.6	1.75
	90–91	2.0	4.5
	215–217	11.3	23.5
	155–156	3.5	23.7
	160–161	2.6–2.7	83
	167–168	1.6	89.5
	130–131	2.0	15.2
	198–200	32.0	11.3
	210–212	90.0	6.5

3.2.4 Sensitivity of Piazselenol Derivatives for ECD

The electron-capture detection (ECD) is very sensitive to the presence of electron-withdrawing groups in organic substances (9). The first GLC-ECD determination of selenium was applied to 5-chloropiazselenol, which was formed from selenium (IV) and 4-chloro-1,2-diaminobenzene (8).

In order to find a more sensitive reagent for selenium, Shimoishi (1977) studied the sensitivities of some 13 derivatives of 1,2-diaminobenzene using a detector temperature of 280°C and carrier gas (nitrogen) flow rate of 29 ml/min. Each piazselenol derivative (at three different concentrations in toluene) was injected into the gas chromatograph at a column temperature of between 120-210°C and the retention time, peak height and peak area were measured. At a constant column temperature, a linear relationship was found between the solute concentration and the peak height or area. The shorter the retention time, the larger the peak height or area. The peak height and area were extrapolated to a constant retention time (3 minutes). The relative sensitivities were calculated from the ratio of the peak height or area of the particular derivative to that of piazselenol at the retention time of 3 minutes (9). The results are given in Table 2.2 (see page 96), the sensitivity depended upon the substituents present and increased in the order H < Cl < Br < NO_2 for the compounds studied. 4,6-Dibromopiazselenol had the highest sensitivity and was capable of detecting a level of 1 ng Se (9).

Dilli and Sutikno (1984) investigated two new fluorinated reagents for the determination of selenium by GLC + ECD and compared their results with those of seven o-diamines (15). The reported sensitivities of eight piazselenols are also listed in Table 2.2.

In the present work a search was made to increase the sensitivity of the determination of selenium (by GLC + ECD). The conditions were maintained constant during the measurement period for some 12 piazselenols following the concepts proposed by Shimoishi. Six of the ligands had previously been reported in the literature, while the other six ligands were new compounds and were being assessed for the first time. The detector temperature was kept at 300°C and the carrier gas (white spot nitrogen) flow rate was kept constant at 30 ml/min. Each piazselenol derivative (at more than three different concentration levels in toluene) was injected into the gas chromatograph at:

a) constant column temperature 200°C,

b) constant retention time (five minutes).

a) Sensitivity at constant column temperature 200°C

The elution of the 12 piazselenols was measured under constant conditions, the only variable factor being the retention time. The peak heights were measured for comparison of the sensitivity. Table 3.4 lists the relative sensitivity and the retention times which ranged from 1 to 90 minutes.

The sensitivity depended upon the substituents present; for mono-substitution the order was:-

$$H < F < CF_3 < Cl < NO_2$$

But for di- or poly-substitutions the sensitivity was as shown in Table 3.4:

dinitro < tetrachloro < 5-chloro-6-fluoro- < dibromo

< 4-bromo-6-fluoro < 4-bromo-6-CF_3 < 4-chloro-6-CF_3

Figures 3.12-3.17 show the calibration traces for some piazselenols at a column temperature of 200°C.

FIGURE 3.12 CALIBRATION CURVE OF PIAZSELENOL AT CONSTANT COLUMN TEMPERATURE OF 200°C

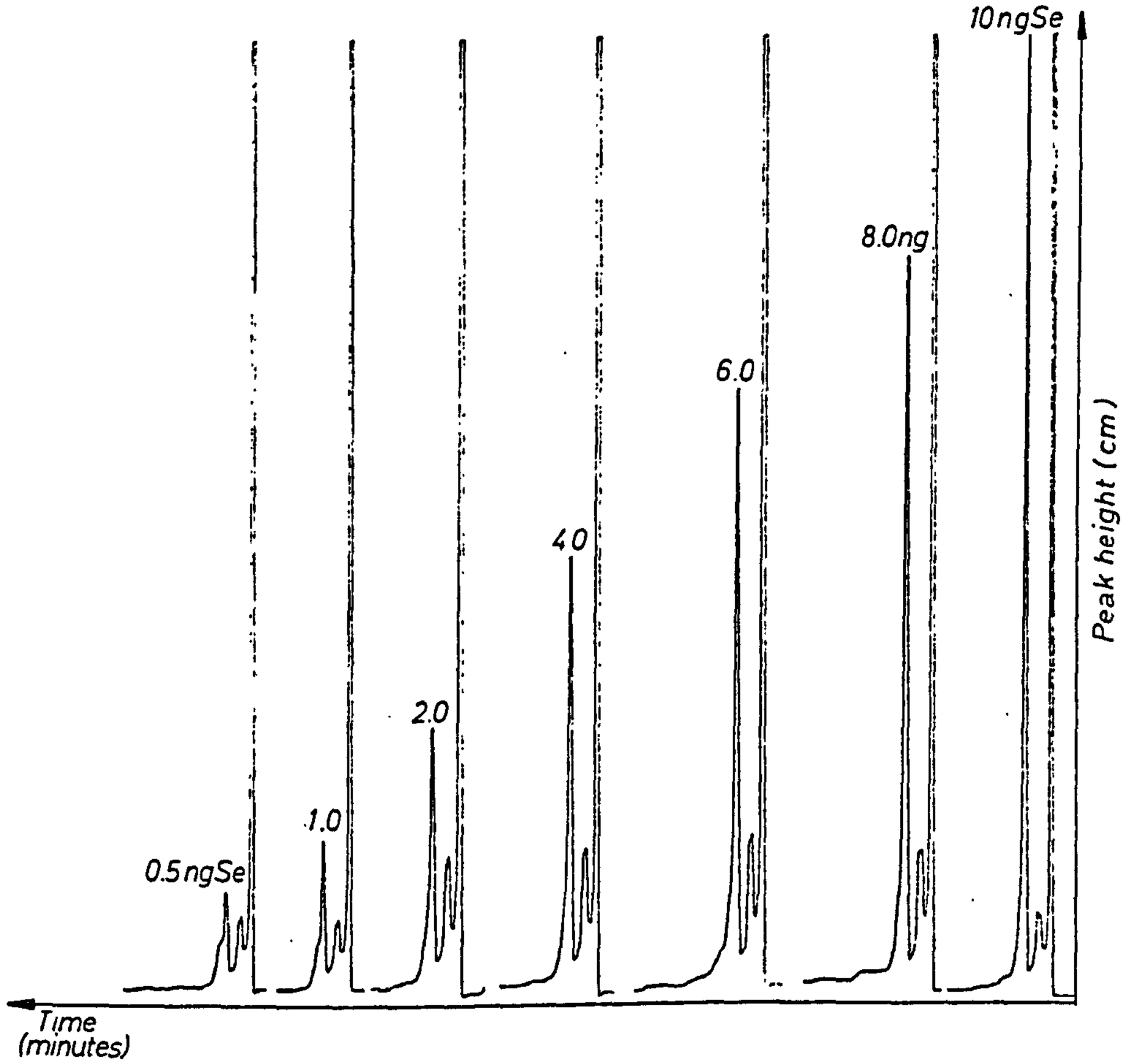


FIGURE 3.13 CALIBRATION CURVE OF 5-CHLOROPIAZSELENOL AT 200°C COLUMN TEMPERATURE

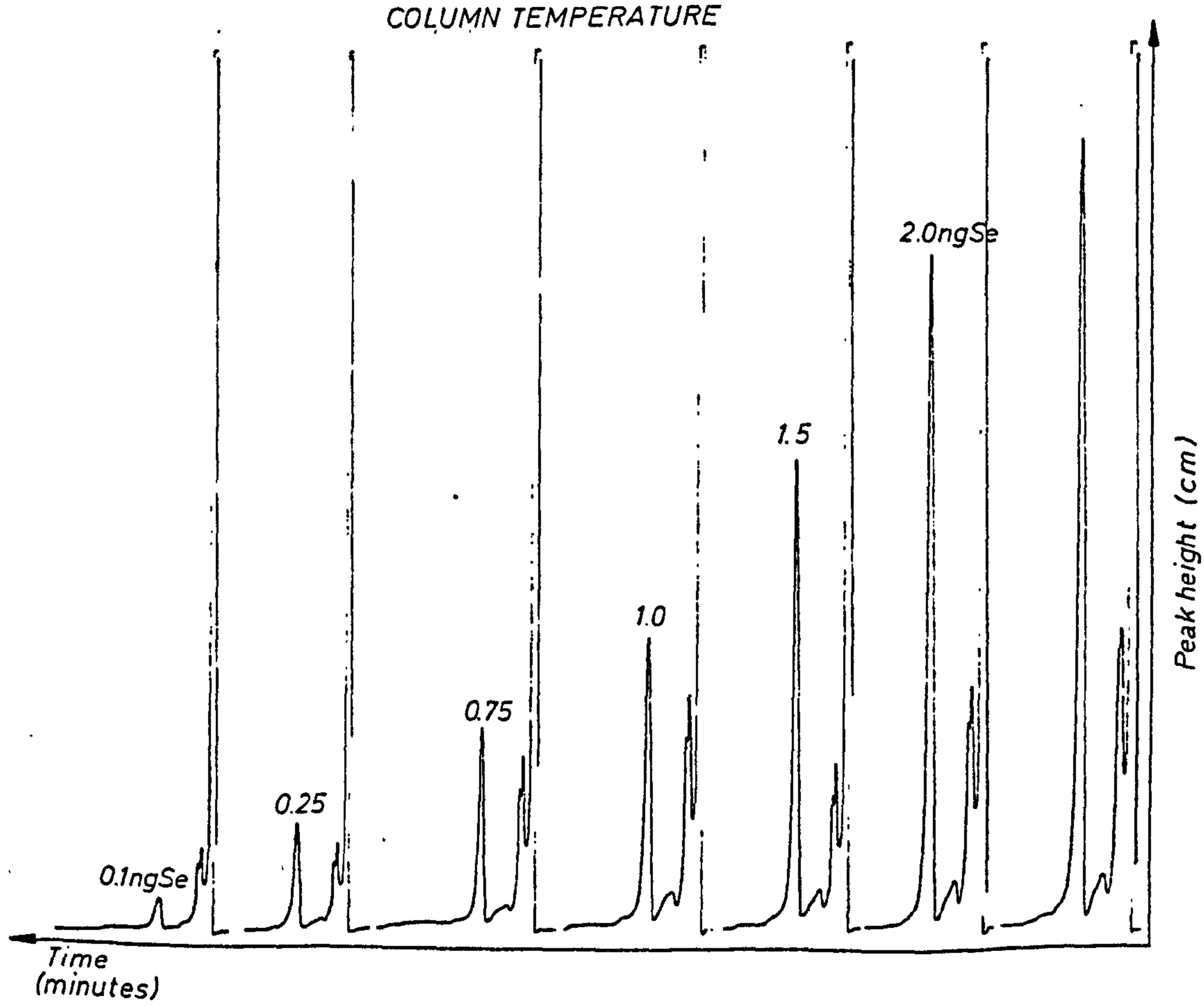


FIGURE 3.14 CALIBRATION CURVE OF 5-NITROPIAZSELENOL AT 200°C COLUMN TEMPERATURE

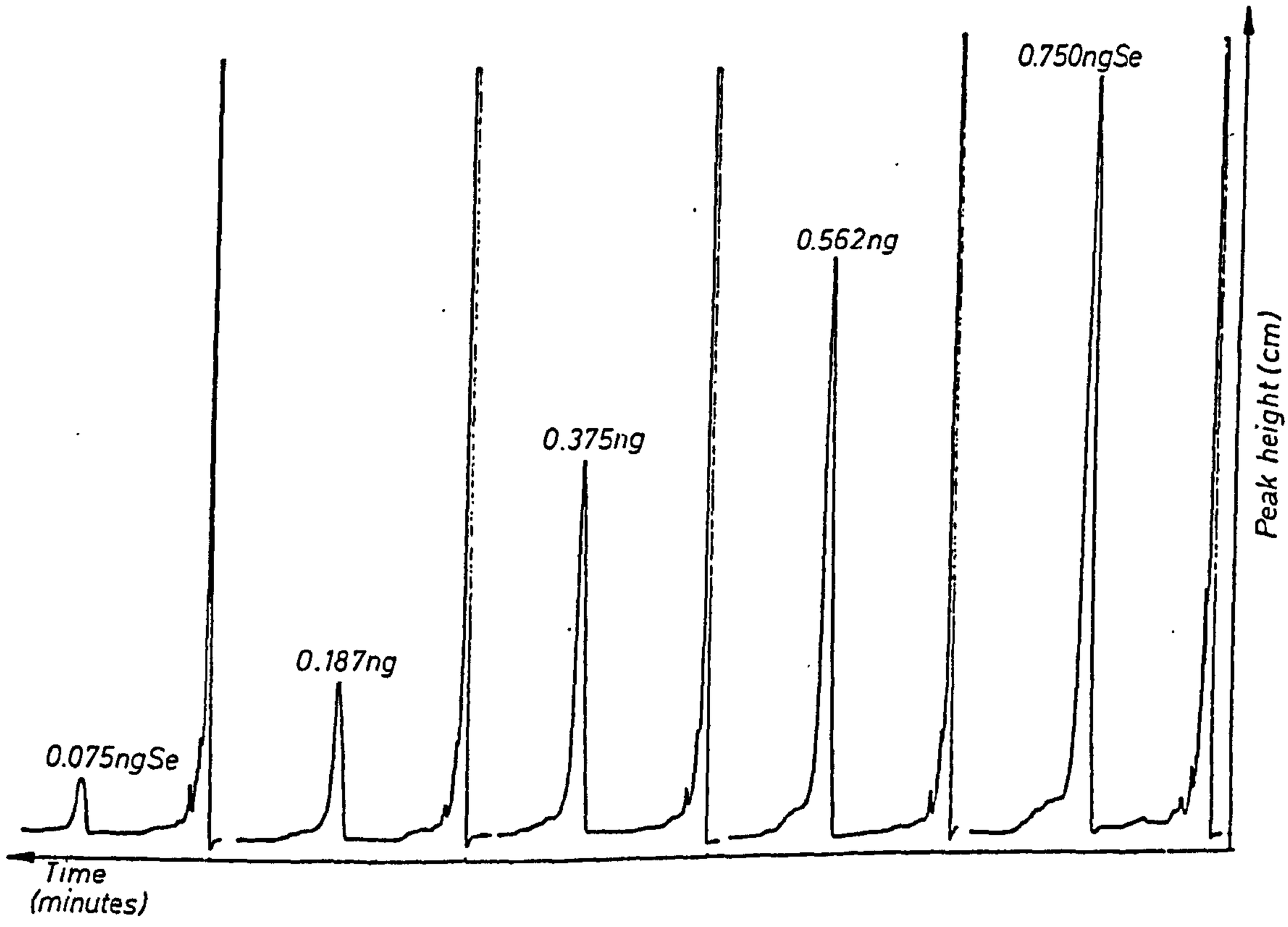


FIGURE 3.15 CALIBRATION CURVE OF 4,6 DI-BROMOPIAZSELENOL AT 200°C COLUMN TEMPERATURE

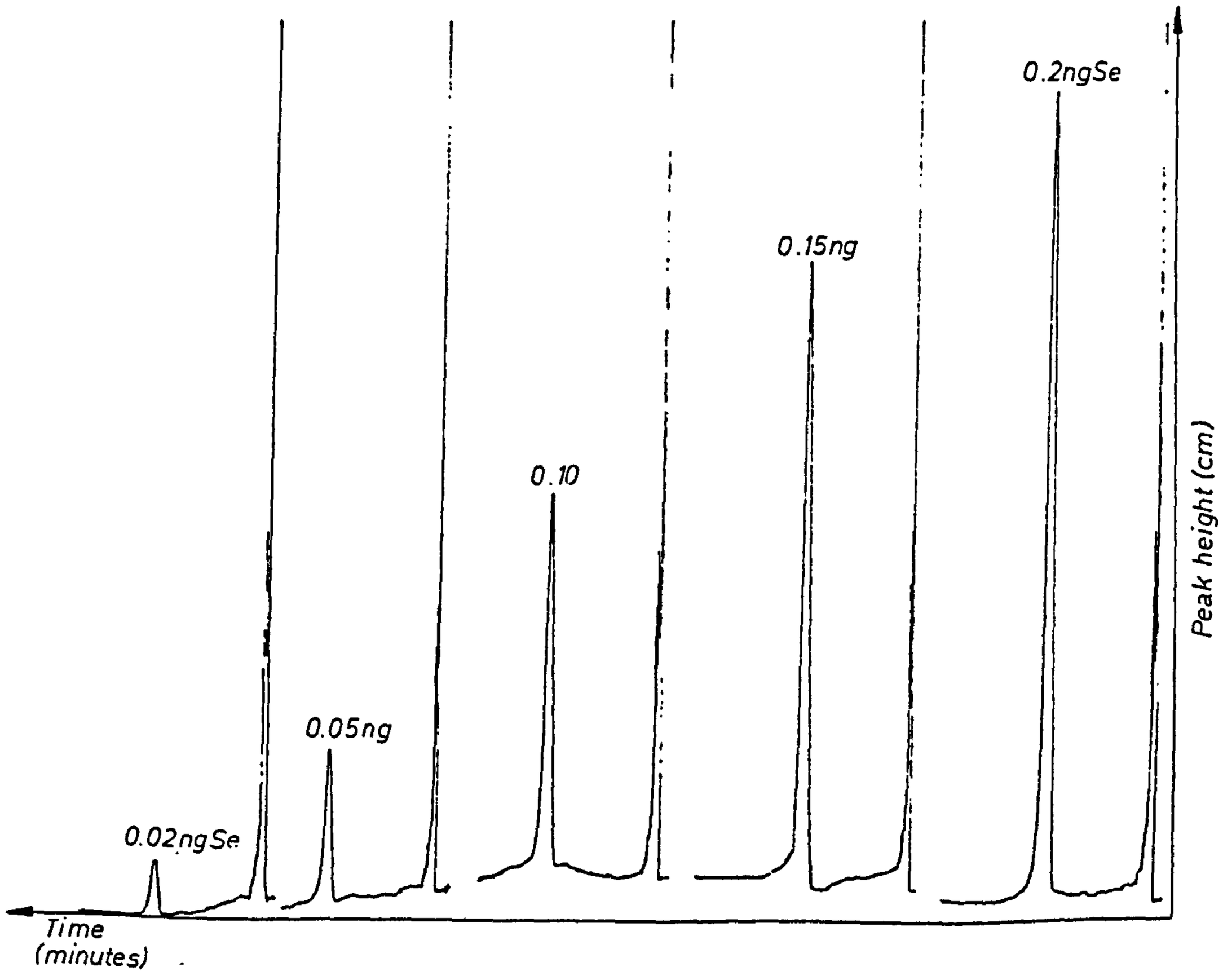


FIGURE 3.16 CALIBRATION CURVE OF 5-CHLORO-6-FLUOROPIAZSELENOL AT 200°C COLUMN TEMPERATURE

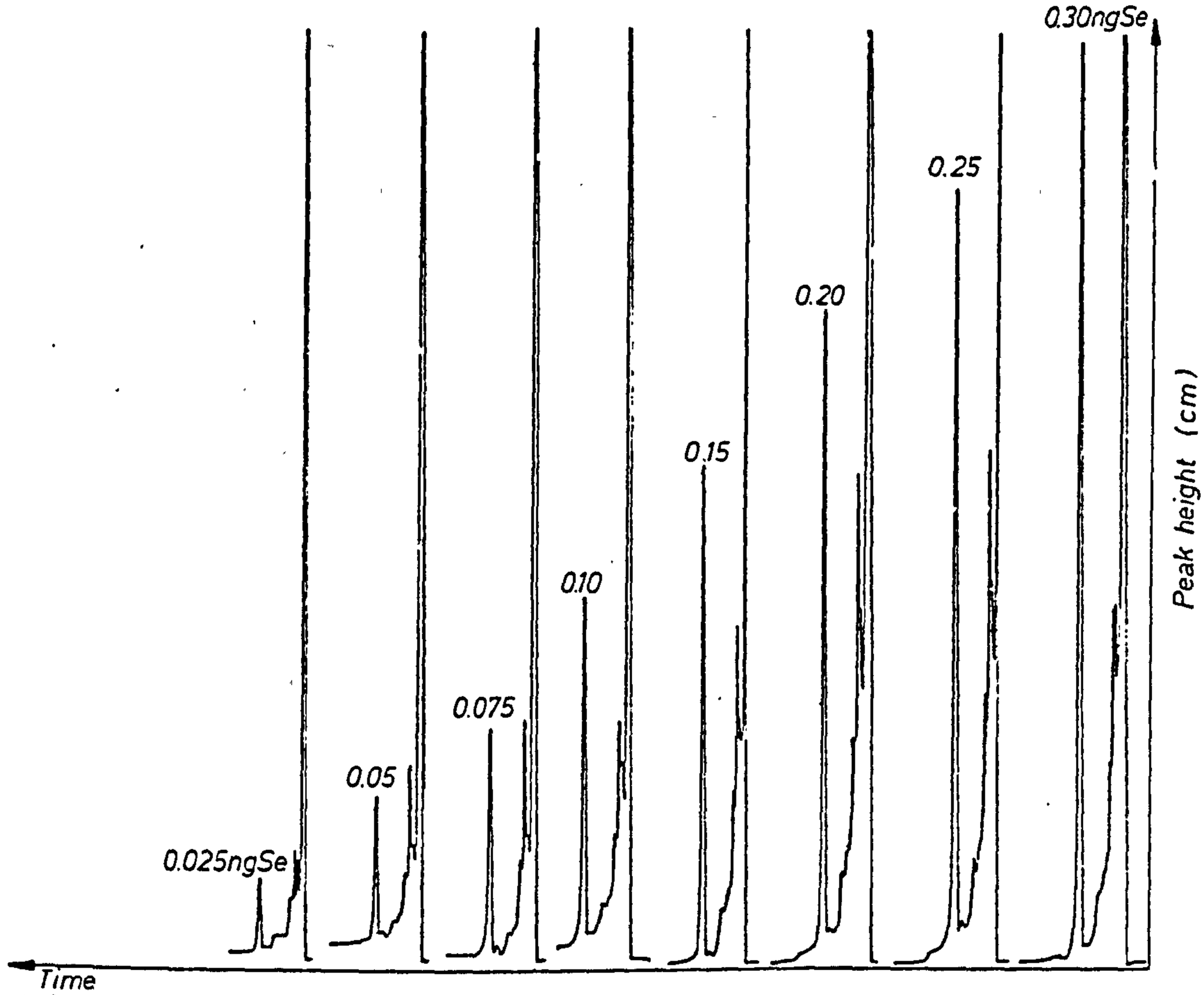
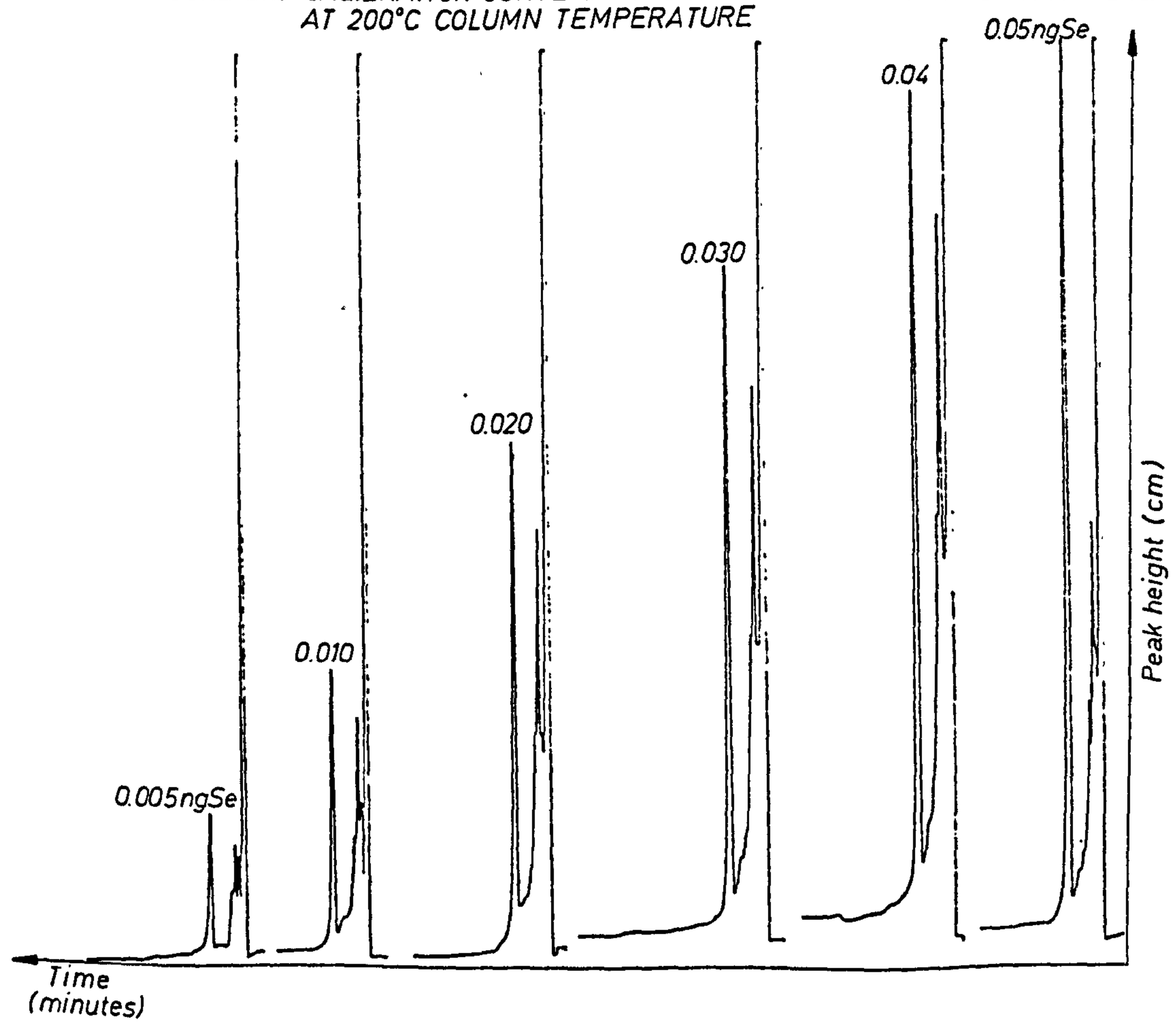


FIGURE 3.17 CALIBRATION CURVE OF 4-CHLORO-6-TRIFLUOROMETHYLPIAZSELENOL AT 200°C COLUMN TEMPERATURE



b) Sensitivity at constant retention time 5 minutes

This method of sensitivity determination was carried out under constant conditions except that the column temperature was changed from 132°C to 230°C so as to allow a constant elution time. Table 3.5 lists the relative sensitivities for all 12 piazselenols. The sensitivity was in the order:

H < F < Cl < Cl & F < NO_2 < CF_3 < F & Br < Cl & CF_3 < tetrachloro < dibromo < Br & CF_3

Figures 3.18 and 3.19 show the calibration traces for two of the piazselenols at constant retention time.

Table 3.5. Relative Sensitivity of Piazselenols

Name of Piazselenol	Sensitivity Found			Literature		
	(1)		(2)			
	Retention Time (min)	Relative Sensitivity at 200°C Column Temperature	At constant Retention Time (5 minutes)	Ref. (9)	Ref. (15)	Ref. (18)
Unsubstituted piazselenol	1.0	1	1	1	1	1
5-Chloropiazselenol	2.25	5.25	3.4	17	7.4	8.2
5-Fluoropiazselenol	1.6	1.75	1.15		3.6	
5-Nitropiazselenol	7.45	9.4	11.3	128	27.6	38.8
5-Trifluoromethyl-piazselenol	2.0	4.5	13.2		23.6	
4,6-Dibromo-piazselenol	11.3	23.5	53.4	363		
4-Bromo-6-fluoro-piazselenol	3.5	23.7	18.0			
4-Bromo-6-trifluoro-methylpiazselenol	2.65	83.0	57.5			
4-Chloro-6-tri-fluoromethyl-piazselenol	1.6	89.5	40.7			
5-Chloro-6-fluoro-piazselenol	2.0	15.2	9.7			
4,6-Dinitro-piazselenol	90.0	6.5	15.3			
4,5,6,7-Tetra-chloropiaz-selenol	32.0	11.3	41.2			

(1) Relative sensitivity at constant column temperature (200°C)

(2) Relative sensitivity at constant retention time (5 minutes)

FIGURE 3.18 CALIBRATION CURVE OF 5-CHLORO-6-FLUOROPIAZSELENOL AT CONSTANT RETENTION TIME (5 MINUTES)

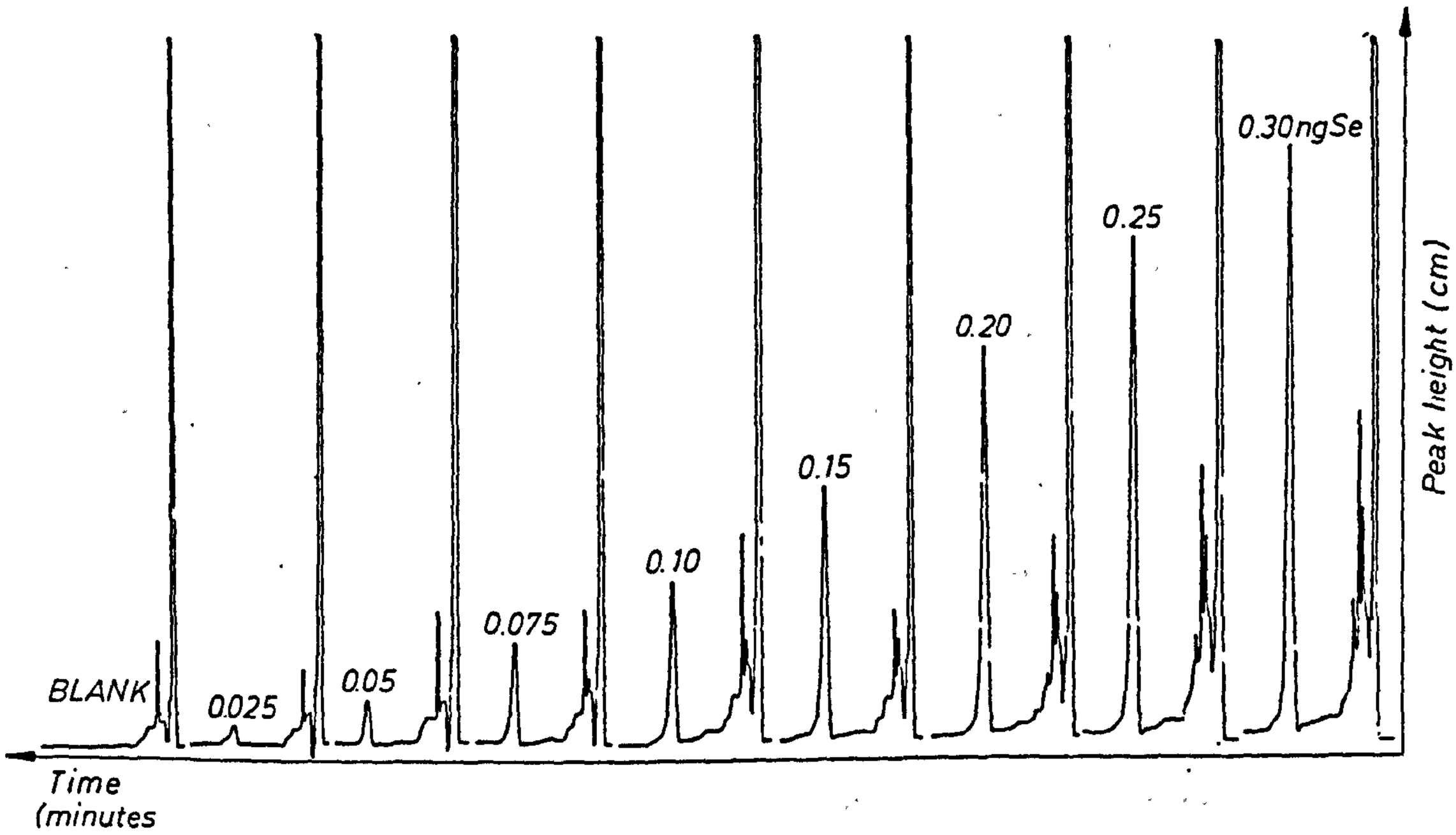
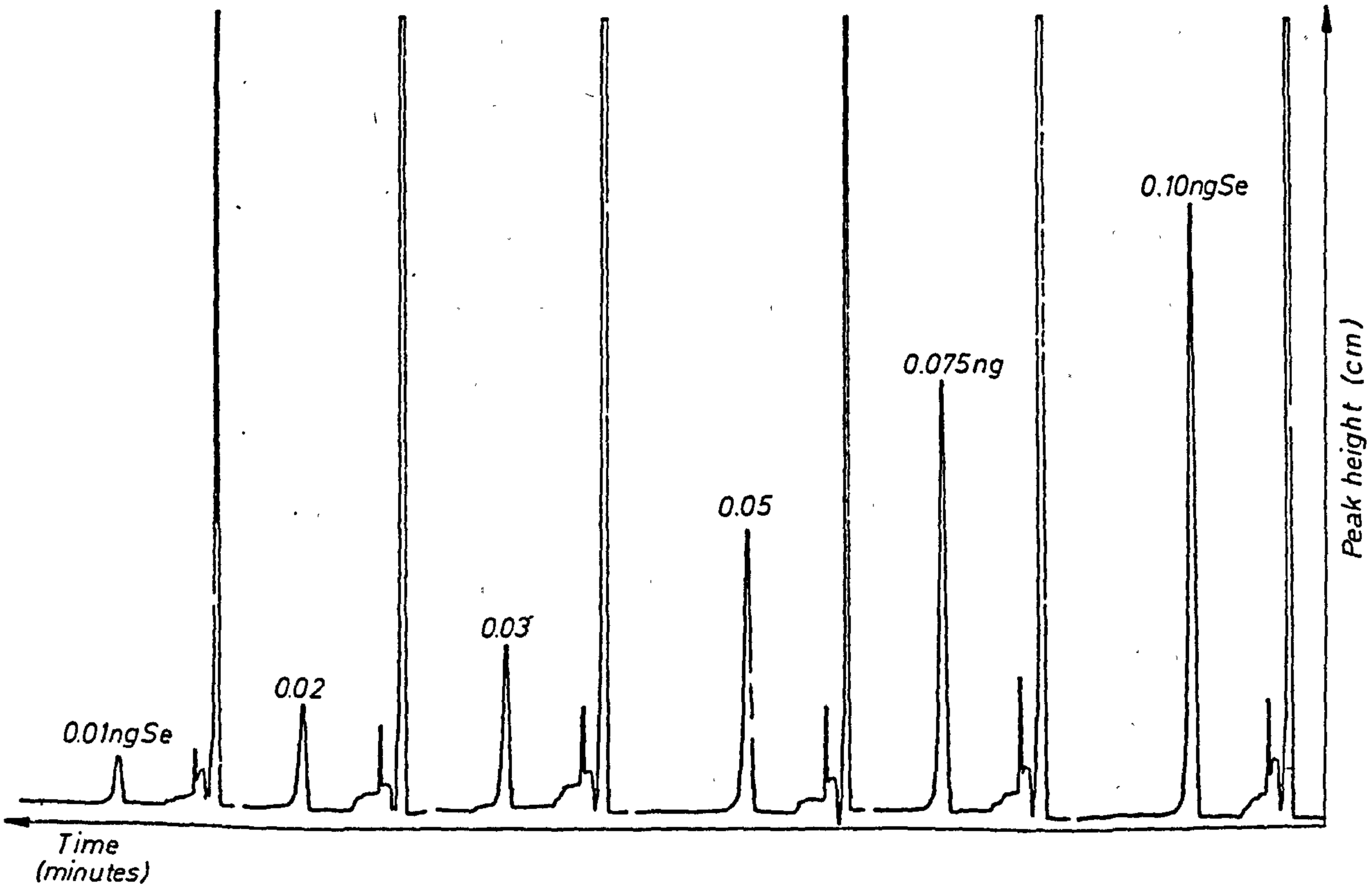


FIGURE 3.19 CALIBRATION CURVE OF 4-CHLORO-6-TRIFLUOROMETHYLPIAZSELENOL AT CONSTANT RETENTION TIME (5 MINUTES)



3.2.5 Statistical Estimation of Detection Limit of Selenium by GLC + ECD

i) Introduction

One of the principal benefits of using instrumental methods of analysis is that they are capable of detecting and determining much smaller quantities of analyte than classical analytical methods. These advantages have led to the appreciation of the importance of trace concentrations of many materials, for example in environmental and biological samples, and thus to the development of many further techniques in which low limits of detection are a major criterion of successful application. It is, therefore, evident that statistical methods for assessing and comparing limits of detection are of importance. In general terms, the limit of detection of an analyte may be described as that concentration which gives an instrument signal significantly different from the 'blank' or 'background' signal. A commonly used definition in the literature of analytical chemistry is that the limit of detection is the analyte concentration giving a signal equal to the blank signal, plus two standard deviations of the blank (19).

Quantitative analysis using GLC is generally performed with a calibration function where the response in peak area or peak height is plotted as a function of concentration; when using ECD the calibration function is a linear relationship.

It is generally agreed that the determination of limit of detection is statistical in nature. Therefore, the limit of detection must be calculated from other available information as the calibration data.

Hubaux and Vos discussed in detail a statistical treatment of linear calibration curves which allowed calculation of the limit of detection (20). Hunter (1981) commented in detail on calibration and

the straight line graph together with the construction of the Working-Hotelling bounds for the true fitted line (21). Liteanu and Rica (22), in their book discussing those aspects of statistical theory and methodology of trace analysis, have presented several detailed methods of calculating or obtaining the detection limit, together with a philosophical approach on the meaning of the terms.

ii) Calibration Graphs and Detection Limit of Selenium as 4-Bromo-6-trifluoromethylpiazselenol

Calibration data for selenium (as 4-bromo-6-trifluoromethylpiaz-selenol) was obtained by making a 1 µl injection of standard solutions, 2.5 ng/ml, 5.0 ng/ml, 10.0 ng/ml, 15.0 ng/ml, 20.0 ng/ml and 25 ng/ml of selenium in toluene. The experiment was repeated four times, the results given in Table 3.6 are the values obtained, with each value being an average of three injections.

Table 3.6. Calibration Data for 4-Br-6-CF_3-Piazselenol

Concentration (ng/µl)	Peak height (cm)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
0.0025	1.2	1.2	1.4	1.2
0.005	3.4	3.3	2.9	3.6
0.010	6.2	6.9	6.3	6.4
0.015	9.5	10.4	9.7	9.8
0.020	13.10	13.6	12.8	13.7
0.025	18.2	18.4	17.9	17.7

where

X = concentration of selenium ng/ul = (µg/ml)

Y = peak height (cm)

Using the LSQPRNT program on a PET microcomputer the correlation coefficient for the calibration line was determined to be 0.996566, which shows a good fit, as shown in Figure 3.20(a).

Using the calibration data, given in Table 3.6, the 95% Confidence Bounds for 90% of the observations, and the Working-Hotelling region, were determined, which by mathematical manipulation yields the value of the limit of detection for selenium in ng/ml (using GLC + ECD).

The statistical procedure that has to be followed in order to ascertain these boundaries and values is presented in (21,23) and allows calculation of the regression line equation, correlation coefficient, the upper prediction limit on the blank, and the limit of detection in terms of concentration.

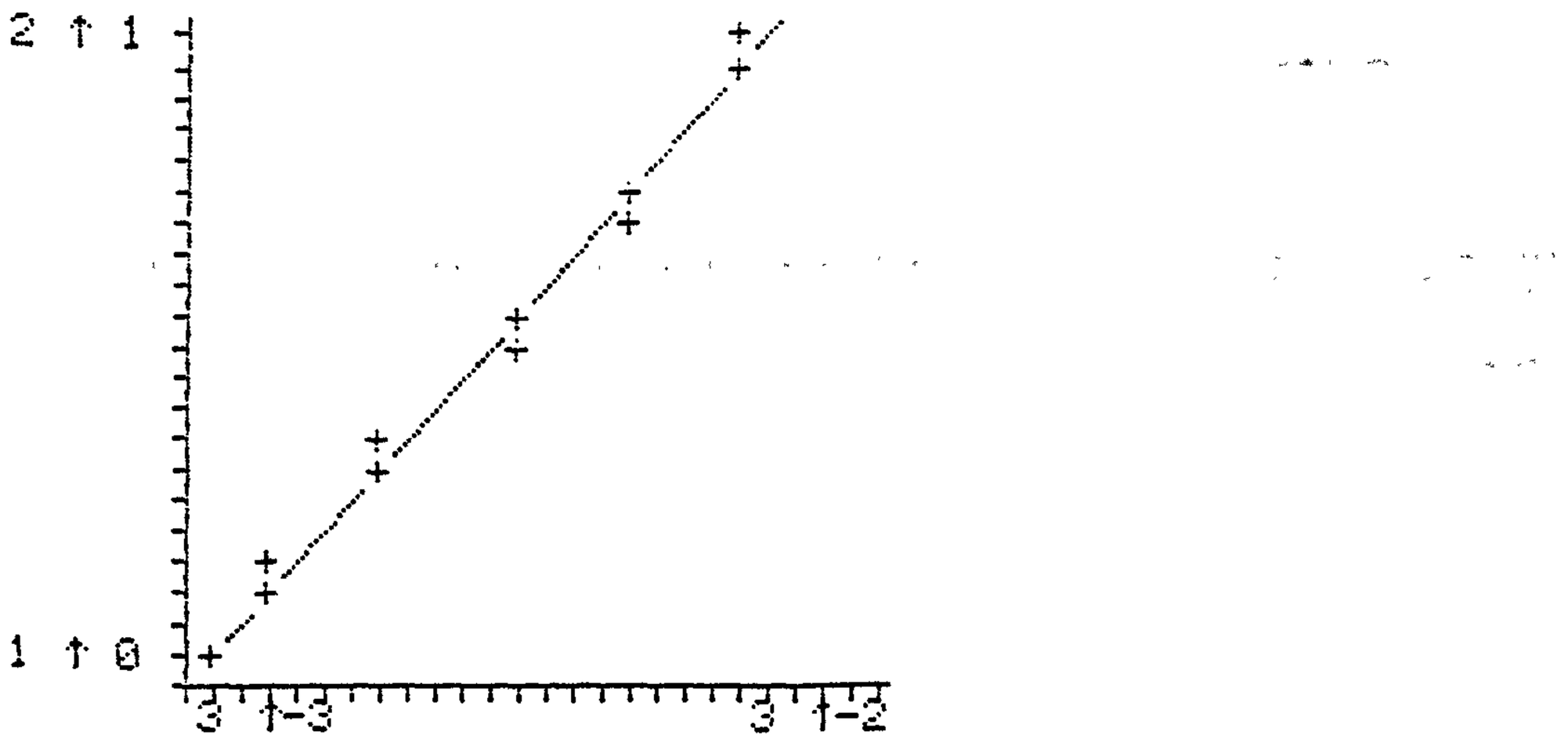
The data and mathematical calculations required in order to obtain the regression equation, 95% confidence bounds and Working-Hotelling region are shown in Table 3.7.

FIGURE 3.20(a) CALIBRATION CURVE OF 4-Br-6-CF_3-PIAZSELENOL

NUMBER OF POINTS= 24

X	Y
2.5E-03	1.2
2.5E-03	1.2
2.5E-03	1.4
2.5E-03	1.2
5E-03	3.4
5E-03	3.3
5E-03	2.9
5E-03	3.6
.01	6.2
.01	6.9
.01	6.3
.01	6.4
.015	9.5
.015	10.4
.015	9.7
.015	9.8
.02	13.1
.02	13.6
.02	12.8
.02	13.7
.025	18.2
.025	18.4
.025	17.9
.025	17.7

GRADIENT= 725.449696
ERROR ON GRADIENT= 12.2605105
CORRELATION= .996566555
INTERCEPT ON Y=-.670391907
MAX.AND MIN.VALUES OF INTERCEPT=-.512026978 &-.828756834



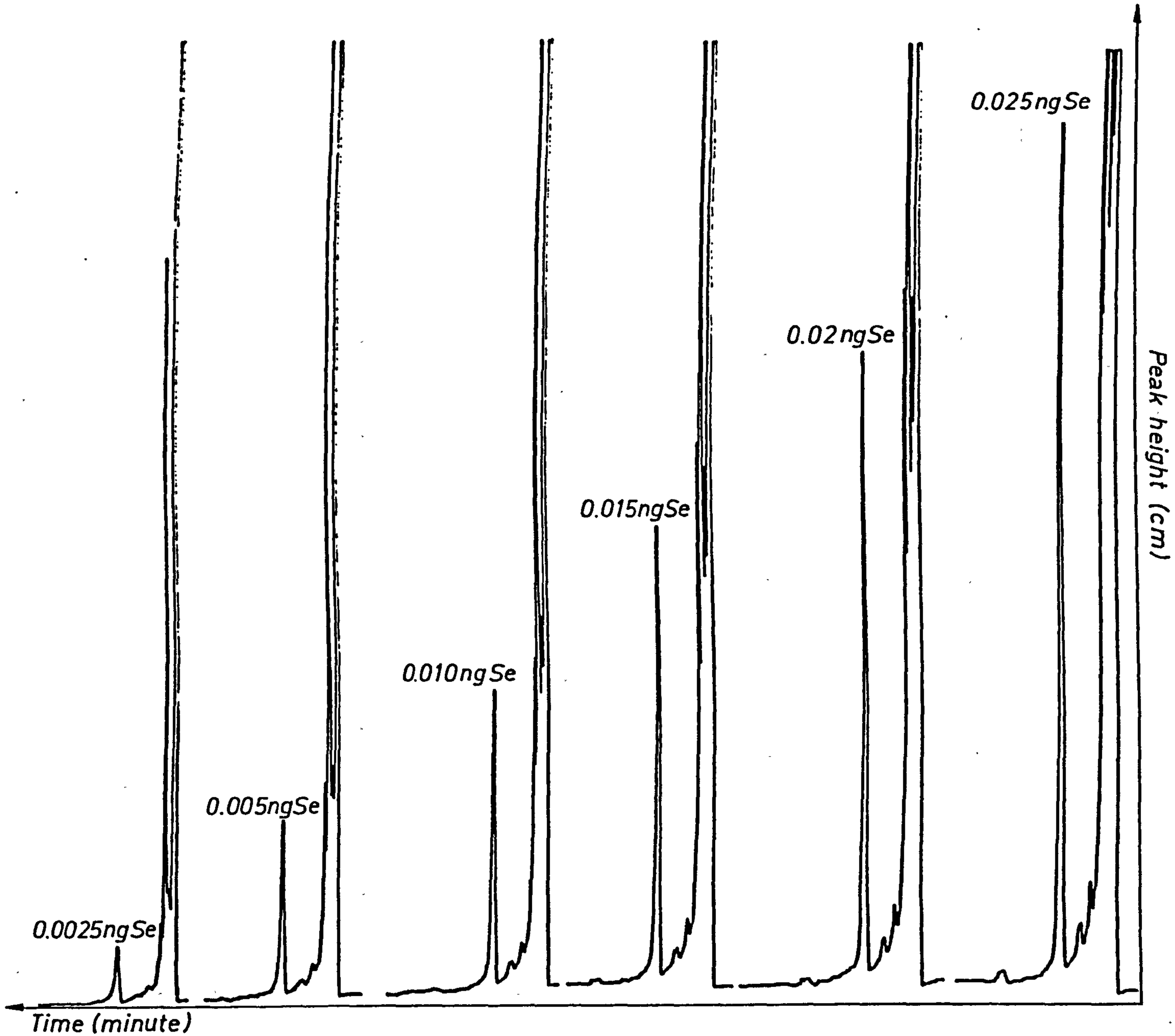


FIGURE 3.20(b) CALIBRATION CURVE OF 4-Br-6-CF_3-PIAZSELENOL AT 200°C CONSTANT COLUMN TEMPERATURE

Table 3.7. Mathematical Calculation for Calibration Curve of 4-Br-6-CF_3-Piazselenol

X	Y	$x=(X-\bar{X})$	$y=(Y-Y)$	x^2	y^2	xy
0.0025	1.2	-0.0104166	-7.5	0.000108505	56.25	0.07812
0.0025	1.2	-0.0104166	-7.5	0.000108505	56.25	0.07812
0.0025	1.4	"	-7.3	"	53.29	0.07403
0.0025	1.2	"	-7.5	"	56.25	0.07812
0.005	3.4	-0.00791667	-5.3	0.6267×10^{-4}	28.09	0.041958
0.005	3.3	"	-5.4	"	29.16	0.04275
0.005	2.9	"	-5.8	"	33.64	0.045916
0.005	3.6	"	-5.1	"	26.01	0.040375
0.010	6.2	-0.00291667	-2.5	0.08507×10^{-4}	6.25	0.007291
0.010	6.9	"	-1.8	"	3.24	0.005250
0.010	6.3	"	-2.4	"	5.76	0.00700
0.010	6.4	"	-2.3	"	5.29	0.006708
0.015	9.5	0.0020833	0.8	0.0434×10^{-4}	0.64	0.001666
0.015	10.4	0.0020833	1.7	0.0434×10^{-4}	2.89	0.003541
0.015	9.7	"	1.0	"	1.0	0.002083
0.015	9.8	"	1.1	"	1.21	0.002292
0.020	13.1	0.0070833	4.4	0.50173×10^{-4}	19.36	0.031166
0.020	13.6	"	4.9	"	24.01	0.034708
0.020	12.8	"	4.1	"	16.81	0.029041
0.020	13.7	"	5.0	"	25.0	0.035416
0.025	18.2	0.0120834	9.5	1.46008×10^{-4}	90.25	0.114792
0.025	18.4	"	9.7	"	94.09	0.117209
0.025	17.9	"	9.2	"	84.64	0.111167
0.025	17.7	"	9.0	"	81.0	0.108750
$\Sigma X=0.31$ $\bar{X}=\frac{0.31}{24}$ $\bar{X}=$ 0.01291667	$\Sigma Y=$ 208.8 $\bar{Y}=8.7$			$\Sigma x^2=$ 0.001520812	$\Sigma y^2=$ 800.38	$\Sigma xy=$ 1.09747

where

$\bar{X}$ = mean of X i.e. = $\frac{\Sigma X}{n}$, n = no. of observations

Σ = sum of

$$\bar{X} = \frac{0.31}{24} = \underline{0.01291667}$$

$$\bar{Y} = \frac{\Sigma Y}{n} = \frac{208.8}{24} = \underline{8.7}$$

b = gradient (slope) of the line = $\frac{\Sigma xy}{\Sigma x^2}$

$$= \frac{1.09747}{0.001520812} = \underline{721.63}$$

a = intercept value

$$a = \bar{Y} - b\bar{X}$$

$$a = 8.7 - (721.63 \times 0.01291667)$$

$$a = \underline{-0.621}$$

Equation of regression line $Y = a + bX$

$$\text{Variance } s^2 = \frac{\Sigma y^2 - b\,\Sigma xy}{(n-2)}$$

$$s^2 = \frac{800.38 - (721.63 \times 1.09747)}{(24 - 2)} = \frac{800.38 - 791.96727}{22}$$

$$s^2 = \underline{0.3823968}$$

For the mean response A_o, at a chosen single value x_o, at the 95% confidence limit:

$$A_o = (b_o + b_1 x_o) \pm t \left[\left(\frac{1}{n} + \frac{(X_o - \bar{X})^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}}$$

and for future single observations $y_o = b_o + x_o$ and $(X_o - \bar{X})^2 = x^2$

therefore

$$A_o = y_o \pm t \left[\left(\frac{1}{n} + \frac{x^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}}$$

t = from t-Tables for correct number of degrees of freedom.

95% Confidence Bounds for 90% of Observations

$$y = a + bx \pm \left[A + Z_p B\right]$$

where $A = \left[2F_{2,v,\alpha/2}\left(\frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma x^2}\right) s^2\right]^{\frac{1}{2}}$

$$B = \left[v\, s^2/\chi^2 v,\ \frac{1-\alpha}{2}\right]^{\frac{1}{2}}$$

Z_p, $F_{2,v,\alpha/2}$ χ^2, v, α: from statistical tables.

$Z_p = 1.29$

$F_{2,v,\alpha/2} = 4.97$

$\chi^2 = 5.01$

$$B = \left(\frac{22 \times 0.3823968}{5.01}\right)^{\frac{1}{2}} = (1.679187)^{\frac{1}{2}} = \underline{1.29583}$$

$$y_1 = a + bx_1 \pm \left[2 \times 4.97\left(\frac{1}{24} + \frac{(0.0025-0.0129166)^2}{0.001520812}\right) 0.38239968\right]^{\frac{1}{2}} + 1.29 \times 1.29583$$

$$y_1 = -0.621 + \left(721.63 \times 0.0025\right) \pm \left(0.6554115 + 1.671626\right)$$

$$\underline{y_1 = 1.183 \pm 2.327}$$

$$y_5 = -0.621 + \left(721.63 \times 0.005\right) \pm \left[2 \times 4.97\left(\frac{1}{24} + \frac{(0.005-0.012966)^2}{0.001520812}\right) \times 0.3823968\right]^{\frac{1}{2}} + 1.29 \times 1.29583$$

$$\underline{y_5 = 2.987 \pm 2.23288}$$

$$y_9 = -0.621 + \left(721.63 \times 0.010\right) \pm \left[2 \times 4.97\left(\frac{1}{24} + \frac{(0.010-0.0129166)^2}{0.001520812}\right) \times 0.382968\right]^{\frac{1}{2}} + 1.29 \times 1.29583$$

$$\underline{y_9 = 6.5953 \pm 2.09546}$$

$$y_{13} = -0.621 + \left(721.63 \times 0.015\right) \pm \left[2 \times 4.97\left(\frac{1}{24} + \frac{(0.015-0.0129166)^2}{0.001520812}\right) \times 0.3823968\right]^{\frac{1}{2}} + 1.29 \times 1.29583$$

$$\underline{y_{13} = 10.2034 \pm 2.082956}$$

$$y_{17} = -0.621 + \left(721.63 \times 0.020\right) \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{(0.020-0.0129166)^2}{0.001520812}\right) \times 0.3823968\right]^{\frac{1}{2}} + 1.29 \times 1.29583$$

$$\underline{y_{17} = 13.8116 \pm 2.2043}$$

$$y_{21} = -0.621 + \left(721.63 \times 0.025\right) \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{(0.025-0.0129166)^2}{0.001520812}\right) \times 0.3823968\right]^{\frac{1}{2}} + 1.29 \times 1.29583$$

$$\underline{y_{21} = 17.42 \pm 2.39499}$$

The Working-Hotelling region is given by:

$$\hat{y} = a + bx \pm \left[2F_{2,v,\alpha} \left(\frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}}$$

$$\hat{y}_1 = -0.621 = 721.63 \times 0.0025 \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{0.000108505}{0.001520812} \right) 0.3823968 \right]^{\frac{1}{2}}$$

$$\hat{y}_1 = 1.183 \pm 0.6554$$

$$\hat{y}_5 = -0.621 + 721.63 \times 0.005 \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{0.00006267}{0.001520812} \right) 0.3823968 \right]^{\frac{1}{2}}$$

$$\hat{y}_5 = 2.987 \pm 0.56126$$

$$\hat{y}_9 = -0.621 + 721.63 \times 0.010 \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{0.08507 \times 10^{-4}}{0.001520812} \right) 0.3823968 \right]^{\frac{1}{2}}$$

$$\hat{y}_9 = 6.5953 \pm 0.42384$$

$$\hat{y}_{13} = -0.621 + 721.63 \times 0.015 \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{0.0434 \times 10^{-4}}{0.001520812} \right) 0.3823968 \right]^{\frac{1}{2}}$$

$$\hat{y}_{13} = 10.2034 \pm 0.411336$$

$$\hat{y}_{17} = -0.621 + 721.63 \times 0.020 \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{0.50173 \times 10^{-4}}{0.001520812} \right) 0.3823968 \right]^{\frac{1}{2}}$$

$$\hat{y}_{17} = 13.8116 \pm 0.53268$$

$$\hat{y}_{21} = -0.621 + 721.63 \times 0.025 \pm \left[2 \times 4.97 \left(\frac{1}{24} + \frac{1.46008 \times 10^{-4}}{0.001520812} \right) 0.3823968 \right]^{\frac{1}{2}}$$

$$\hat{y}_{21} = 17.42 \pm 0.72137$$

The values of the Working-Hotelling region and the confidence bounds are given in Table 3.8.

Table 3.8. The Values of the Working-Hotelling Region and the Confidence Bounds

x (ng/µl)	y (cm)	Working-Hotelling Region	Confidence Bounds
0.0025	1.25	0.5276 or 1.8384	-1.144 or 3.51
0.005	3.30	2.42574 or 3.54826	0.75412 or 5.2199
0.010	6.45	6.17146 or 7.01914	4.50 or 8.691
0.015	9.85	9.792064 or 0.614736	8.120 or 12.2864
0.020	13.30	13.27892 or 14.34428	11.607 or 16.059
0.025	18.05	16.69863 or 18.14137	15.025 or 19.815

Figure 3.21 illustrates the calibration line and then the boundaries. From the graph, the limit of detection can be determined.

From Figure 3.21, it can be seen that the Working-Hotelling system offers a lower detection limit than the 95% Confidence Bounds.

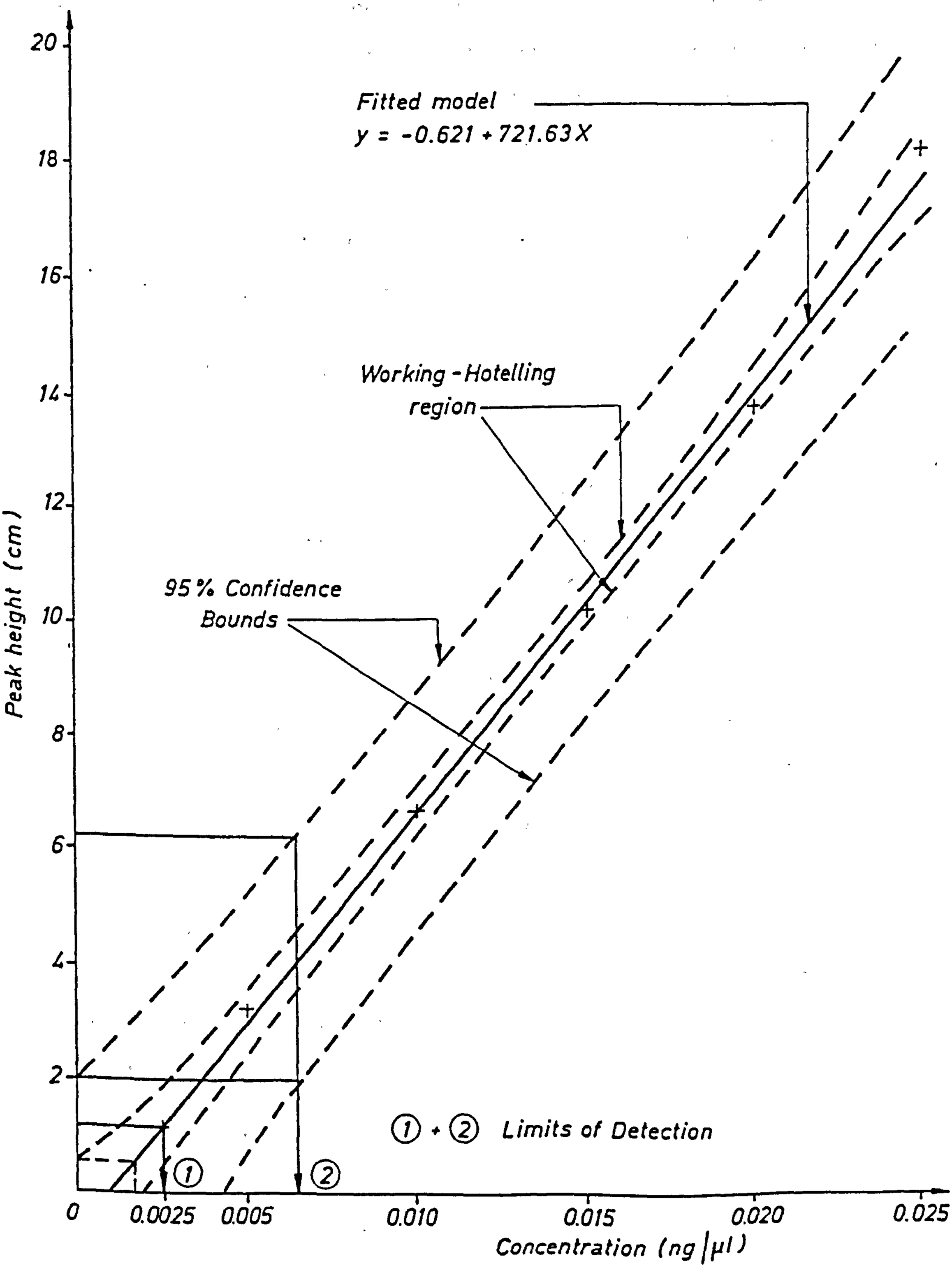
Limit of Detection Values :-

95% Confidence Bounds : 0.0067 ng/ul = 6.7 ng/ml

Working-Hotelling : 0.0025 ng/ul = 2.5 ng/ml

The lowest amount in units of concentration that can be measured above the blank. The value of limit of detection obtained using the Working-Hotelling bounds is closer to the real values than the values from the 95% confidence bounds, which seems rather high reflecting the rather conservative nature of the calculations.

FIGURE 3.21 CALIBRATION GRAPH, 95% CONFIDENCE BOUNDS AND WORKING-HOTELLING REGION FOR 4-Br-6-CF_3-PIAZSELENOL



iii) Determination of the Lower Limit of Detection X_{LD} (23)

The limit of detection was calculated by comparing two prediction limits. The upper prediction limit of the blank was calculated and lower prediction limits on areas for given concentration were calculated. The lower prediction limits were compared with the upper prediction limit on the blank until a lower prediction limit was found which was greater than or equal to the upper prediction limit on the blank (23,24).

The prediction limits for an area for a given concentration were calculated by the following equation:

$$\hat{y} \pm \left[t_{n-2,\alpha} \; s_{y,x} \sqrt{1 + \frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma x^2}} \right]$$

where $\hat{y} = a + bx$

Y_{UB}, the 99% upper prediction limit on the blank, was calculated by using the positive portion of the above equation as follows:

$$Y_{UB} = a + bx + \left[t_{n-2,\alpha} \; s_{y,x} \sqrt{1 + \frac{1}{n} + \frac{(X)^2}{\Sigma x^2}} \right]$$

In this work, 99% confidence is selected ($\alpha = 0.01$) and 22 degrees of freedom. The appropriate value for t_{n-2} is 2.819 (from Statistical Tables).

$$Y_{UB} = -0.621 + 721.63 \times 0 + \left[2.819 \times 0.61838 \times \sqrt{1 + \frac{1}{24} \frac{(1.6684 \times 10^{-4})}{0.001520812}} \right]$$

$$Y_{UB} = \underline{1.2495}$$

Y_L, the 99% lower prediction limit on the expected area at a given concentration, was calculated using the following equation:

$$Y_L = a + bx - \left[t_{n-2,\alpha} s_{y,x} \sqrt{1 + \frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2}} \right]$$

where X are values substituted into the above equation.

1) at X = 0.0025

Y_L = $\underline{-0.6553}$ i.e. $< Y_{UB}$

2) at X = 0.005

Y_L = $\underline{1.0995}$ i.e. $< Y_{UB}$

3) at X = 0.010

Y_L = $\underline{4.81137}$ i.e. $> Y_{UB}$

$\underline{X_{DL} = 0.010 \text{ ng}/\mu\text{l} = 10.0 \text{ ng Se/ml}}$

The peak height corresponding to X_{DL} was referred to as Y_Q,

$Y_Q = a + bx$

$Y_Q = -0.621 + 721.63 \times 0.01$

$\underline{Y_Q = 6.595 \text{ cm}}$ = $\underline{6.6 \text{ cm}}$.

iv) Estimation of Detection Limit of Selenium (as Piazselenol by GLC + ECD) by Using Computer

The detection limit for selenium as the piazselenol (using GLC + ECD) was also estimated using the method suggested by Liteanu and Rica (22). The method is similarly based on the use of the calibration function, employing the appropriately constructed confidence bands based on the normality of the errors and minimising the sum of the squares in the traditional manner. The detailed calculations were performed using a software package developed by C. Scott of these laboratories, who was interested in developing such a system for indirect photometry detection in HPLC. Figure 3.22 illustrates the parameters which are used for estimation of the detection limit by this method, while Figure 3.23 depicts the relevant parameters for the 4-bromo-6-trifluoromethyl piazselenol being used as an example. The data was as listed below:

i) Equation of calibration line

$$y = -0.398 + 687.26x$$

ii) Limits

a) For signal, y (cm)

1) upper limit = 2.2

2) lower limit = 0.702

b) For concentration, C (µg/ml)

1) upper limit = 0.004114

2) lower limit = 0.000997

c) Detection limit = 0.00261 µg/ml = 2.61 ng/ml

FIG. 3.22 ESTIMATION OF DETECTION LIMIT OF SELENIUM AS PIAZSELENOL BY THE LITEANU & RICA (22) METHOD

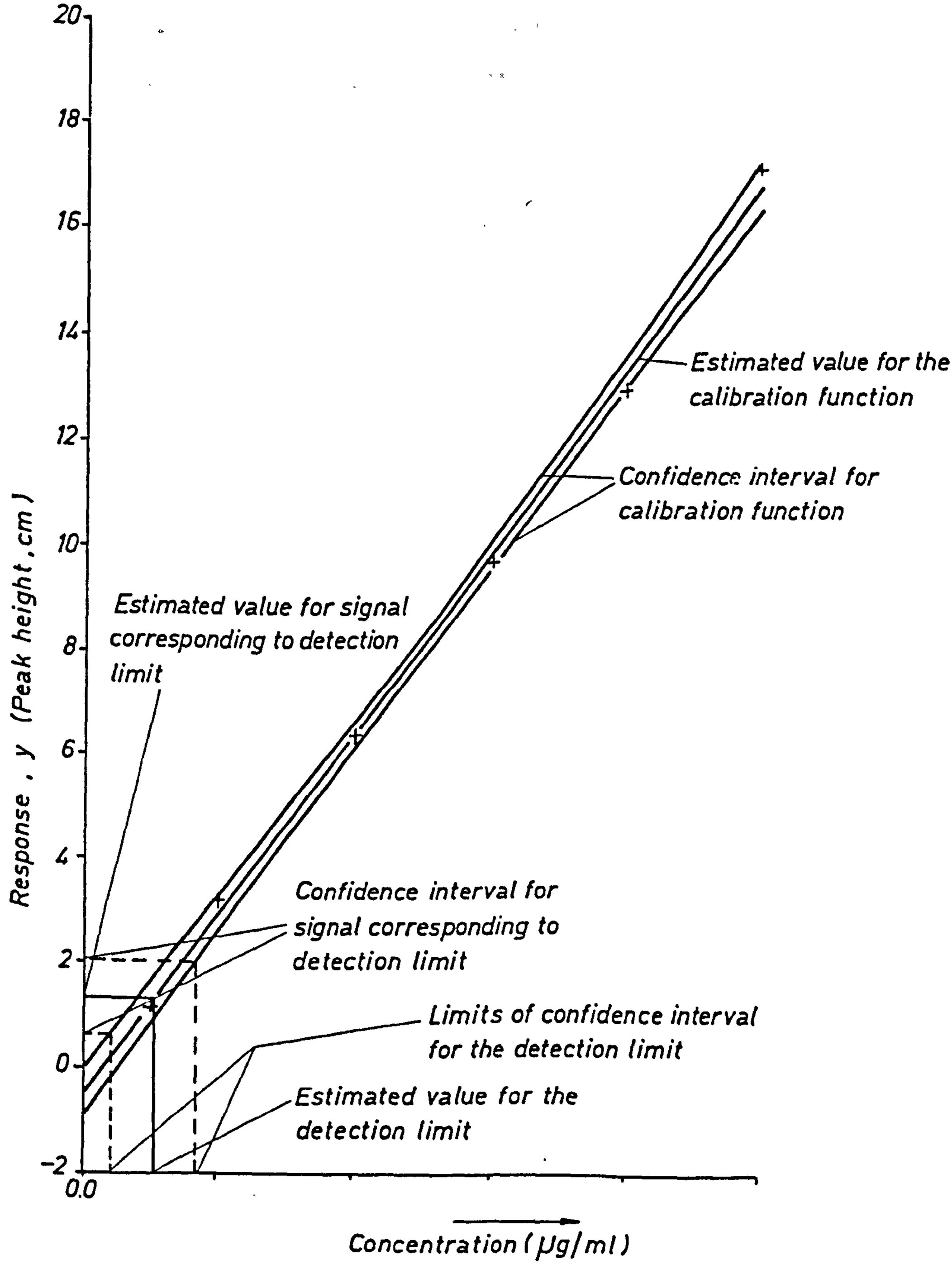
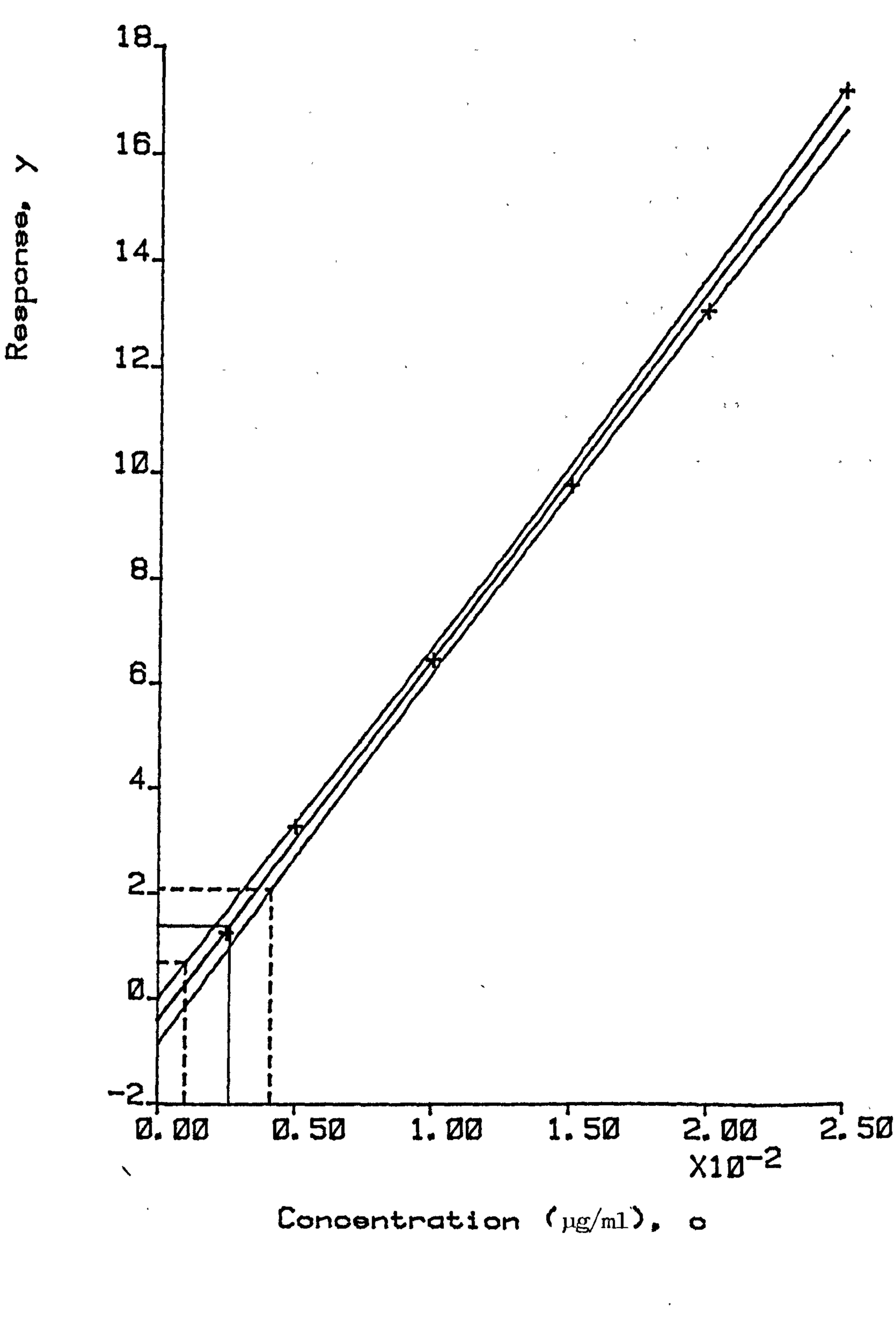


FIGURE 3.23 DETECTION LIMIT FOR 4-Br-6-CF3-PIAZSELENOL

y = -0.398 + (.687E 3) * conc

95.0% Confidence Limits

Detection Limit = 0.00261 µg/ml



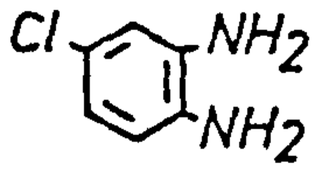
3.3 DISCUSSION

i) Mass Spectroscopy

The mass spectra of a series of the diamine derivatives (ligands) are shown in Figures 3.1a to 3.11a inclusive and illustrate the presence of the molecular ion of the ligand and the fragmentation pattern observed under the influence of 70 eV. The mass spectra of the corresponding selenium complexes (piazselenols) are similarly shown in Figures 3.1b to 3.11b inclusive.

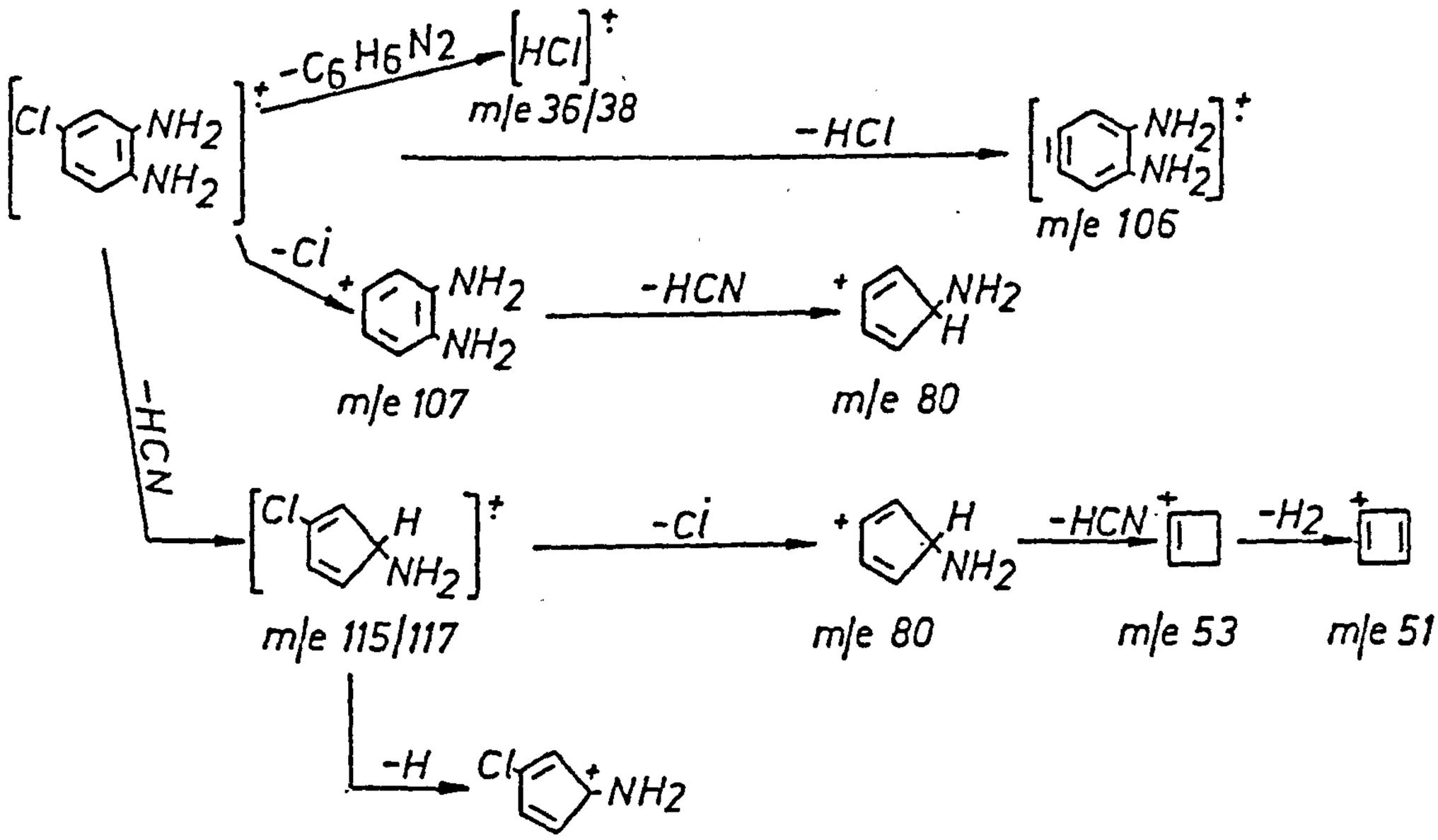
However, to illustrate the form of the fragmentation pattern observed, four selected examples of ligands and the analogous selenium complexes are examined in further detail. The examples chosen were a, c, e and g, the chloro, nitro, dibromo and bromo plus trifluoromethyl as substituent groups on the basic ligand while b, d, f and h are the corresponding selenium complexes. Generally, all the mass spectra recorded confirmed the molecular weight of the diamine or complex with a fairly normal fragmentation pattern through well established mechanisms and resultant ions.

a) 4-Chloro-1,2-diaminobenzene (14)



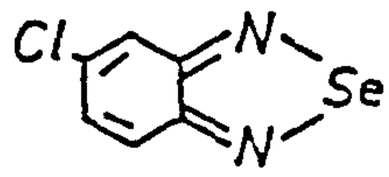
Molecular weight: 142/144

Figure 3.2a illustrates the molecular weight of the diamine as 142, 144 and the fragmentation pattern as follows:-



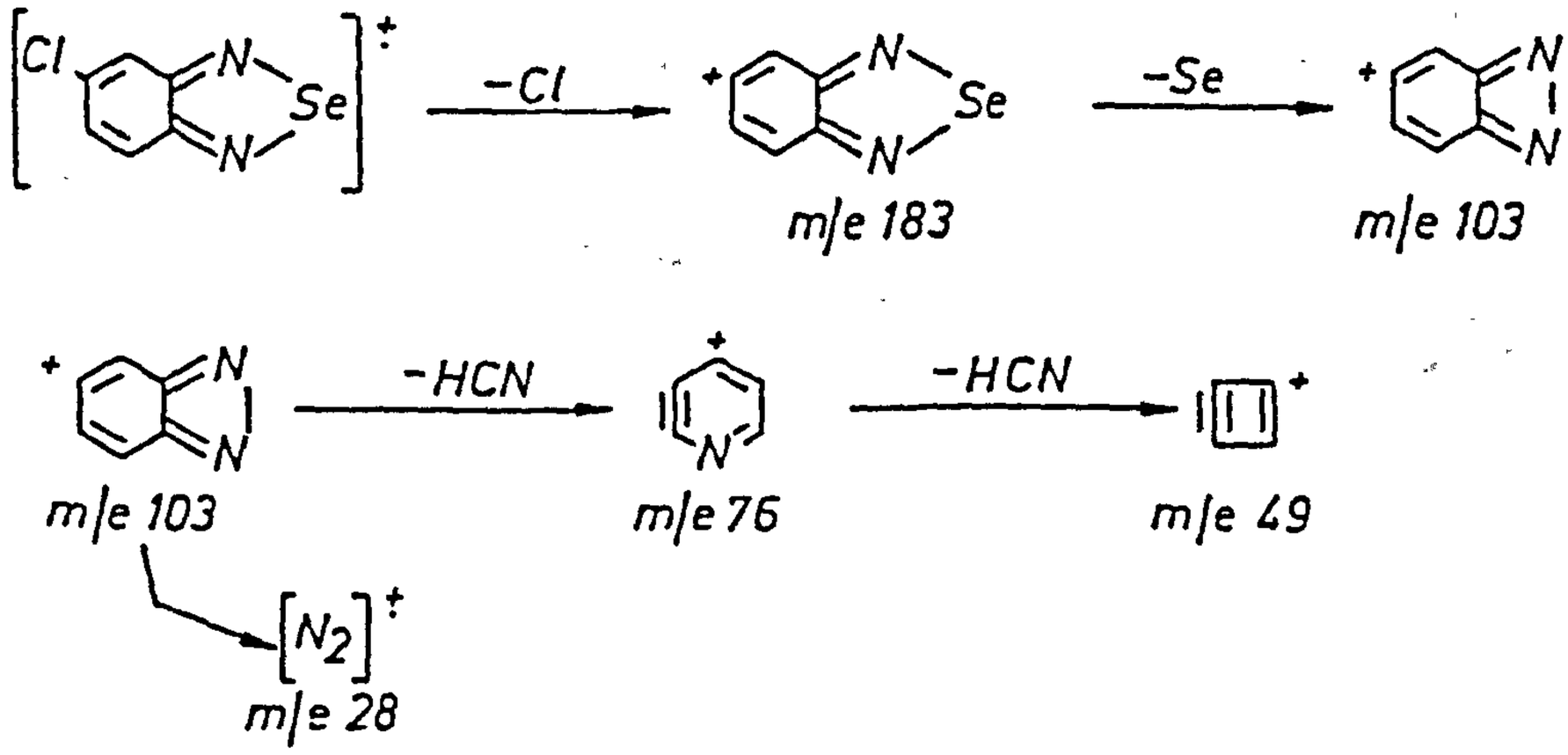
where: m/e 36/38 = 36 and 38

b) 5-Chloropiazselenol (14)

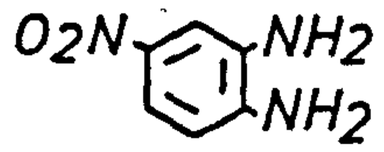


Molecular weight: 218/220

Figure 3.2b illustrates the molecular weight of 5-Cl-piazselenol and the fragmentation pattern as follows:-

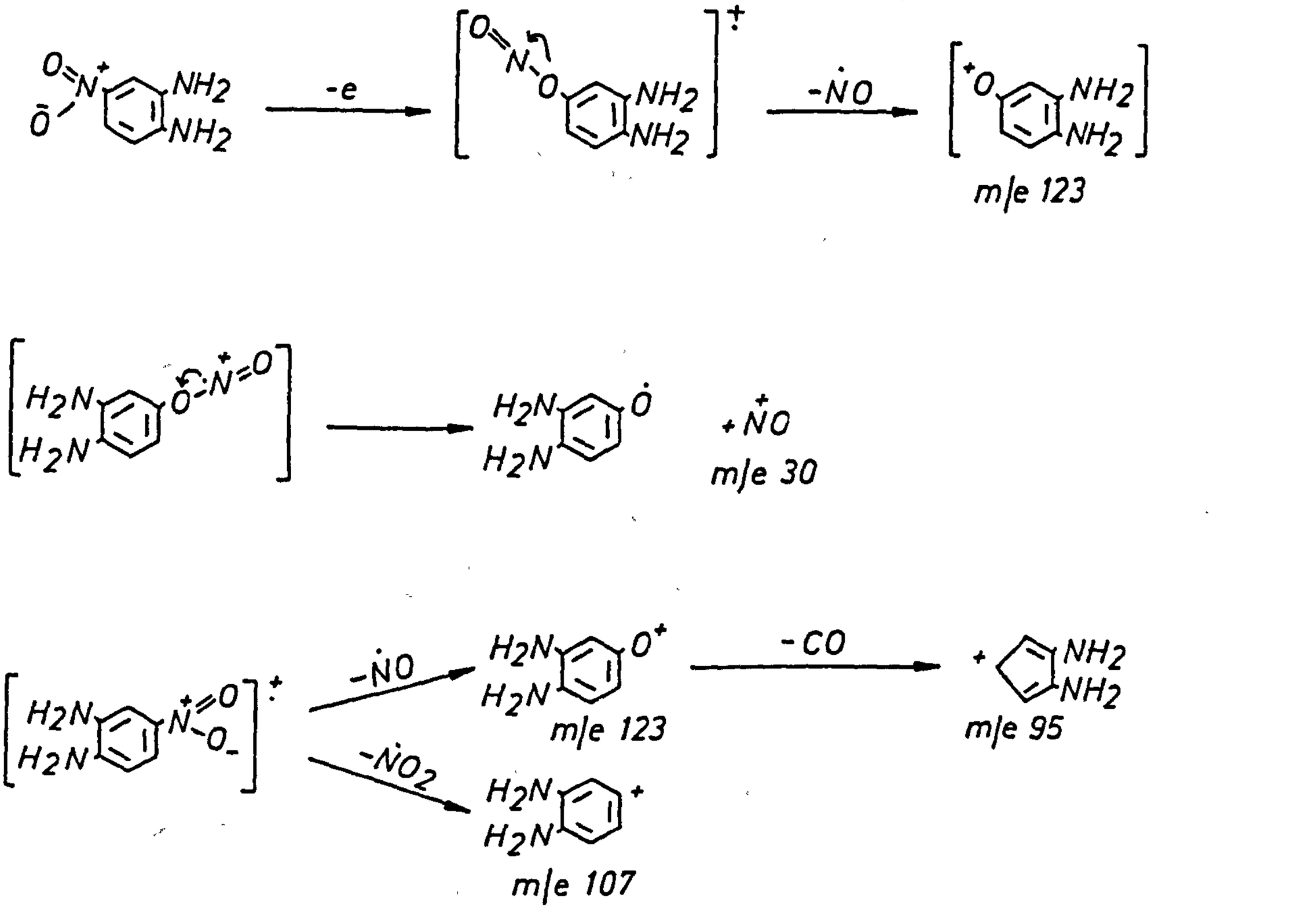


c) 4-Nitro-1,2-diaminobenzene (14)

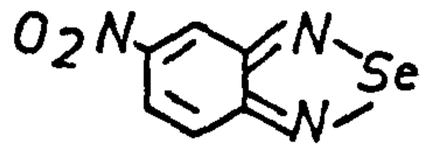


Molecular weight: 153

Figure 3.3a illustrates the molecular weight of the diamine and fragmentation pattern as follows:-

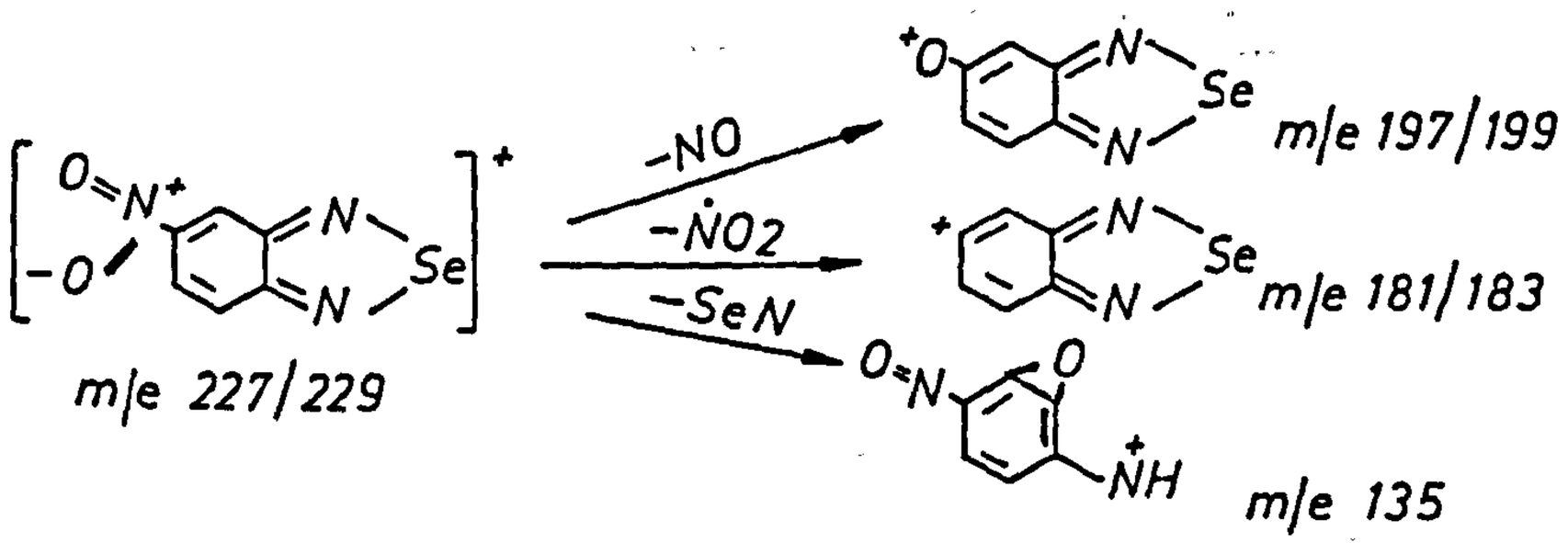


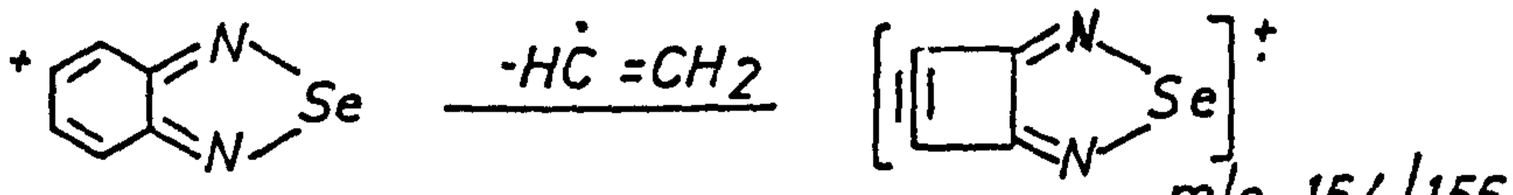
d) 5-Nitropiazselenol (14)

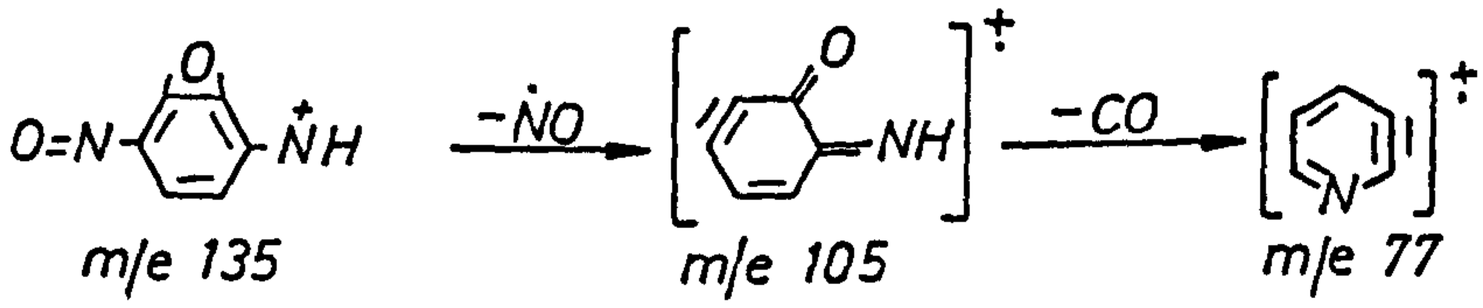


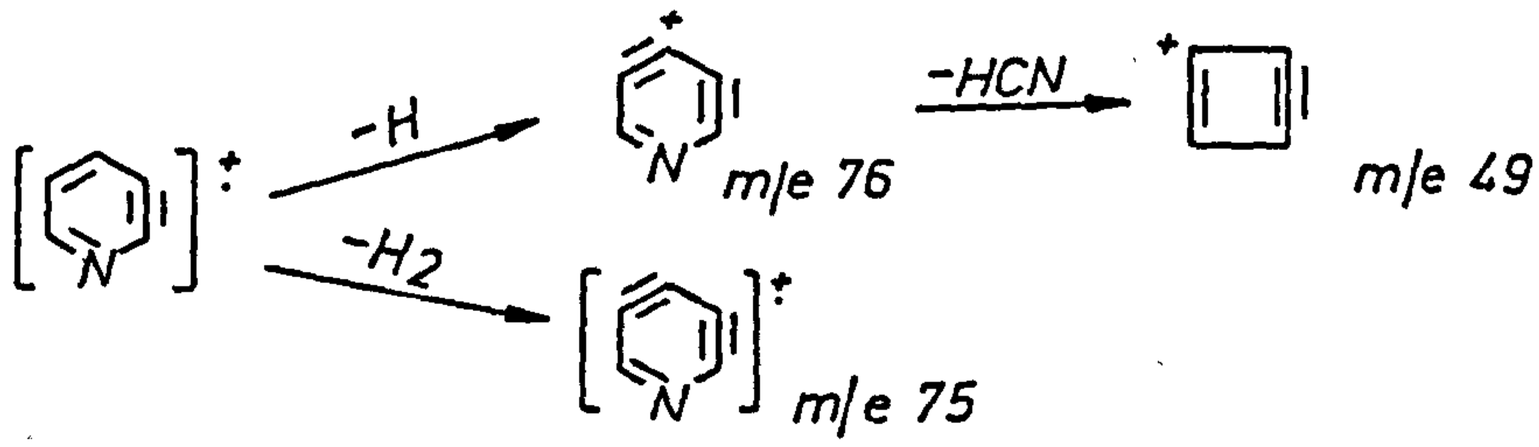
Molecular weight: 229

Figure 3.3b illustrates the molecular weight and fragmentation pattern as follows:-

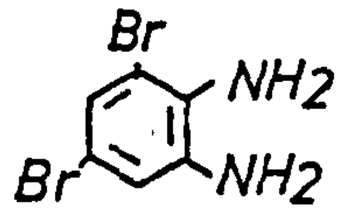




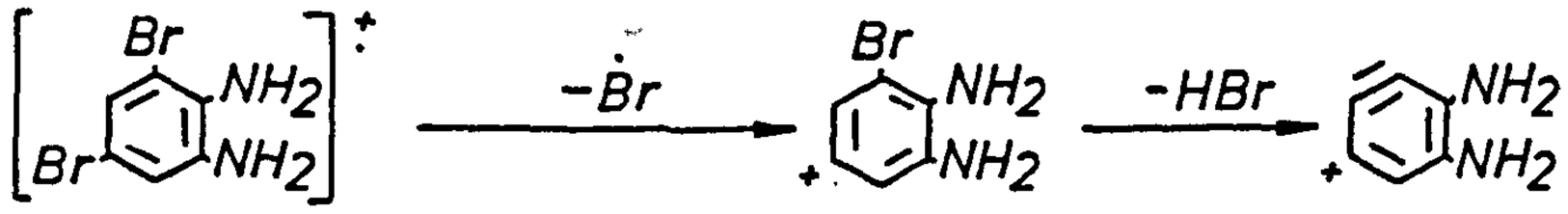


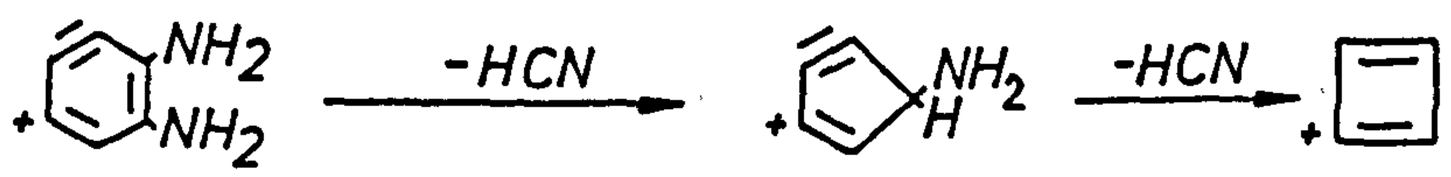


e) 3,5-Dibromo-1,2-diaminobenzene

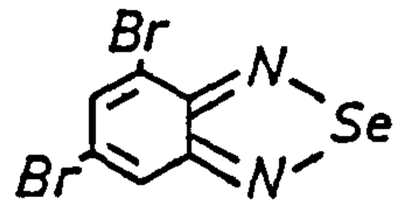


Molecular weight: 264/268



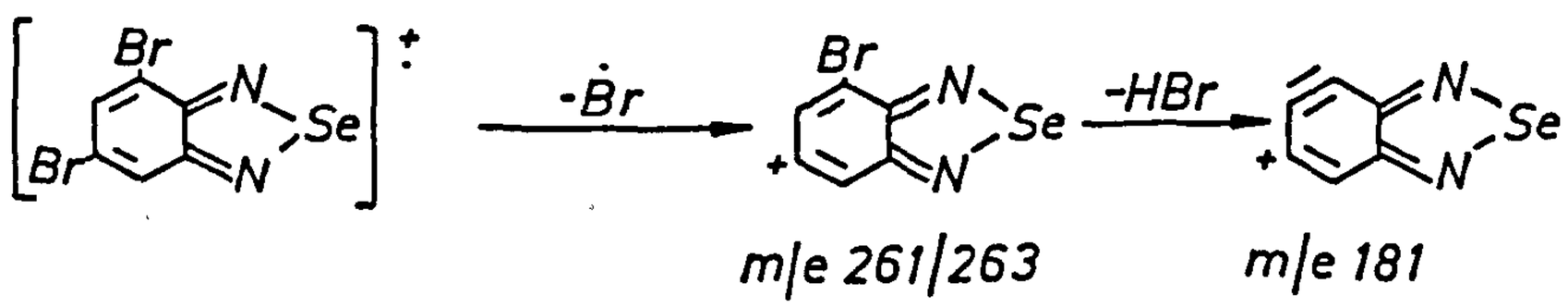


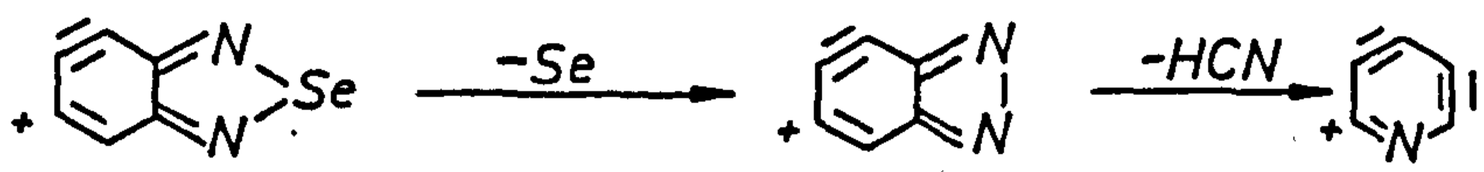
f) 4,6-Dibromopiazselenol



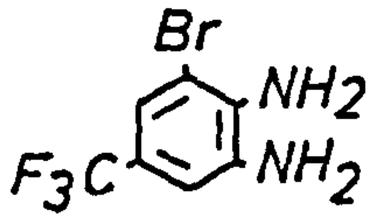
Molecular weight: 340/344

Figure 3.6b illustrates the molecular weight of the 4,6-dibromopiazselenol and the fragmentation pattern as follows:-



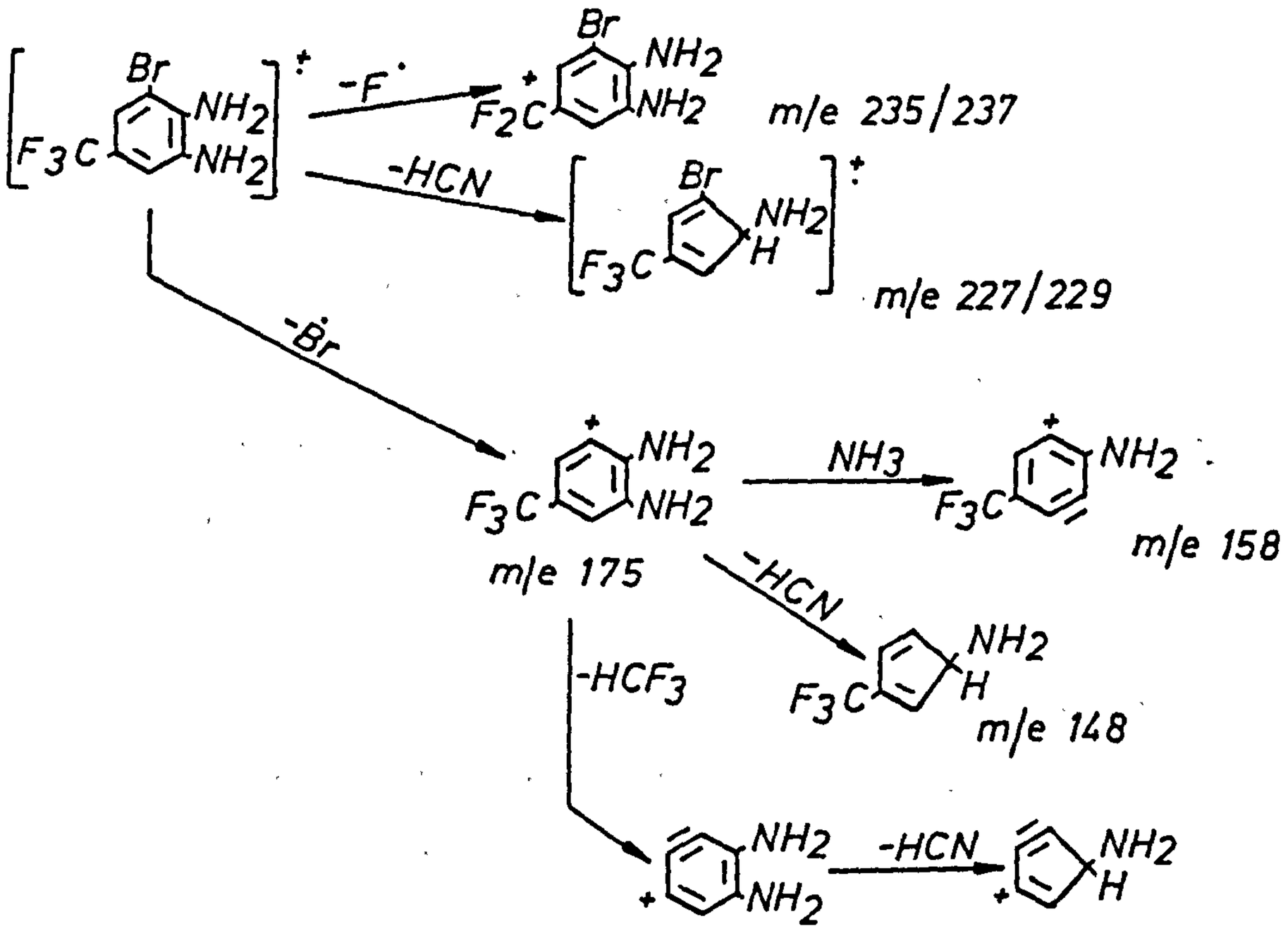


g) 3-Bromo-5-trifluoromethyl-1,2-diaminobenzene

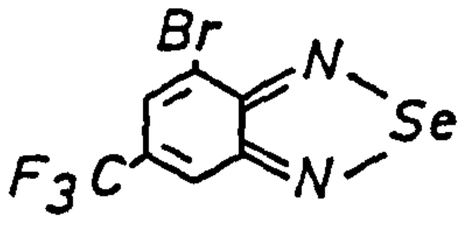


Molecular Weight: 254/256

Figure 3.8a illustrates the molecular weight of the diamine and the fragmentation pattern as follows:-

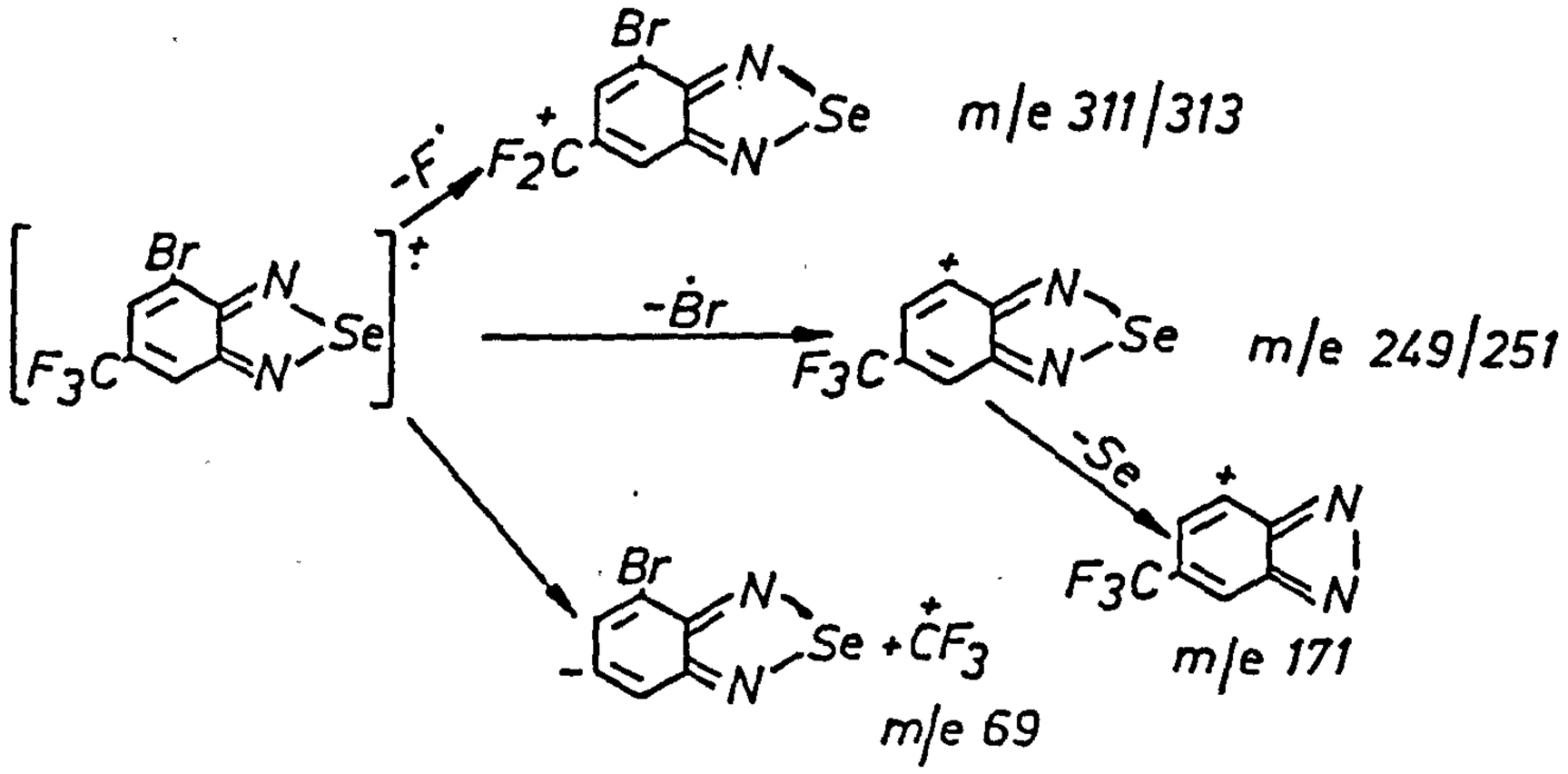


h) 4-Bromo-6-trifluoromethylpiazselenol



Molecular Weight: 330/332

Figure 3.8b illustrates the molecular weight of piazselenol and the fragmentation pattern as follows:-



(ii) "Clean-up" Methods

In the present work, only two methods of "clean-up" or complex purification were examined. Both of them involved using separate aliquots (3 ml volume) to wash the toluene layer, a total of three times, with mineral acids.

Figure 3.24 shows the results where the procedure adopted involved 5-chloro-6-fluoropiazselenol when perchloric acid or hydrochloric acid was used. Figure 3.24A shows the formation of the complex (containing 0.2 ng Se) direct from solution, while Figure 3.24B depicts the result when using perchloric acid as washing agent. Notice that some degradation of the complex occurs, especially when compared to the peak height response obtained from the injection of a pure sample of the piazselenol (by weighing) of 0.2 ng of the purified complex. The effect is even more noticeable when hydrochloric acid was used instead of perchloric acid, see Figure 3.24C. Clearly the latter result is unacceptable and was not used as a clean-up method, both in terms of extra peaks created and loss of selenium response.

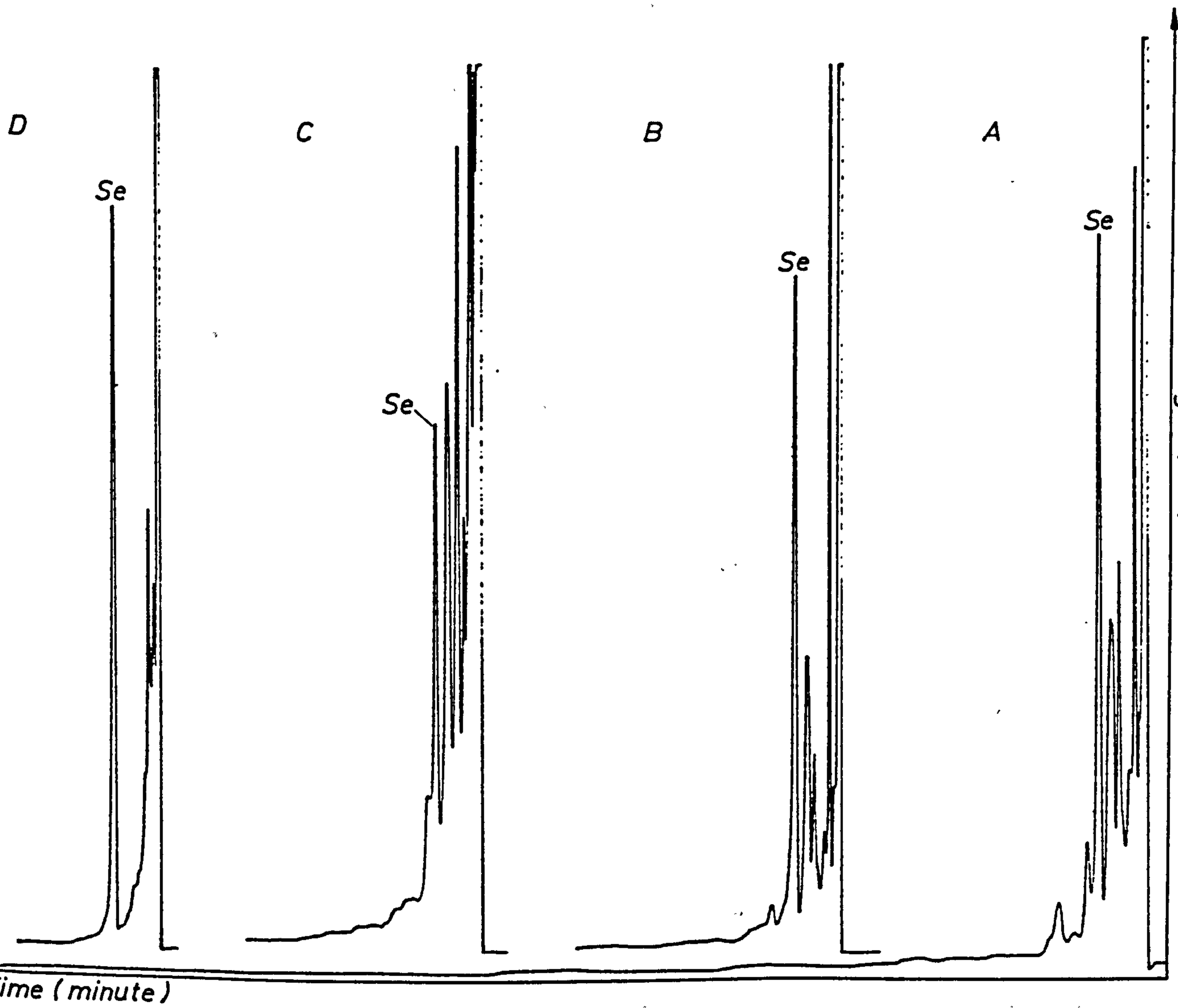
Another example is shown in Figure 3.25, which depicts the chromatograms obtained following the "clean-up" of the 4-bromo-6-fluoro-piazselenol after formation of the complex. Without clean-up, Figure 3.25A, although the peak due to the Se complex is fairly well separated, it is surrounded by much larger peaks with greater and lesser retention times. Figure 3.25B shows the resultant blank where some of these extra peaks are missing, following clean-up by washing with perchloric acid. The later eluting peaks seen in Figure 3.25A were absent in Figures 3.25C and D. (The region of the chromatogram is not shown in order to save space.) Figure 3.25C shows a similar effect for the complex, but again some degradation is observed (compare the response

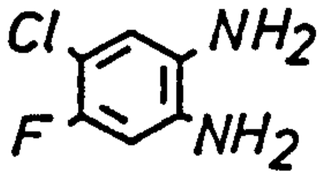
in Figure 3.25E for the same weight of complex). The same purification procedure but using HCl is better than for the 5-chloro-6-fluoropiazselenol, but several major peaks are still associated with the solvent peak.

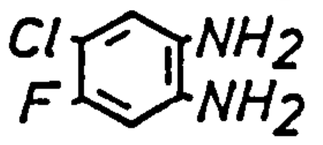
An extreme case is given for the 4-bromo-6-trifluoromethyl complex, where its signal is completely swamped when using injection directly from the solution. For example, compare Figure 3.26A with Figure 3.26D which is the peak produced by 0.025 ng/Se complex. However, by washing with perchloric acid, a relatively clean trace is obtained. Although little degradation of the complex is seen when using HCl as a washing agent, there are a greater number of interfering peaks still remaining.

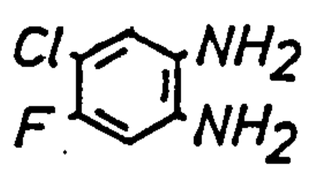
Purification using perchloric acid is clearly more efficient than with hydrochloric acid and in spite of the greater danger was used throughout the remainder of the work.

FIGURE 3.24 EC D RESPONSES SHOWING PEAKS IN CHROMATOGRAMS OF 5 - CHLORO - 6 - FLUORO PIAZSELENOL EXTRACTS AND CLEAN - UP



A = 0.2ng Se(IV) +  without clean - up

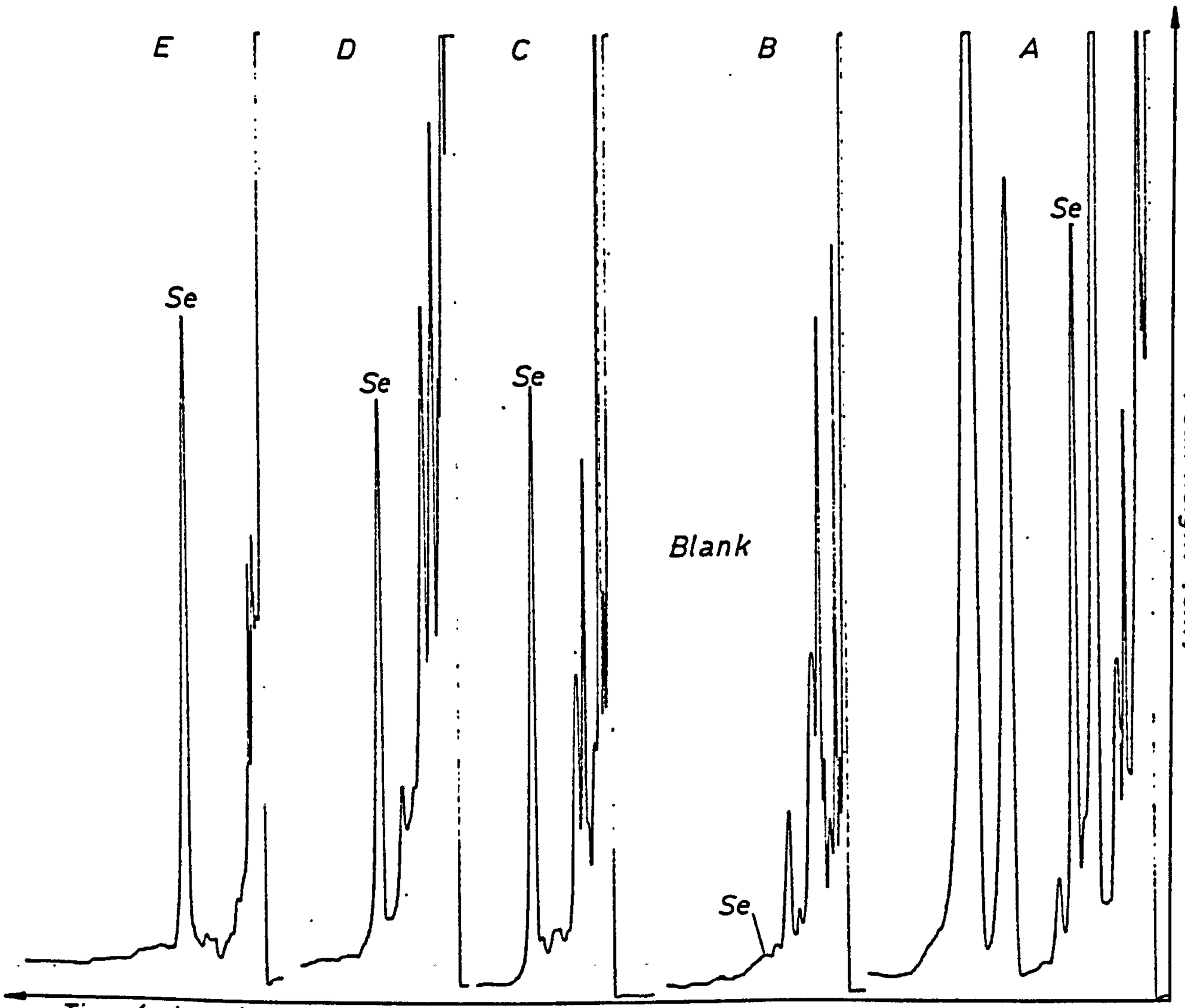
B = 0.2 ng Se (IV) +  + washing three times with $HC10_4$ (6M)*

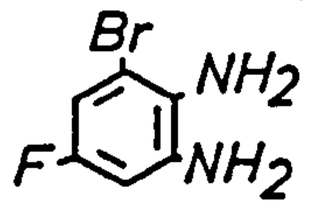
C = 0.2ng Se (IV) +  + washing three times with HC1 (9M)

D = 0.2ng Se as standard 5 - Chloro - 6 - Fluoro piazselenol (weighed)

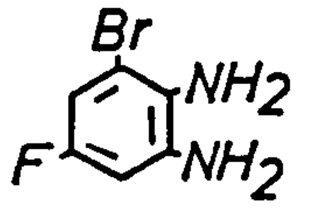
* 6M Perchloric acid = (1 : 1) volume with water

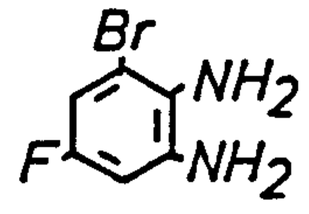
FIGURE 3.25 ECD RESPONSES SHOWING PEAKS IN CHROMATOGRAMS OF 4-BROMO-6-FLUORO PIAZSELONOL EXTRACTS AND CLEAN-UP



A = 0.100 ng Se (IV) +  without clean-up

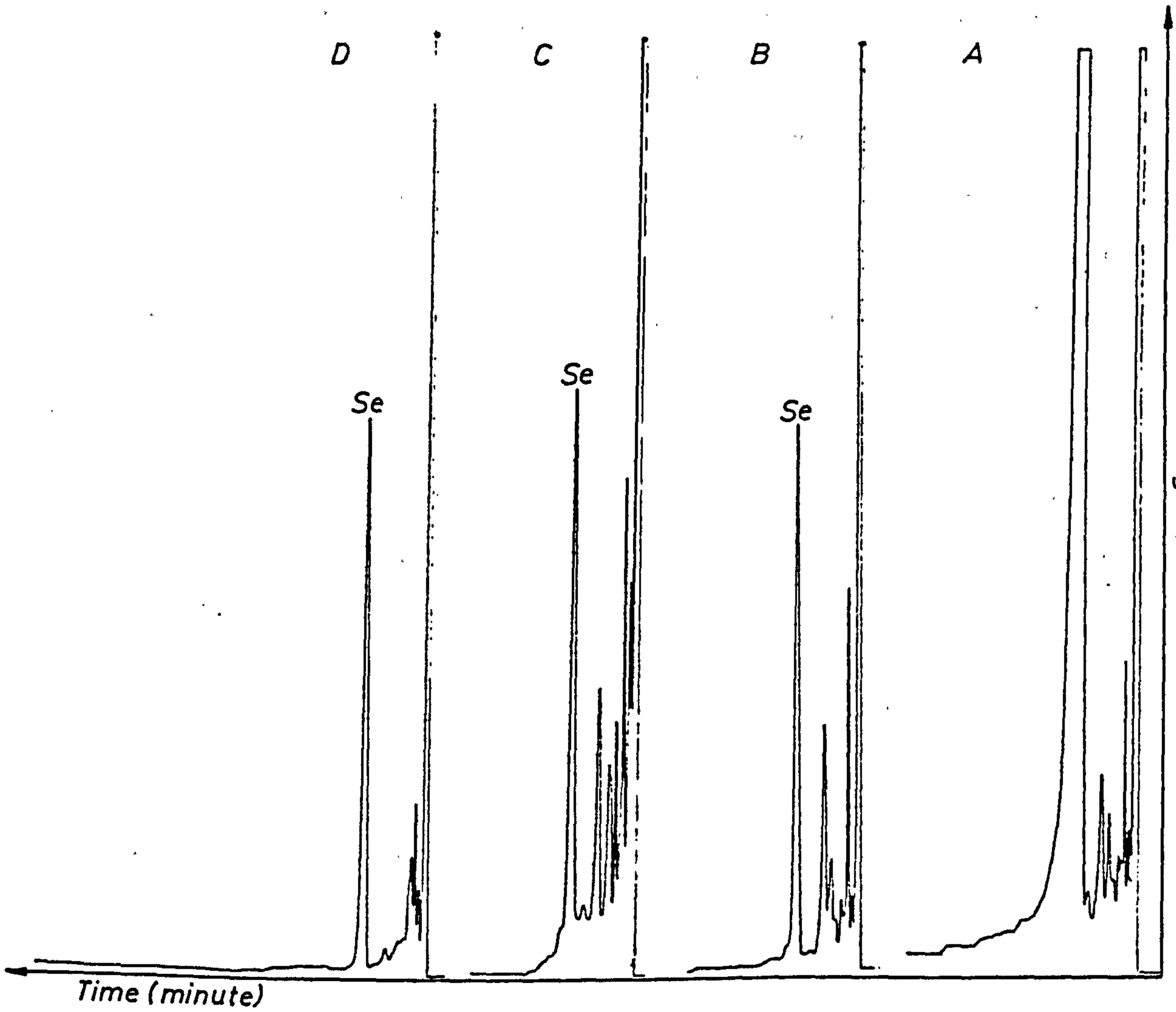
B = Blank washed with three times $HClO_4$ (1:1)

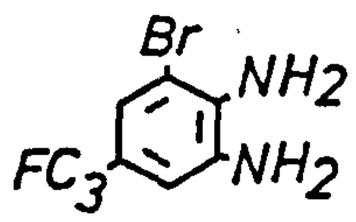
C = 0.100 ng Se (IV) +  washed three times with $HClO_4$ (6M)

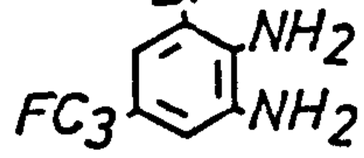
D = 0.100 ng Se (IV) +  washed three times with HCl (9M)

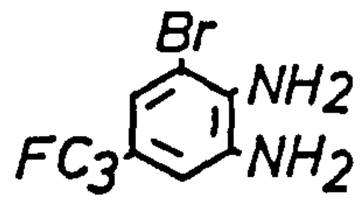
E = 0.100 ng Se as piazselenol standard (weighed)

FIGURE 3.26 ECD RESPONSES SHOWING PEAKS IN CHROMATOGRAMS OF 4-BROMO-6-TRIFLUOROMETHYLPIAZSELENOL AND CLEAN-UP



A = 0.025 ng Se (IV) +  without clean-up

B = 0.025 ng Se (IV) +  + washing three times with $HC1O_4$ (6M)

C = 0.025 ng Se (IV) +  + washing three times with HC1 (9M)

D = 0.025 ng Se as piazselenol standard (weighed)

iii) Gas Chromatographic Properties of Piazselenol

a) The stationary phase

Elution of the piazselenols was examined only on the moderately polar stationary phase OV-17, which is composed of the poly-phenyl methyl silicone; 50% phenyl, and has an upper temperature limit of 300°C (25). However, OV-17 is a stationary phase in very common usage and gives reasonably short retention times for these compounds (piazselenols) and so coupled with a relatively high upper temperature limit offers the advantage that any column could be used for many months continuous work.

Table 3.4 lists the retention times for 12 piazselenols and eight of them have retention times of less than four minutes, even though these retention times are measured at an isothermal column temperature of 200°C.

b) The relative sensitivity of piazselenols

Relative sensitivities for the twelve piazselenols were determined under the same experimental conditions, and carried out by two methods:

a) at an isothermal column temperature of 200°C,

b) at a constant retention time (of five minutes).

Table 3.5 lists the relative sensitivities when determined under the two methods and lists the retention time recorded at a column temperature of 200°C. For the mono-substituted and dibromopiaszelenol (six piazselenols), the most sensitive was the dibromo complex using both methods of sensitivities. Figure 3.27(a) illustrates the calibration curves for the six piazselenols and confirmed the sensitivities recorded in the literature as shown in Table 3.5 (drawn point by point, for comparison of sensitivities, not used for calibration purposes).

However Table 3.5 and Figure 3.27b illustrate that the disubstituted fluorinated piazselenols are more sensitive by both two methods. Another advantage of these new piazselenols is their shorter retention times which are all less than four minutes compared to the eleven minutes for the dibromopiazselenol.

iv) Detection Limit of Selenium by GLC + ECD

The suggested detection limits obtained using both the Working-Hotelling confidence band concept and the Liteanu and Rica systems were very close in value. Thus, if 4-bromo-6-trifluoromethyl-piazselenol is again taken as an example, the respective values were:

Detection limit from Working-Hotelling = 0.0025 µg/ml

Detection limit from Liteanu and Rica = 0.0026 µg/ml

Similarly the boundaries for the signal response y (in cm) were:

a) Upper limit = 2.2 Liteanu and Rica

Upper limit = 2.0 Working-Hotelling

b) Lower limit = 0.70 Liteanu and Rica

Lower limit = 0.60 Working-Hotelling

For this particular ligand an alternative method proposed by Bailey et al. (23) called the concept of the lower limit of detection (X_{LD}) but also based on concentrations produced from a calibration graph suggested that the minimum peak height (in cm) suitable for determination purposes was 6.59 cm. Clearly such a value which occupies approximately 30% of the full scale deflection on a recorder (20 cm FSD) available to an experimenter as an estimate of the "limit of detection" is far too large and was not pursued further. The reason

FIGURE 3.27(a) CALIBRATION CURVES FOR PIAZSELENOLS

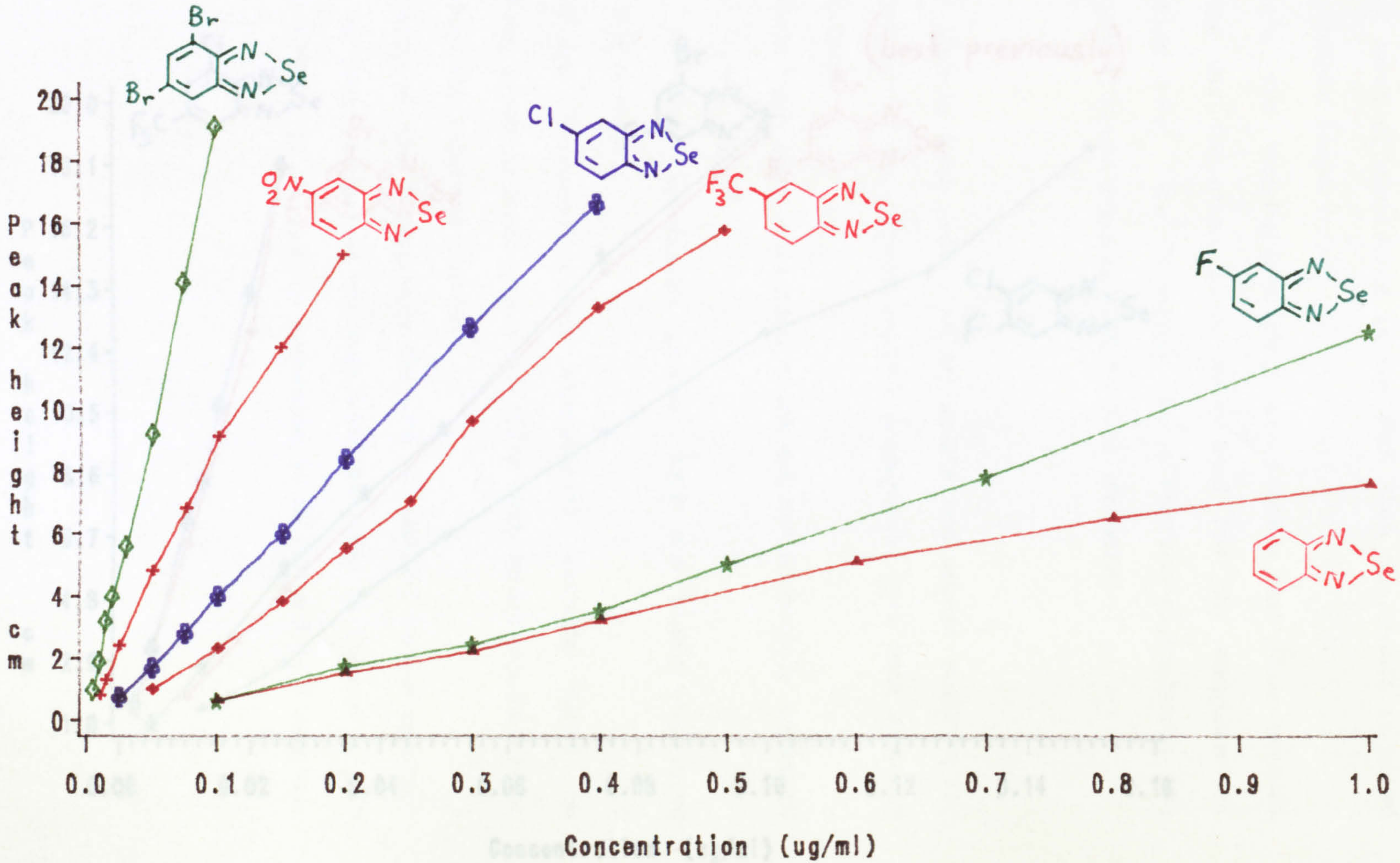
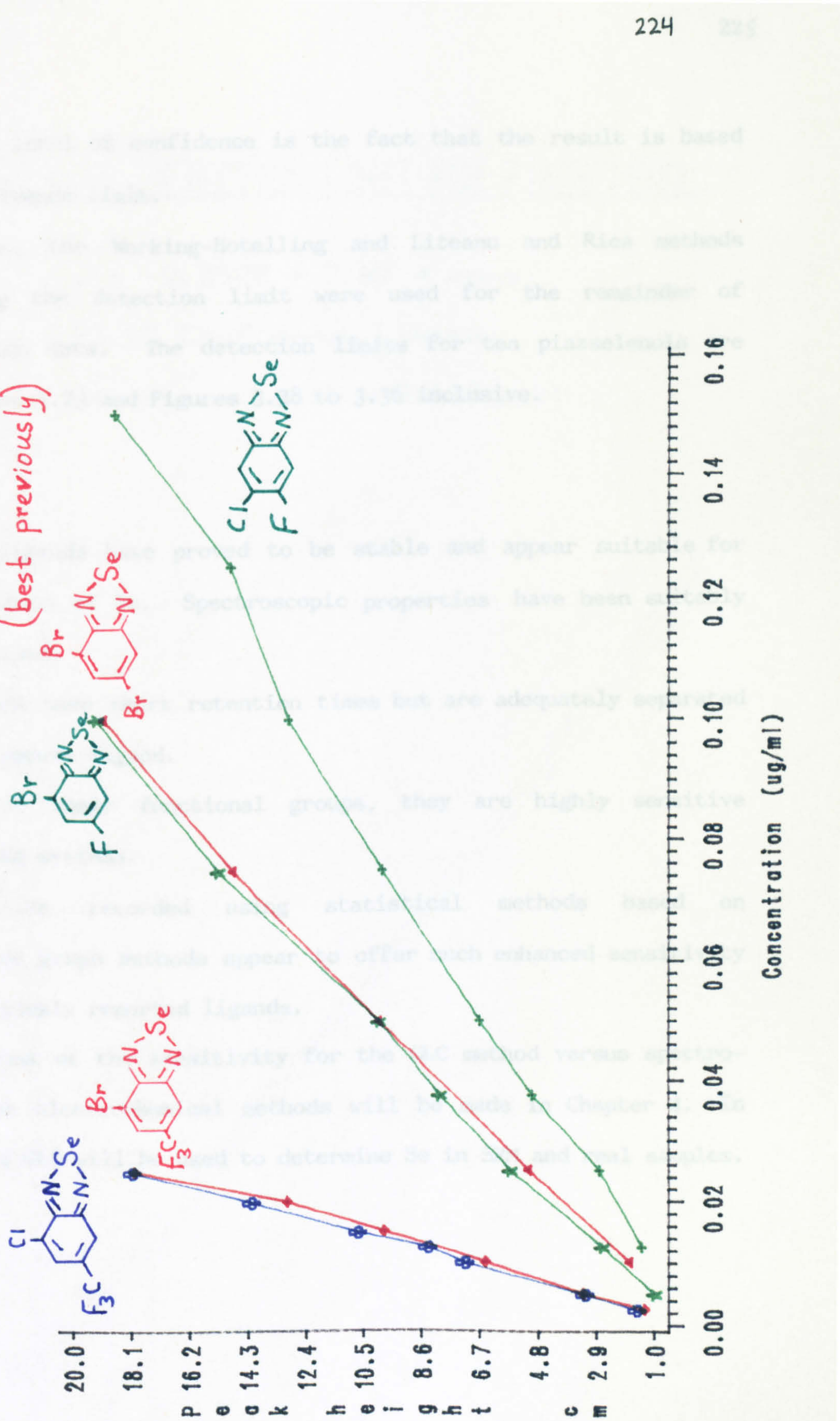


FIGURE 3.27 (b) CALIBRATION CURVES FOR DIBROMO- & NEW PIAZSELENOLS



for the high level of confidence is the fact that the result is based on a 99% confidence limit.

Therefore, the Working-Hotelling and Liteanu and Rica methods of estimating the detection limit were used for the remainder of the calibration data. The detection limits for ten piazselenols are shown in Figure 3.23 and Figures 3.28 to 3.36 inclusive.

Summary

1. The new ligands have proved to be stable and appear suitable for GLC detection of Se. Spectroscopic properties have been suitably characterised.
2. The ligands have short retention times but are adequately separated from the parent ligand.
3. Because of their functional groups, they are highly sensitive towards ECD systems.
4. Sensitivities recorded using statistical methods based on calibration graph methods appear to offer much enhanced sensitivity over previously reported ligands.
5. Comparisons of the sensitivity for the GLC method versus spectroscopic or electrochemical methods will be made in Chapter 4. In Chapter 4 GLC will be used to determine Se in SRM and real samples.

FIGURE 3.28 DETECTION LIMIT FOR PIAZSELENOL

y = -0.0520 + 7.97 * conc

95.0% Confidence Limits

Detection Limit = 0.116 µg/ml

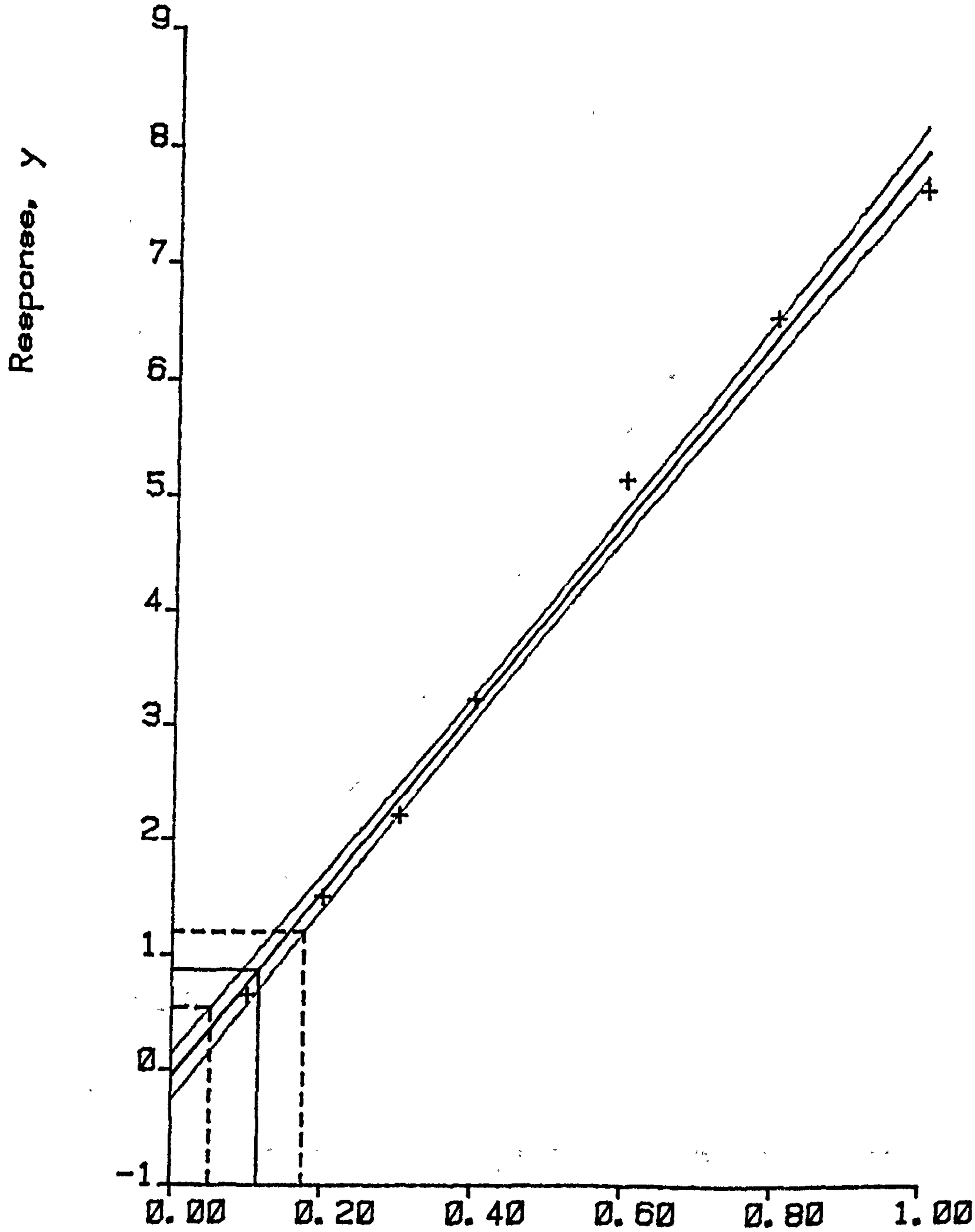


FIGURE 3.29 DETECTION LIMIT FOR 5-CHLOROPIAZSELENOL

y = -0.369 + 43.0 * conc

95.0% Confidence Limits

Detection Limit = 0.0190 µg/ml

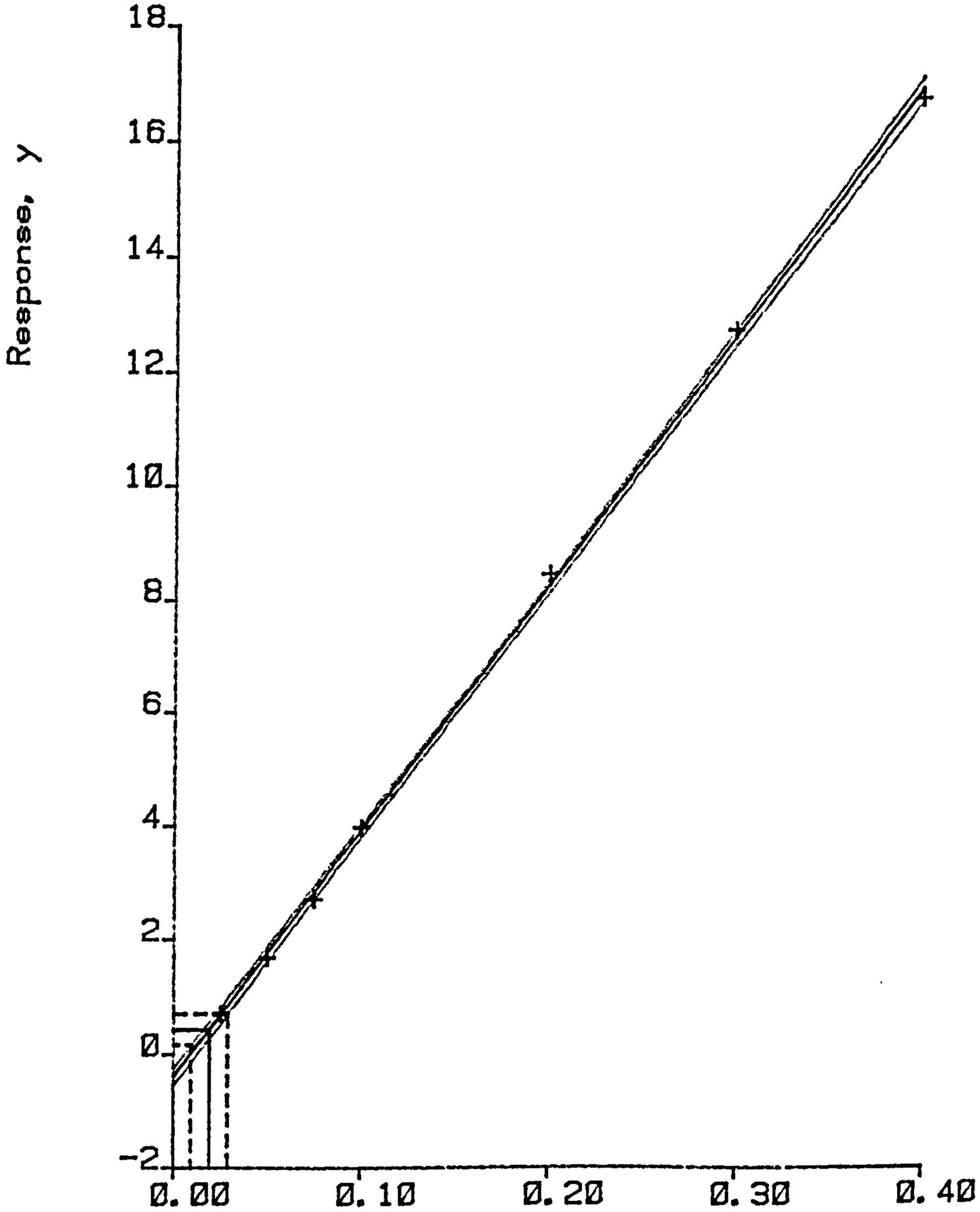


FIGURE 3.30 DETECTION LIMIT FOR 5-NITROPIAZSELENOL

y = 0.613 + 74.0 * conc

95.0% Confidence Limits

Detection Limit = 0.0239 µg/ml

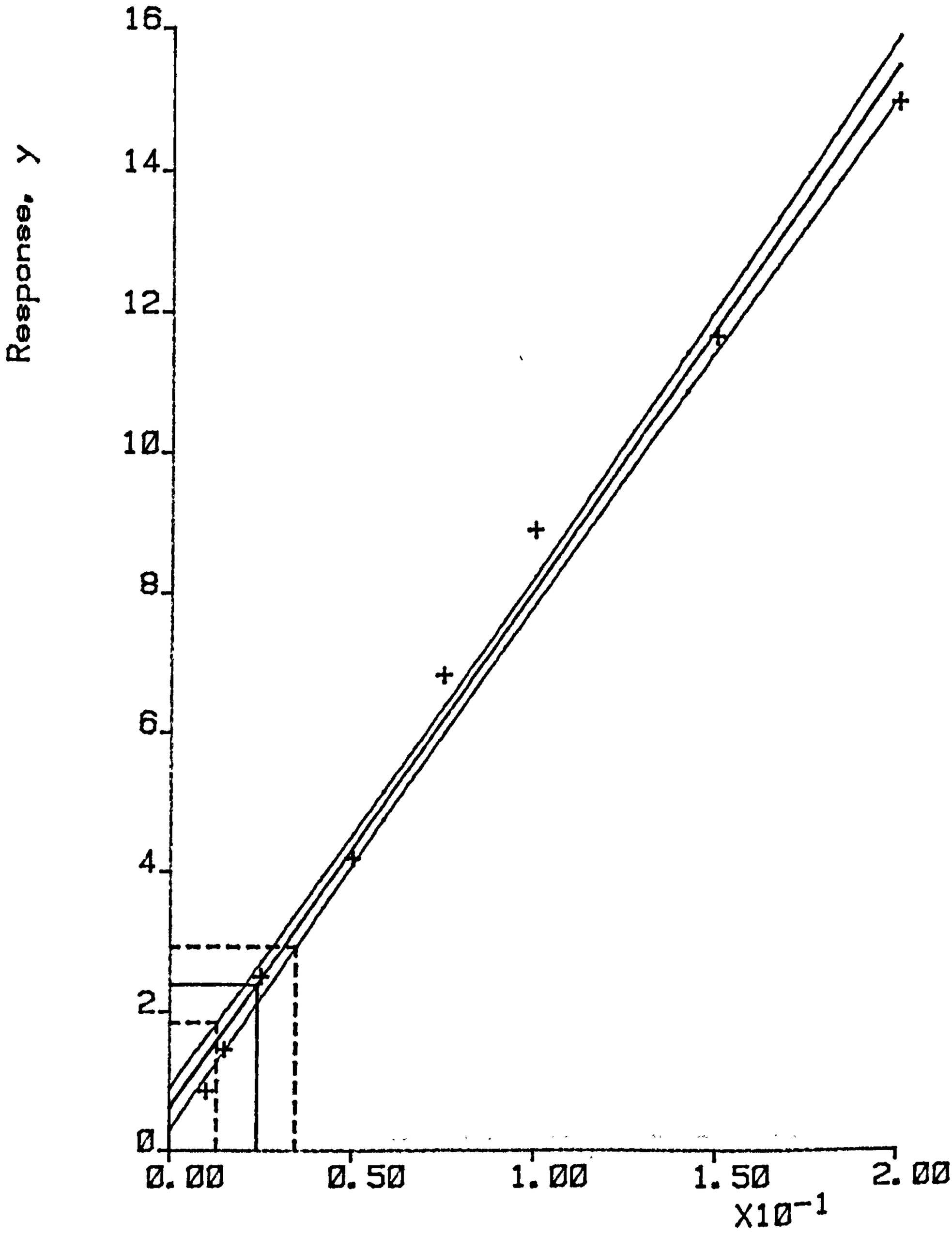


FIGURE 3.31 DETECTION LIMIT FOR 5-FLUOROPIAZSELENOL

y = -1.46 + 13.6 * conc

95.0% Confidence Limits

Detection Limit = 0.130 µg/ml

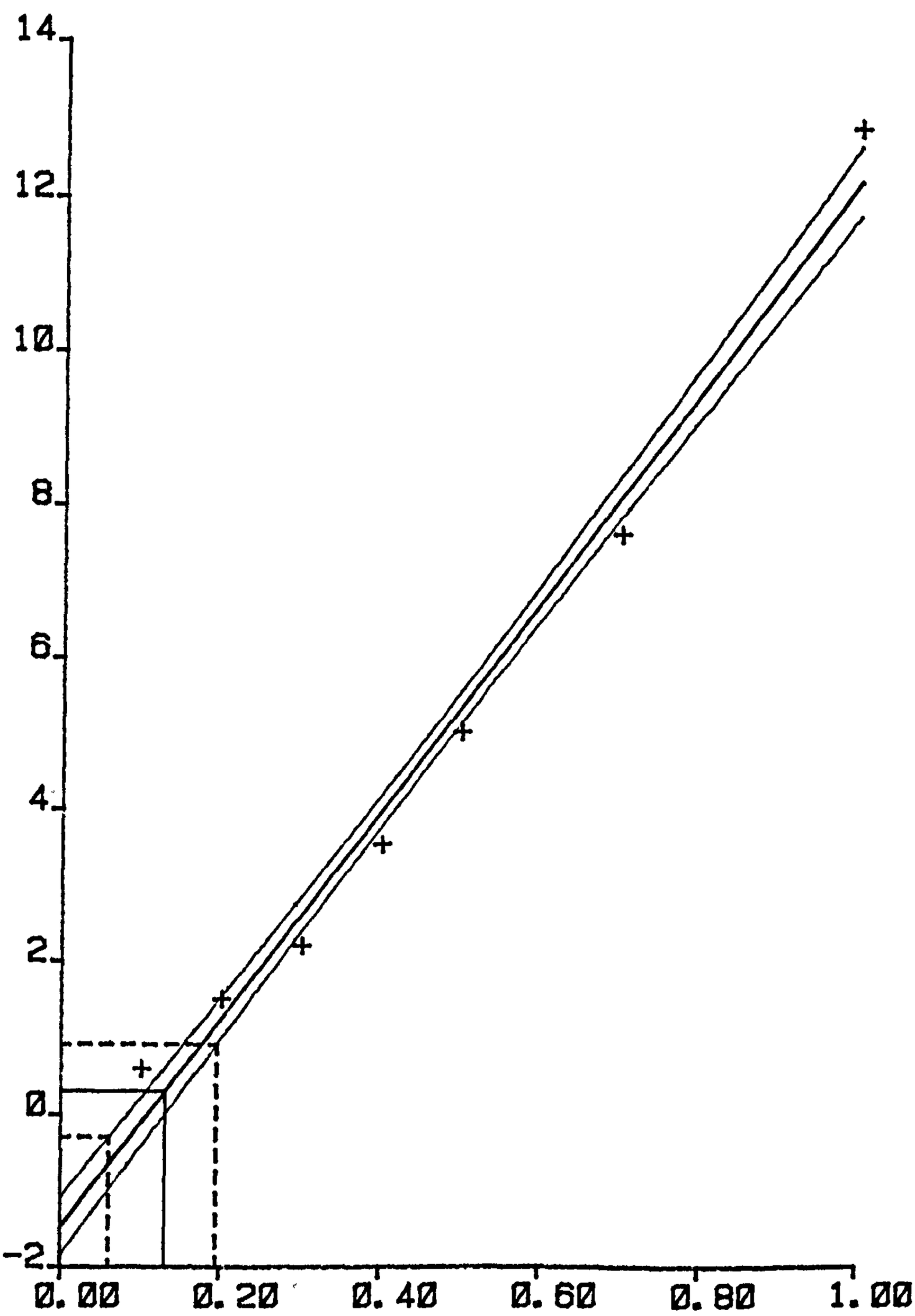


FIGURE 3.32 DETECTION LIMIT FOR 5-CF3-PIAZSELENOL

y = -1.34 + 35.8 * conc

95.0% Confidence Limits

Detection Limit = 0.0419 µg/ml

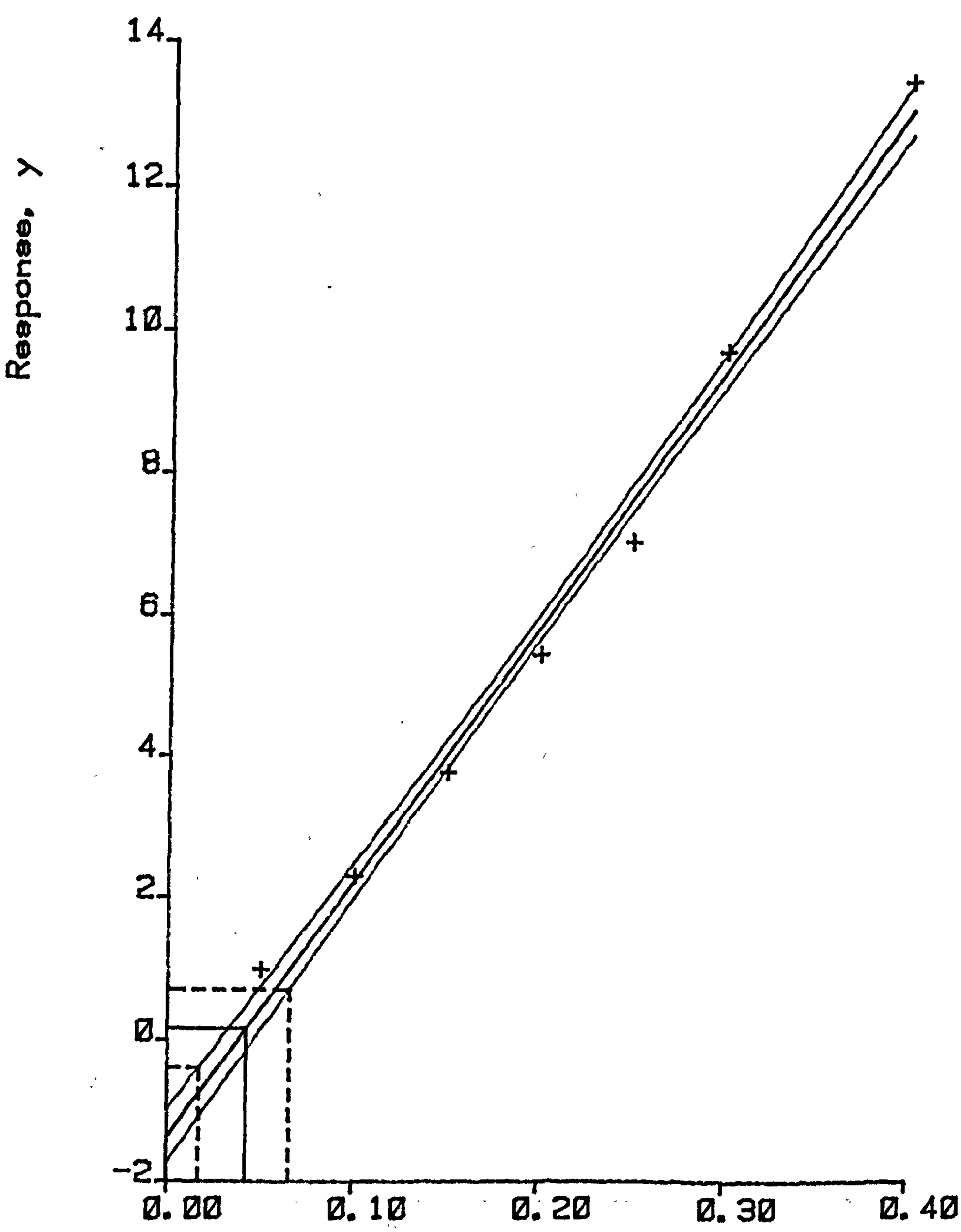


FIGURE 3.33 DETECTION LIMIT FOR 4,6-DIBROMOPIAZSELENOL

y = 0.149 + (.186E 3) * cono

95.0% Confidence Limits

Detection Limit = 0.00593 µg/ml

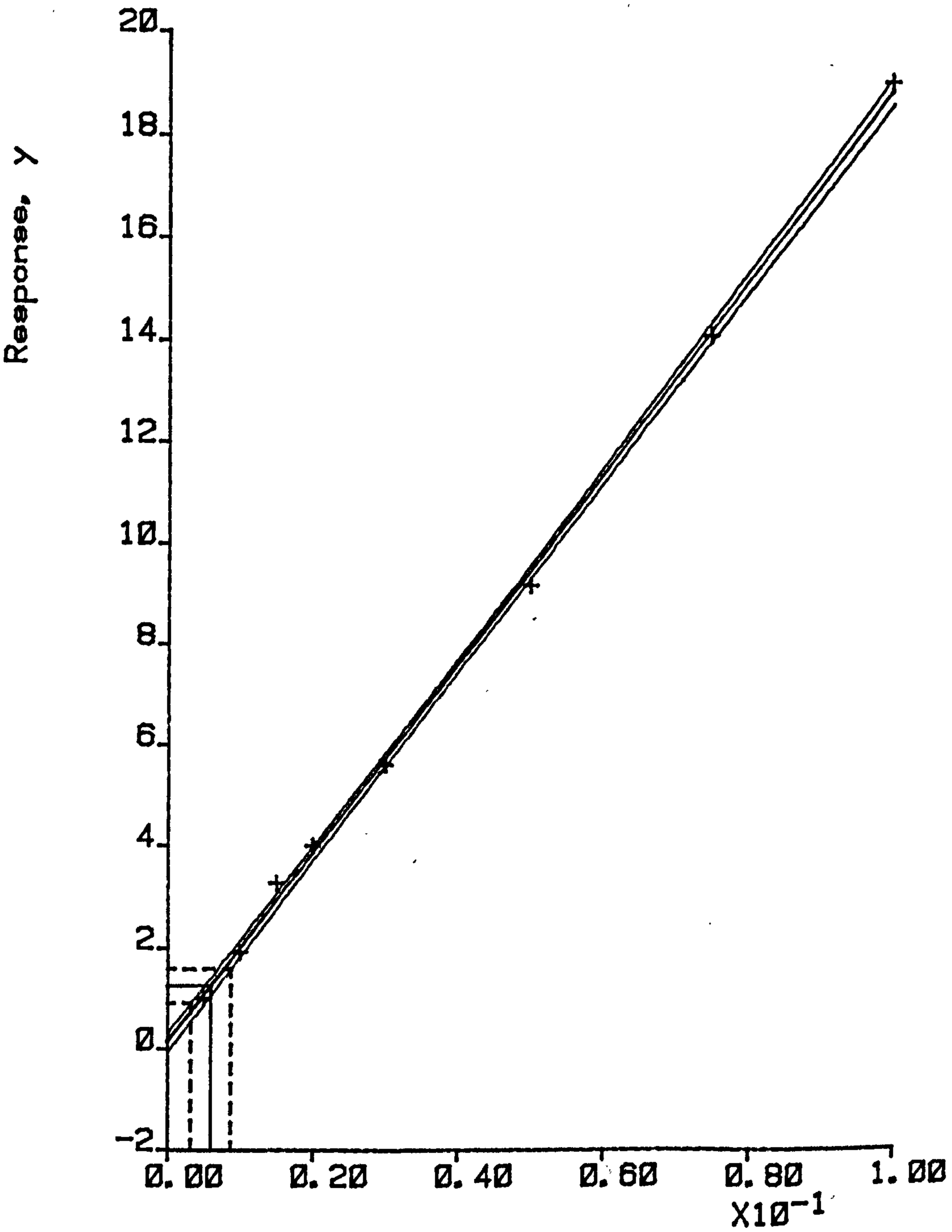


FIGURE 3.34 DETECTION LIMIT FOR 4-Br-F-PIAZSELENOL

y = 0.392 + (.190E 3) * conc

95.0% Confidence Limits

Detection Limit = 0.00770 µg/ml

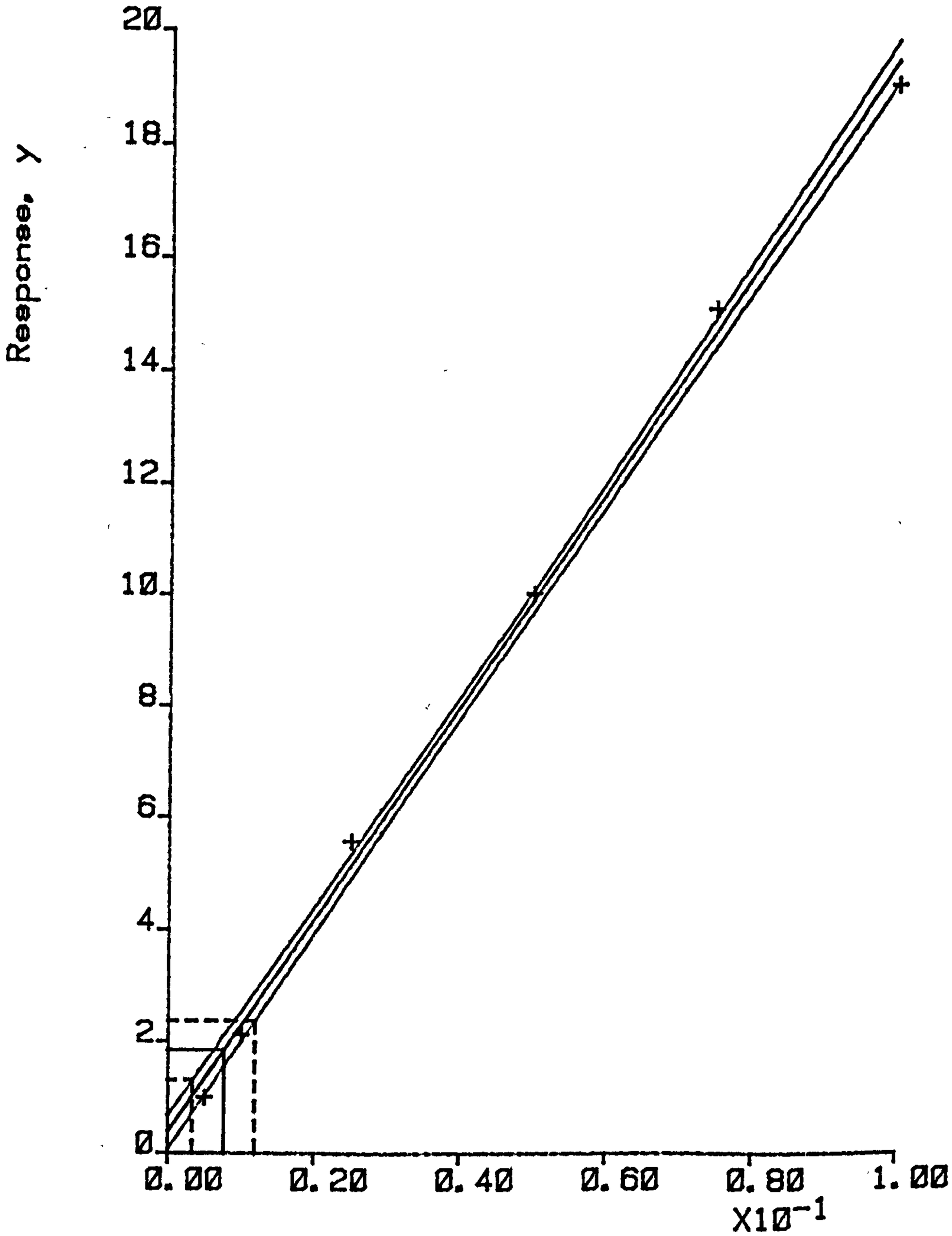


FIGURE 3.35 DETECTION LIMIT FOR 4-Cl-6-CF3-PIAZSELENOL

y = -0.320 + (.728E 3) * conc

95.0% Confidence Limits

Detection Limit = 0.00221 µg/ml

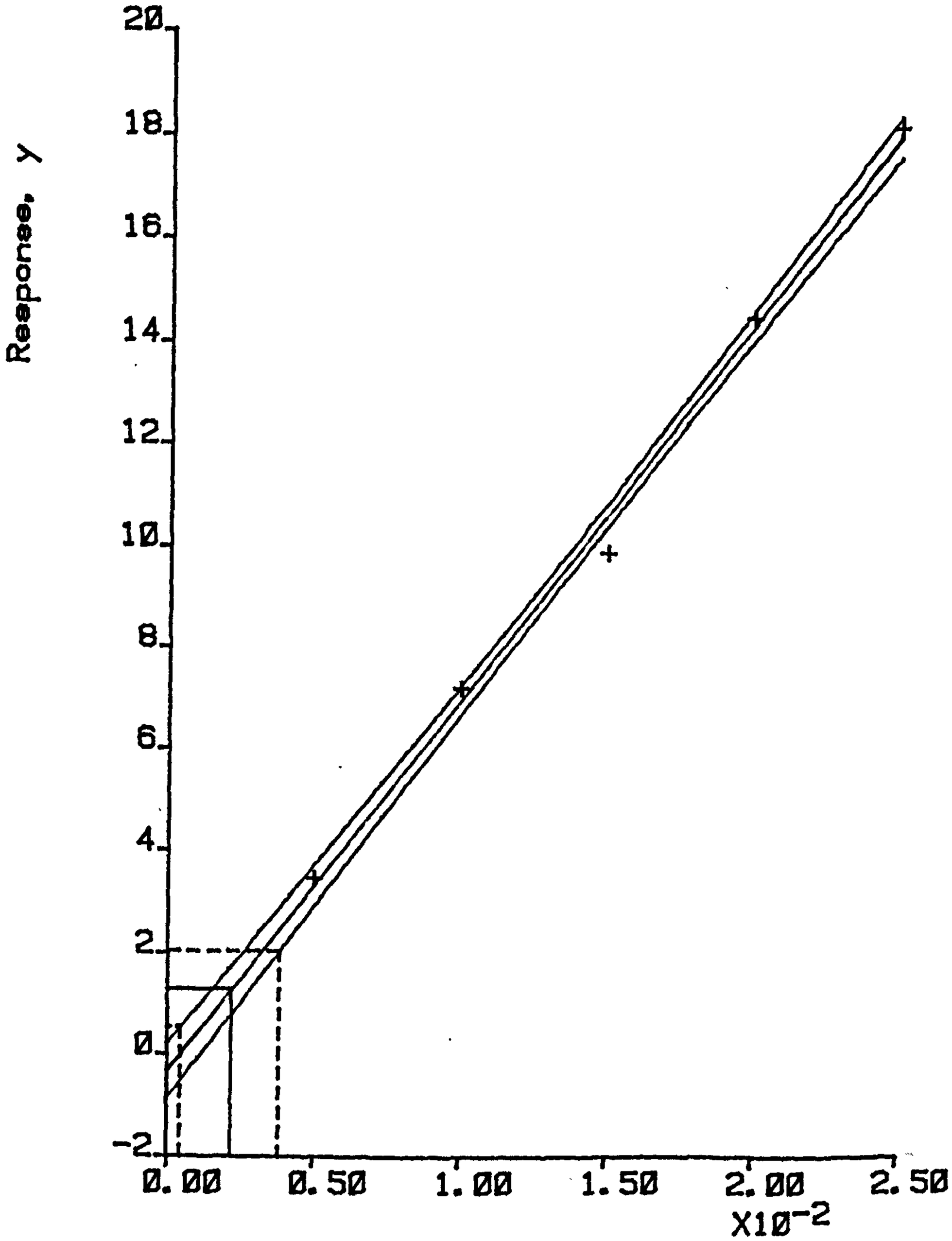
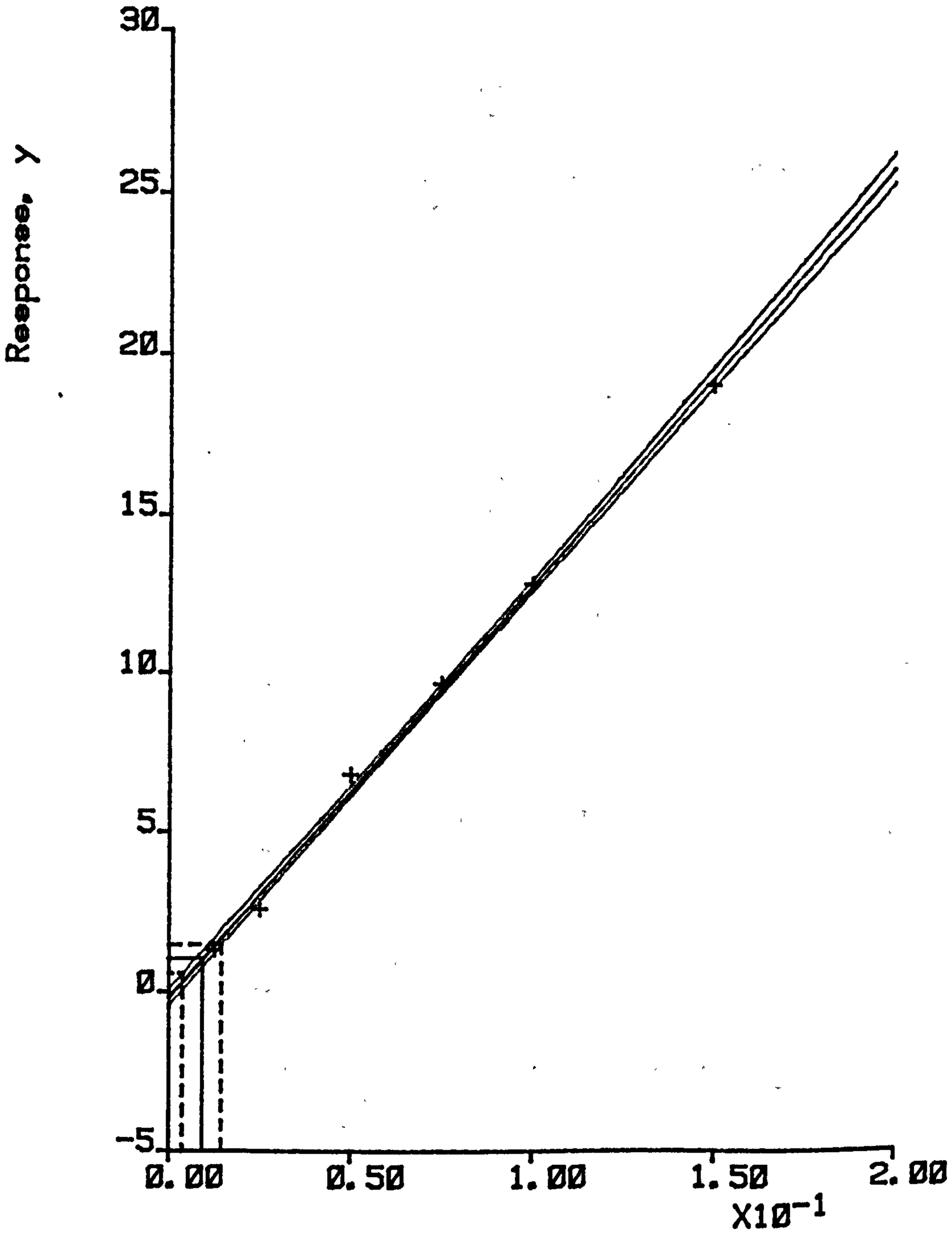


FIGURE 3.36 DETECTION LIMIT FOR 5-Cl-6-F-PIAZSELENOL

y = -Ø.149 + (.129E 3) * oono

95.Ø% Confidence Limits

Detection Limit = Ø.ØØ926 μg/ml



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CHAPTER 4

DETERMINATION OF SELENIUM

CONTENTS

4.1 INTRODUCTION

There is increasing recognition that selenium is an important metalloid of industrial, environmental, biological and toxicological significance. Selenium is an essential element in many biological species, including humans. The toxicology of selenium and its compounds is often conflicting and controversial. While the carcinogenicity of selenium has been reported, the prevention (by addition of sodium selenite) of several chemically induced cancers in animals has also been described. Additionally, sodium selenite is an anticarcinogenic, antimutagenic, anticlastogenic agent (1).

Selenium is widely distributed in the environment (waters, soil and air) albeit generally in very low concentrations ($\leqslant$ 1 μg/ml). The concentration of selenium in most human biological materials is between 0.01 ug/ml (e.g. in milk, urine) and 1 ug/ml (hair, nails, kidney) (2,3).

Many analytical techniques are now available for the determination of selenium at the trace level (ng/g). In a review (2) of the determination of selenium in environmental and biological materials, the frequency of the use of various analytical methods over the period 1975-1979 was stated to be: neutron activation analysis (NAA), 35%; atomic absorption spectrophotometry (AAS), 22%; gas chromatography (GC), 12%; spectrophotometry, 4%; X-ray fluorescence spectrometry, 4%; and others, including fluorimetric and electroanalytical methods such as potentiometry, polarography, voltammetry, 22%. Since then, these figures have changed, particularly for methods other than NAA and especially for methods based on GC (4).

The determination of selenium in environmental and biological

samples has been reviewed (2-9). Generally GLC + ECD and atomic absorption spectrometry are the more popular methods for determination of selenium.

The methods usually employed for determining selenium in biological and environmental materials initially involve the destruction of organic constituents with concurrent oxidation of the element to the tetravalent or hexavalent state and its subsequent determination by a variety of techniques (such as AAS and GLC + ECD (1).

1,2-Diaminobenzene (o-phenylenediamine) and its derivatives react selectively and quantitatively with selenium (IV) to form piazselenols that are both volatile and stable. Trace levels of selenium in materials as diverse as plant and animal tissues, metals and natural waters have been determined successfully as piazselenols by means of gas chromatography with electron capture detection (10). These analyses have been reviewed by Toei et al. (7) and Dilli et al. (4). In Chapter 3 of this Dissertation methods concerned with the use of 1,2-diaminobenzenes for the determination of selenium have been reviewed.

The use of atomic absorption spectrometry (AAS) has increased during the last two decades and now covers a large fraction of the total number of selenium determinations. Robberecht and Van Grieken (8) have recently reviewed AAS determinations of selenium in water samples, and Verlinden et al. (6) reviewed the AAS-procedure for determination of selenium in various materials.

Allan in 1962 (11) was the first to describe the determination of selenium by AAS; he used a hollow-cathode lamp (HCL) and the less sensitive 204 nm line. For aqueous standards and an air-acetylene flame a 5 ppm selenium solution gave 1% absorption. Rann and Hambly (1965) measured the absorption of selenium at 196 nm (a more sensitive

line) using an air-acetylene flame, and achieved a sensitivity of about 1 ppm (12).

The most intense resonance line of selenium (196.03 nm) corresponds to a wavelength near the vacuum ultraviolet. In addition, the most frequently used air-acetylene flame absorbs about 55% of the radiation intensity of the light source. If electrodeless discharge lamps are used with air-acetylene flame, a lower detectability level of 0.2 µg/ml can be achieved. These levels can be extended down to 0.1 µg/g by using a deuterium lamp for background correction (5). Kahn and Schallis (1968) used an argon-hydrogen (entrained air) flame to increase sensitivity, but the system also increases interferences (13) because of the lower temperature.

Although the flame methods lack sensitivity for submicrogram quantities of selenium, advances in flameless AAS were able to increase markedly the sensitivity of the selenium method (5).

Graphite furnace atomic absorption spectroscopy (GFAAS) is especially suitable for direct analysis because of its high sensitivity (detection limit 90 ρg of selenium), but it is not simple, nor free from interferences or volatilisation losses (9).

Many problems have been reported in the determination of selenium. Due to severe matrix effects, many procedures have utilised the method of standard additions or digestion and extraction (14). Spectral interferences are also a problem in the determination of Se. In 1978, Manning (15) found that the presence of a large amount of iron resulted in correction errors in the determination of Se using continuum background correction. Fernandez and Giddings (16) showed that this interference could be eliminated with the Zeeman background correction system.

Ediger (17) was the first to propose the "matrix modification" technique: adding nickel salts which stabilised the inorganic selenium up to 1200°C, probably as nonvolatile nickel selenide. The technique is now very popular (18-20), the concentration of nickel being between 0.03 and 1%, typically 0.1 to 0.2% (3). The added nickel does not necessarily eliminate all the interferences (3,21), but it produces major improvements, namely (3):

a) higher ashing temperature;

b) levelling off of the enhancement effect of other ions;

c) sensitivity improvement (by a factor of 1.9 to 6); and

d) better precision.

Several matrix modifiers have been recommended for determination of selenium, such as silver (20), copper (17,20), magnesium nitrate and nickel (22,23) and palladium (24). The use of palladium (Pd) as a matrix modifier is not new. It was first used in 1979 by Shan in the determination of mercury. Palladium has also been used in the determination of tellurium, bismuth, arsenic and thallium (14).

After some deliberation, it was decided to design the experimental work to answer, if possible, the following questions:-

i) What parameters in the GLC + ECD system give the optimum signal (peak height) for selenium determination? The observed optimum parameters were then to be applied in the determination of selenium in water, sediments and plant samples.

ii) What is the best reagent (ligand) for selenium determination especially in water samples?

The quantity of selenium in natural waters is so small that many analytical techniques including neutron activation analysis often necessitate preconcentration. However, the GLC

+ ECD method using 3,5-dibromo-1,2-diaminobenzene as reagent allows the detection of selenium levels as low as 2 ng/L, with preconcentration (7). Investigations (25-27) have reported that the dibromo ligand was the best, afforded the highest sensitivity and was capable of detecting a level of 1 ng selenium.

iii) What parameters in GFAAS give the optimum response for selenium determination in plant and sediment samples?

iv) What is the total selenium present in the river sediments? Very little work on the presence of selenium in such systems has been reported (10).

v) To determine the level of selenium in the plant growth experiments (to be described in Chapter 6).

4.2 EXPERIMENTAL AND RESULTS

4.2.1 Site Description and Sample Collection

Selenium has a toxic effect on man and animals comparable with that of arsenic, giving rise to similar symptoms. Selenium also has been suspected of causing dental caries in man and of being a carcinogenic agent.

The selenium concentration of most drinking waters is less than 10 µg/L. Concentrations exceeding 500 µg/L are rare and limited to seepage from seleniferous soils. The sudden appearance of selenium in a water supply might indicate industrial pollution. Since selenate and selenite are both found in soils, it is reasonable to expect that both may be present in seleniferous water. Water contaminated with wastes may contain selenium in any of its four valence states (28).

For determination of selenium levels as Se(IV) and Se(VI) in the River Avon, samples were collected in 28th June 1984 from three points. Reybridge on the River Avon was thought to be a good sampling point because of the presence of a factory in Chippenham known to use selenium in one of its basic processes. The waste from the factory is despatched as an effluent to Chippenham sewage works, where after treatment the final effluent from the sewage works is sent to the River Avon. The two other points were one prior to (Kellaways), and one much further downstream (Melksham) of the point of contamination.

On 24 July 1984 samples of river water were collected from a further nine sites along the River Avon from Malmesbury in the upper reaches of the River Avon to the last site, Keynsham, near Bristol. Shortly afterwards the River Avon becomes tidal at St. Annes. The location of these sample sites is shown in Figure 4.1.

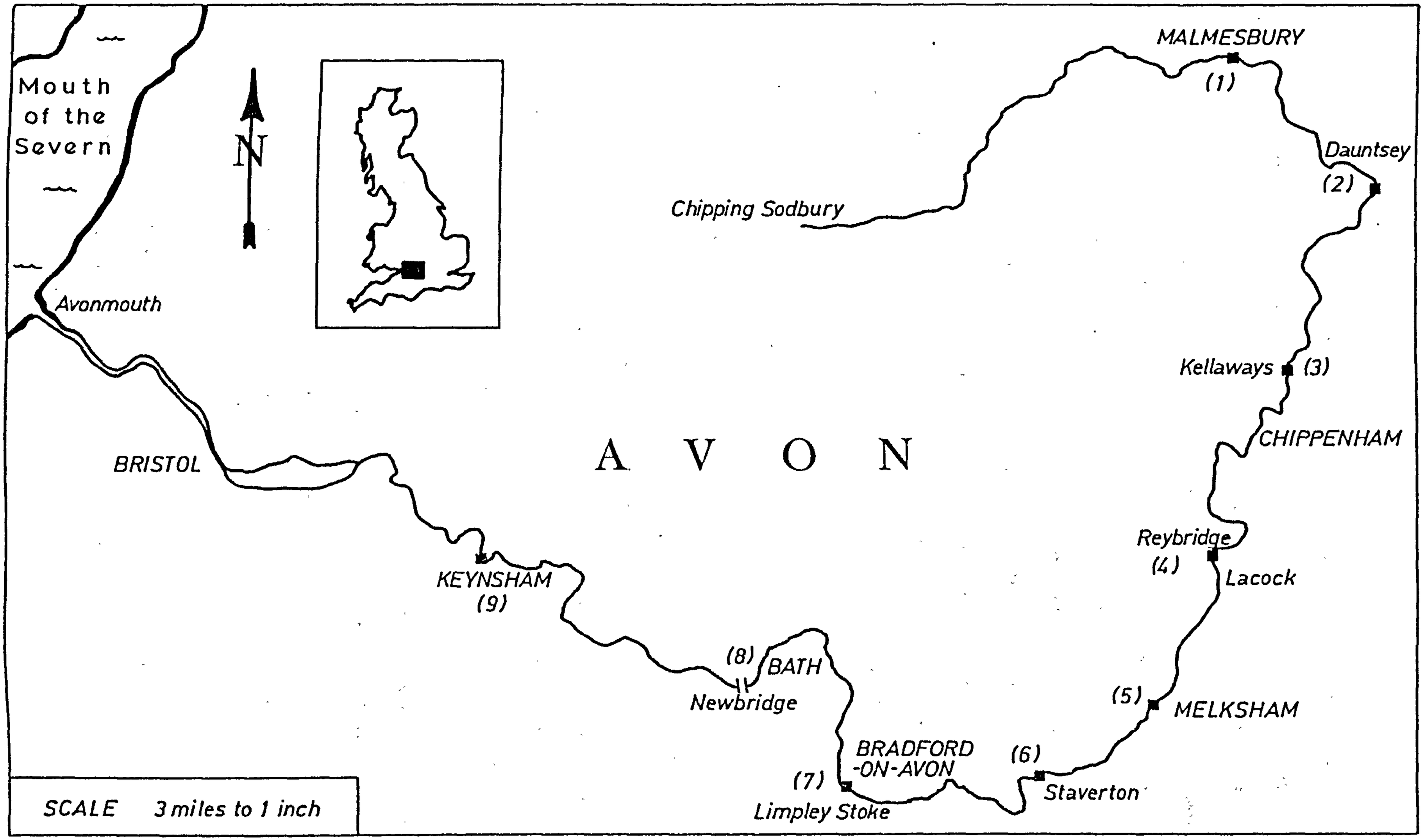


FIG. 4.1 SAMPLING SITES ON RIVER AVON

On 23rd August 1984, samples of river water and river sediments were collected from six sites starting from Kellaways to Newbridge, Bath. The location of these six sample sites is also shown in Figure 4.1. Figure 4.1 shows all nine sites, namely: 1. Malmesbury; 2. Dauntsey; 3. Kellaways; 4. Reybridge; 5. Melksham; 6. Staverton; 7. Limpley Stoke; 8. Newbridge, Bath; and 9. Keynsham.

The water samples were collected in polyethene bottles, previously rinsed with nitric acid and again with DDW. The polyethene bottles (2.5 L) were rinsed three times with the sample water prior to collection. After return to the laboratory, the water samples were acidified with concentrated nitric acid to a pH around 2.

4.2.2 Experimental Preparation

i) Reagents

AnalaR and AristaR reagents were used whenever possible, these were:- hydrochloric, hydrobromic, nitric, perchloric, and sulphuric acids, bromine, magnesium nitrate hexahydrate, metallic selenium, selenium dioxide, selenious acid standard solution (for atomic absorption spectroscopy), and sodium selenate were obtained from BDH, Poole, U.K.

Reagent-grade 1,2-diamino-4-chloro-5-fluorobenzene, 1,2-diamino-3-bromo-5-trifluoromethylbenzene were obtained from Maybridge (Trevillett, Tintagel, Cornwall, U.K.). The synthesis of 1,2-diamino-3,5-dibromobenzene hydrochloride was reported in Chapter 3 (page161).

ii) Standard solutions

Selenium (IV), stock solution 1000 µg/ml and Se(VI) stock solution 1000 µg/ml were prepared as in Chapter 3.

iii) Ligand Solutions

a) 0.1 g of 3,5-dibromo-1,2-diaminobenzene was dissolved in 100 ml concentrated hydrochloric acid.

b) 0.25 g of 4-chloro-5-fluoro-1,2-diaminobenzene was dissoled in 100 ml of 10% (v/v) aqueous hydrochloric acid solution.

c) 0.2 g of 3-bromo-5-trifluoromethyl-1,2-diaminobenzene was dissolved in 100 ml of 10% (v/v) aqueous hydrochloric acid solution.

The ligand solutions were stored in a refrigerator at 4°C. New solutions were prepared every two weeks, or when needed.

iv) Instrumentation

All samples (water, sediment and plant) for the determination of selenium by GLC + ECD were carried out with a Pye-Unicam Series 104 chromatograph using an electron capture detector Ni^{63}. All the operational parameters are described in Chapter 3.

4.2.3 Determination of Selenium by GLC + ECD in Water, Sediment and Plant Samples

i) Water Samples Determination and Results

a) Sample Treatment

In the laboratory samples were filtered by using Millipore membrane filters of 0.45 μm pore size to remove suspended matter. The water samples were examined as soon as possible. The selenium present in the double distilled water (DDW) was used as a blank for the river samples.

b) Reduction of Selenium (VI) to Selenium (IV)

The reduction of Se(VI) to Se(IV) is often necessary since several of the determination methods are specific to Se(IV). These include gas chromatography, fluorimetry (the 1,2-diaminobenzene type complexing agents only react with Se(IV) (29)), and electro-analytical methods because Se(VI) is an electroinactive species, i.e. it is not reduced on a mercury electrode, while Se(IV) is reduced to selenide Se(-II) on the mercury electrode (30).

The most convenient method for reducing selenate to selenite is by means of hydrochloric acid, provided the process is carefully controlled to avoid loss of selenium as various volatile species. Generally, the reduction step is carried out by heating with a concentrated hydrochloric acid (5 ml) by boiling gently for 10 minutes at 100°C (4,25).

If the reduction is not carried out then the amount of Se(IV) present is found, while the quantity determined after reduction is the total selenium present, Se(IV) plus Se(VI), so the difference is Se(VI).

c) Determination of Selenium in Water Samples

Selenium was determined in all the water samples collected from the River Avon (as shown in Figure 4,1 for location sites). The procedure used for the determination of Se(IV) and total Se was by means of GLC + ECD.

1) Determination of Se(IV) (Procedure 1)

In a 1 L separating funnel, 500 ml of pretreated sample water and 20 ml of concentrated hydrochloric acid were added and mixed well. The solution was shaken vigorously with 25 ml of toluene to saturate it with

toluene and to remove toluene-soluble matter. The aqueous phase was transferred into a further 500 ml separating funnel after the solvent phase separation. An aqueous solution 0.10% (w/v) of 3,5-dibromo-1,2-diaminobenzene hydrochloric solution (10 ml) was added; the solution allowed to stand for 2 hours. Toluene (5 ml) was then added, the solution was vigorously shaken for 5 minutes and the complex (4,6-dibromopiazselenol) so formed was extracted. The organic extract was washed three times with 3 ml of perchloric acid 6M (i.e. 1:1 volume with water), while an aliquot of the extract was injected into the gas chromatograph, and the peak height of 4,6-dibromopiazselenol was measured (27). Procedure 1 was repeated two or three times and an aliquot from each sample treatment injected three times each (for reproducibility) into the glc, and the mean of the peak heights was measured. The result was then compared with the calibration curve of Se(IV) plus 3,5,-dibromo-1,2-diaminobenzene as shown in Figure 4.2; using the LSQPRNT program on a PET microcomputer to calculate the concentration of selenium present in the water samples. The results of Se(IV) concentration for all the water samples are illustrated in Table 4.1 and Figure 4.3, while Figure 4.4 reproduces the type of traces for Se(IV) in the river water samples obtained when using 3,5,-dibromo-1,2-diaminobenzene as ligand. However, the Melksham and Staverton samples (collected on 24th July 1984) were extracted in 10 ml tolune while the remainder were each extracted with 5 ml toluene. Because the Staverton level was very high, only 2 ul aliquots were injected but for all other sites, 3 ul aliquots were used.

Procedure 1 was also used to assess the performance in a real situation of two of the new ligands. Figures 4.5 and 4.6 illustrate the determination of Se(IV) in the samples of river water collected on the same day using:

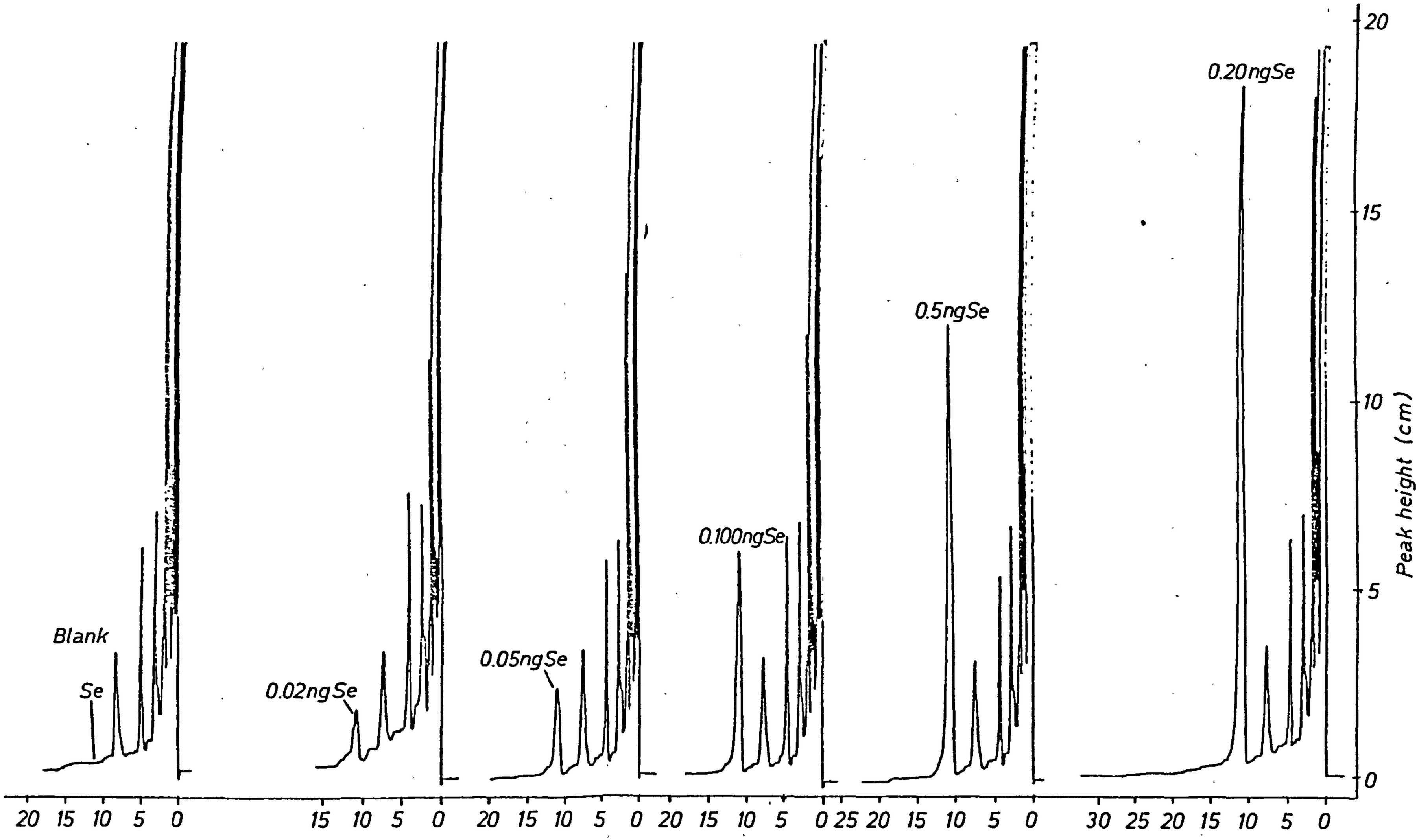


FIG.4.2 CALIBRATION CURVE OF Se (IV) PLUS 3, 5-DIBROMO-1, 2-DIAMINOBENZENE AT 200°C COLUMN TEMPERATURE

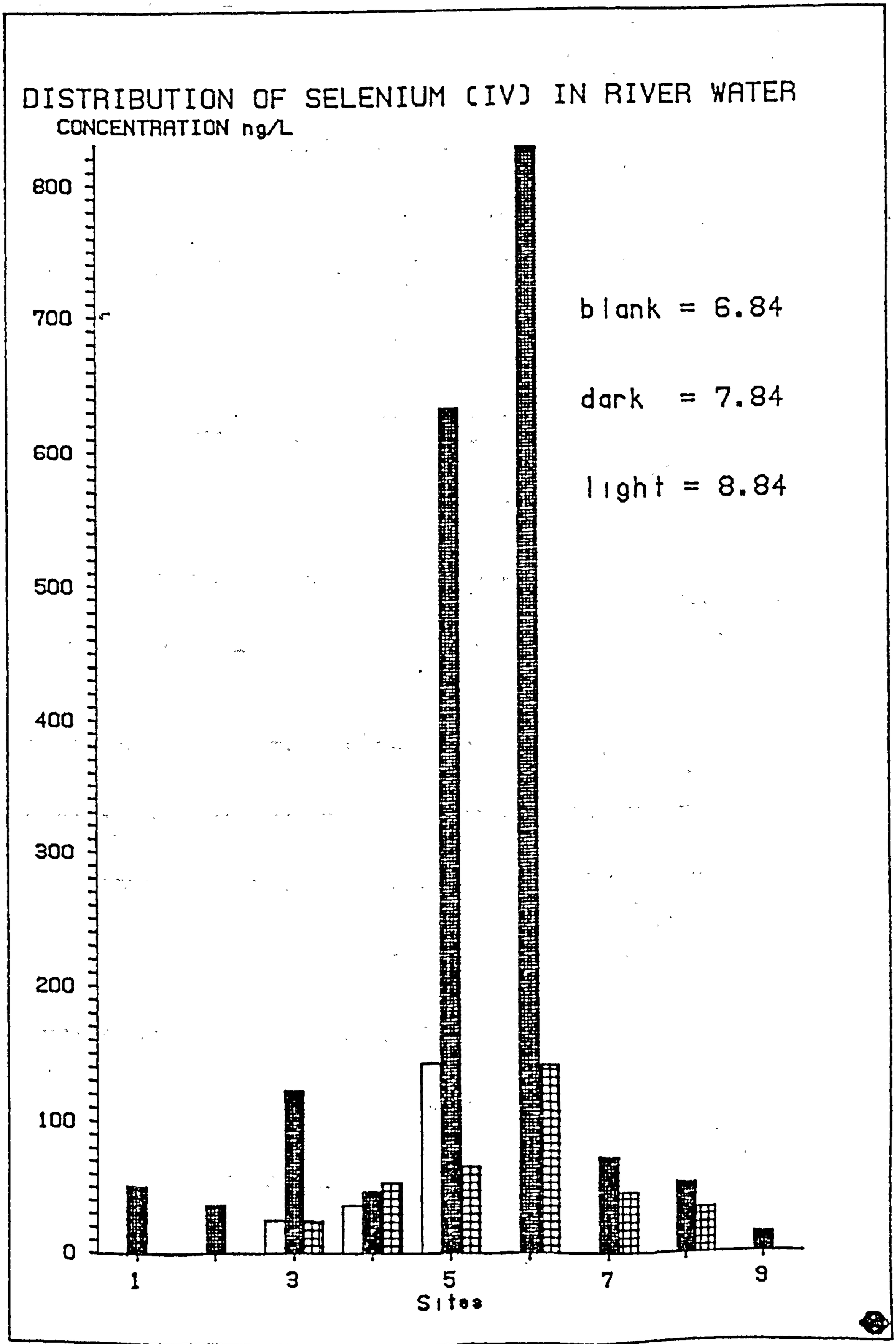
Table 4.1: Distribution of Se(IV) in River Water Samples Using 3,5,-dibromo-1,2-diaminobenzene as Ligand.
Concentration of Se(IV) ng/L.

Sample Site	Collected on		
	28/6/84	24/7/84	23/8/84
Malmesbury		51.3	
Dauntsey		36.8	
Kellaways	24.5	123.3	24.7
Reybridge	36.15	46.7	54.5
Melksham	143.0	632.3	61.8
Staverton		827.9	157.6
Limpley Stoke		72.1	46.4
Newbridge, Bath		54.6	35.25
Keynsham		14.9	

Table 4.2: Selenium (IV) in River Water Samples Using Three Diamino Derivatives

Sample Site	Concentration ng/L		
	3,5-Dibromo-	3-Bromo-5-CF_3	3-Bromo-5-fluoro-
Malmesbury	51.3	54.1	55.6
Dauntsey	36.8	35.6	41.3
Kellaways	123.3	119.8	115.7
Reybridge	46.7	50.2	49.8
Melksham	632.3	641.8	653.2
Staverton	827.9	835.9	848.3
Limpley Stoke	72.1	67.1	68.5
Newbridge, Bath	54.6	52.7	50.7
Keynsham	14.9	14.1	16.8

FIGURE 4.3


DISTRIBUTION OF SELENIUM (IV) IN RIVER WATER
CONCENTRATION ng/L
800
700
600
500
400
300
200
100
0
1
3
5
7
9
Sites
blank = 6.84
dark = 7.84
light = 8.84

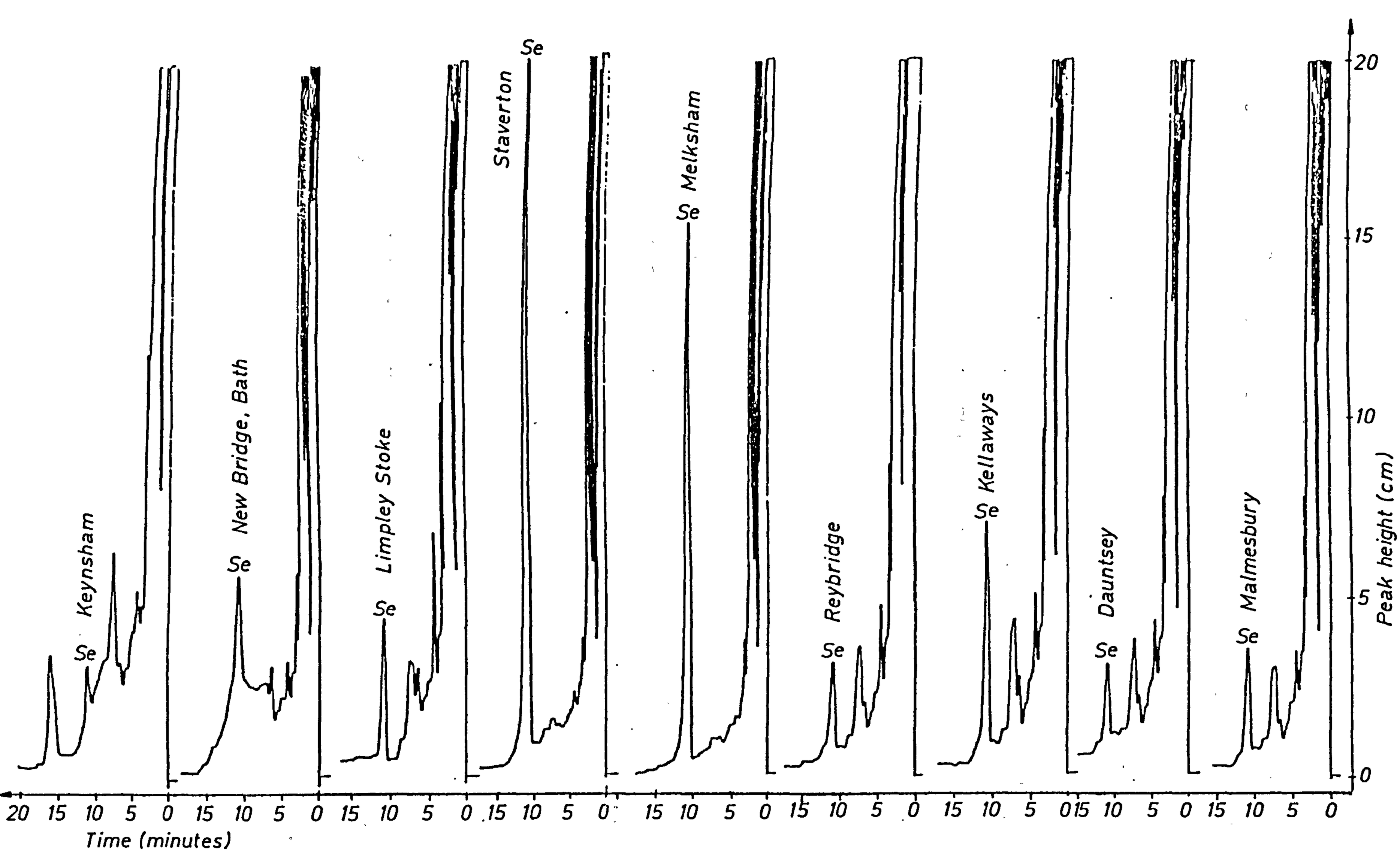


FIG. 4.4 TRACES OF Se (IV) IN RIVER WATER SAMPLES BY USING 3, 5 - DIBROMO - 1, 2 - DIAMINOBENZENE

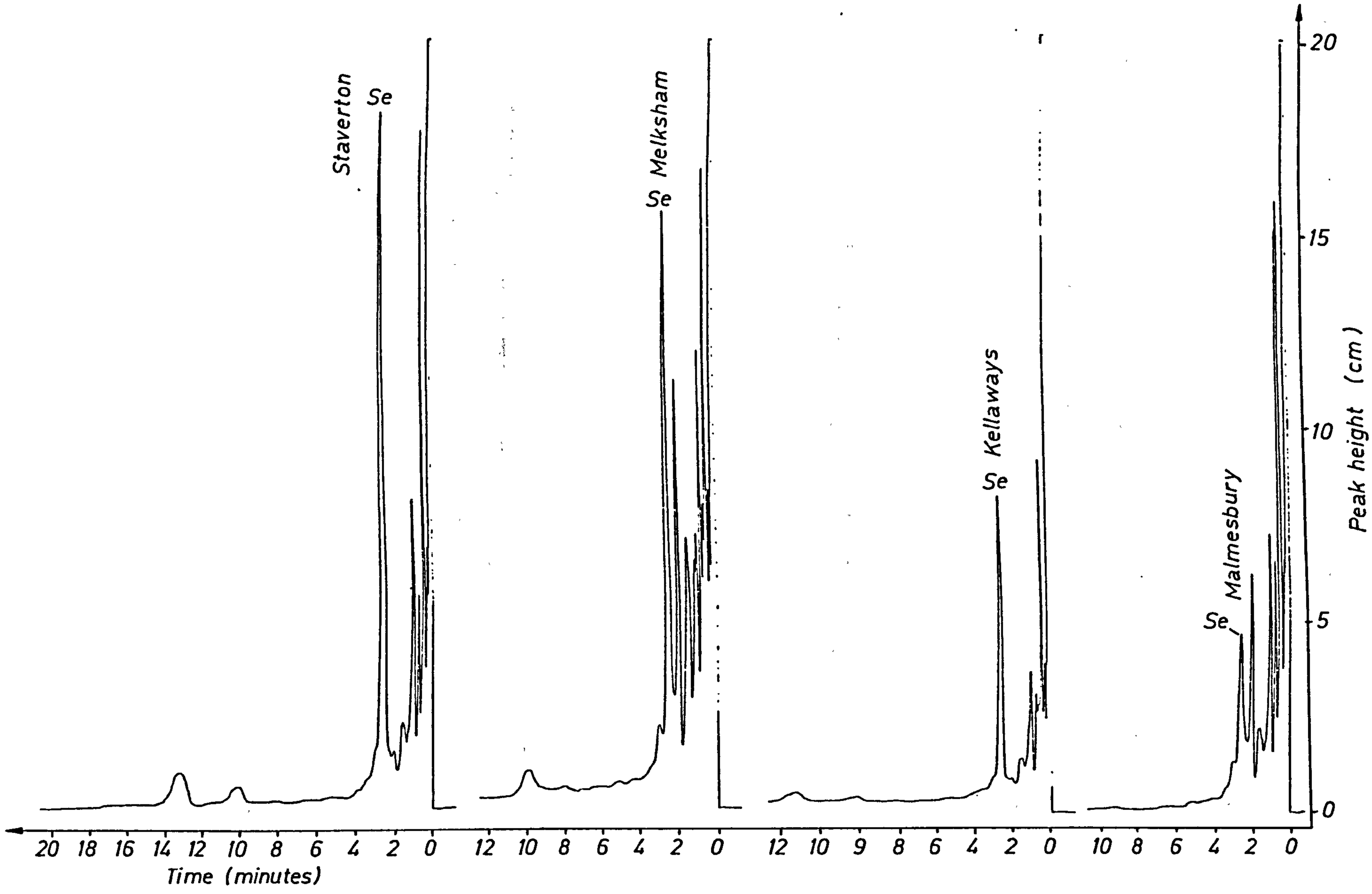


FIG.4.5 TRACES OF Se (IV) IN RIVER WATER SAMPLE BY USING 3-BROMO-5-TRIFLUOROMETHYL-1,2-DIAMINOBENZENE

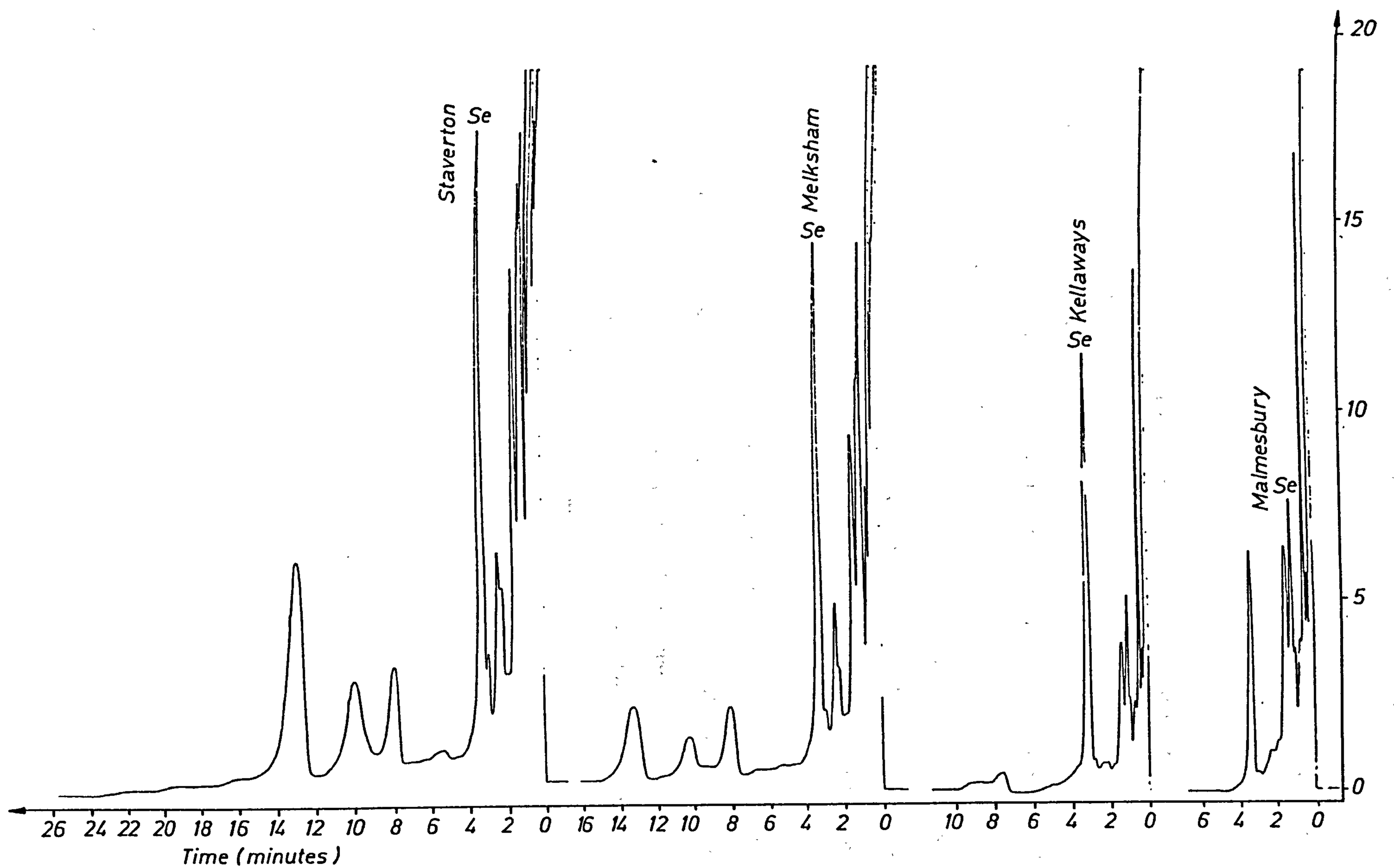


FIG.4.6 TRACES OF Se (IV) IN RIVER WATER SAMPLES BY USING 3-BROMO-5-FLUORO-1, 2-DIAMINOBENZENE

(i) 3-bromo-5-trifluoromethyl-1,2-diaminobenzene (Figure 4.5), and (ii) 3-bromo-5-fluoro-1,2-diaminobenzene (Figure 4.6).

Table 4.2 lists the Se(IV) values found using the three ligands.

2) Determination of Total Selenium (Procedure 2)

For total Se, in water samples, the recommended method for the reduction step carried out on the pretreated sample water (500 ml) involved heating with a mixture of concentrated hydrochloric acid (50 ml), 47% v/v hydrobromic acid (25 ml), and 3% w/v bromine solution (0.5 ml) by boiling gently for 15 minutes (at 100°C). Using this treatment, Se(-II,0) is oxidised to Se(IV) (if any of Se(-II) or Se(0) or both exist in such water samples) while Se(VI) is reduced to Se(IV). Thus, all selenium is now in the quadrivalent state. After the solution was cooled to room temperature, 2 ml of 1M hydroxylammonium chloride ($NH_2OH.HCl$) was added to the solution to reduce the excess bromine. The solution was washed with 25 ml of toluene and the total selenium determined by the same method as for Procedure 1 (27). Table 4.3 lists the Se(IV) and total selenium found in the river water samples using 3,5,-dibromo-1,2-diaminobenzene as ligand.

Table 4.3: Selenium (IV) and Total Selenium in River Water Samples Using Dibromo Ligand

Sample Site	Concentrations in ng/L					
	28/6/84		24/7/84		23/8/84	
	Se(IV)	Total Se	Se(IV)	Total Se	Se(IV)	Total Se
Malmesbury			51.3	95.8		
Dauntsey			36.8	91.2		
Kellaways	24.5	59.5	123.3	318.2	24.7	61.2
Reybridge	36.1	320.2	46.7	306.9	54.5	283.1
Melksham	143.0	391.2	632.3	2145.3	61.8	480.0
Staverton			827.9	2958.6	157.6	605.3
Limpley Stoke			72.1	288.4	46.4	204.8
Newbridge, Bath			54.6	215.7	35.2	156.0
Keynsham			14.9	52.2		

Figure 4.7 Se (IV) and Total Se in River Water Samples

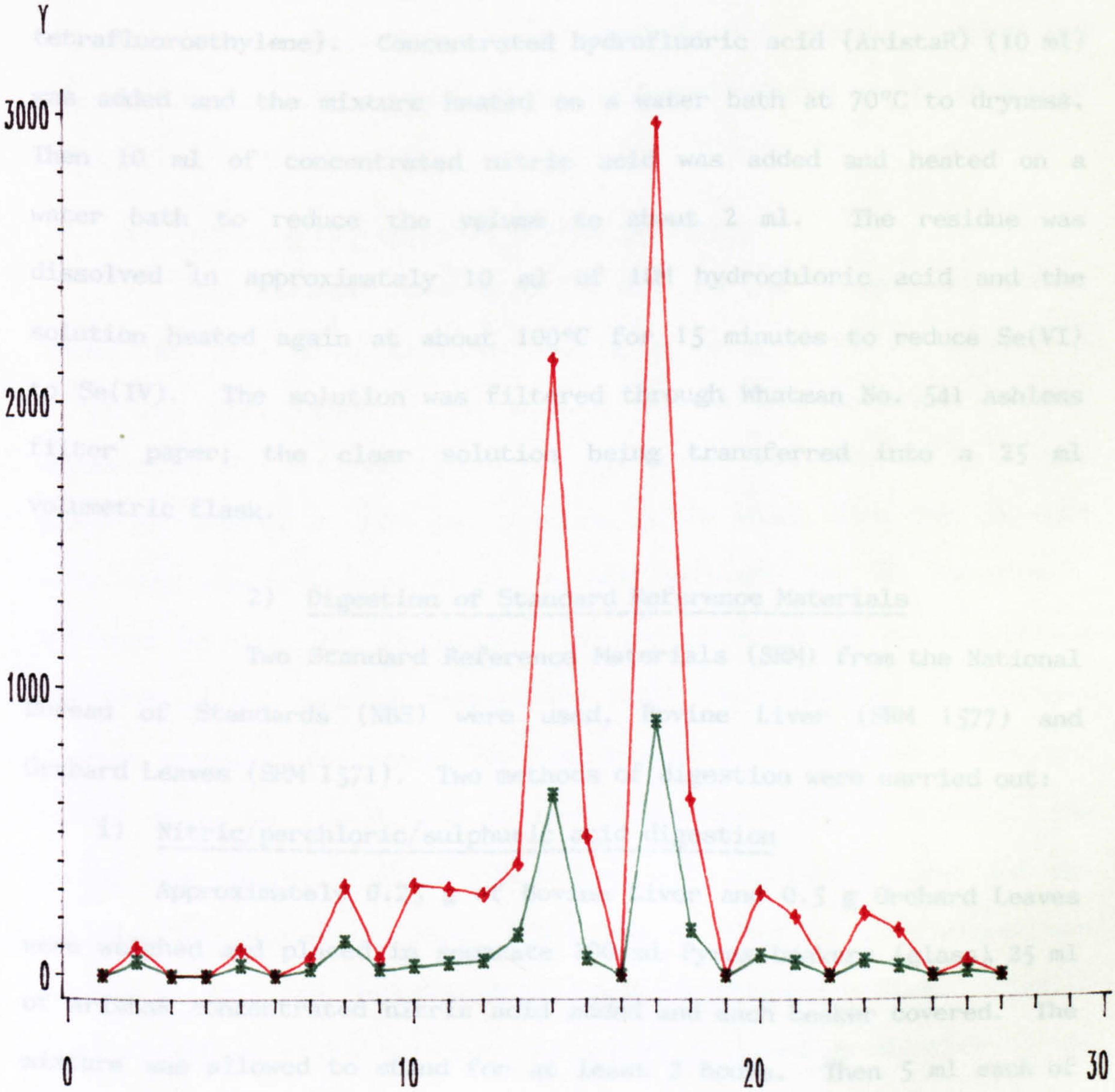
x = Sample site

Y = Concentration (ng/l)

Diamond = Total Selenium

Star = Selenium (IV)

every three divisions of x equal to one site



ii) Sediment Samples Treatment and Results

a) Sample Treatment

The collected river sediments from six sites starting at Kellaways and finishing at Newbridge, Bath, were oven dried at 50°C for 48 hours; the samples were finely ground by means of a Tema mill. The determinations were performed on the 105 mesh portion of sample.

b) Digestion Methods

1) Sample Digestion for Sediment

A 1.0 g sample was placed in a Teflon beaker (poly-tetrafluoroethylene). Concentrated hydrofluoric acid (AristaR) (10 ml) was added and the mixture heated on a water bath at 70°C to dryness. Then 10 ml of concentrated nitric acid was added and heated on a water bath to reduce the volume to about 2 ml. The residue was dissolved in approximately 10 ml of 10M hydrochloric acid and the solution heated again at about 100°C for 15 minutes to reduce Se(VI) to Se(IV). The solution was filtered through Whatman No. 541 ashless filter paper; the clear solution being transferred into a 25 ml volumetric flask.

2) Digestion of Standard Reference Materials

Two Standard Reference Materials (SRM) from the National Bureau of Standards (NBS) were used, Bovine Liver (SRM 1577) and Orchard Leaves (SRM 1571). Two methods of digestion were carried out:

i) Nitric/perchloric/sulphuric acid digestion

Approximately 0.25 g of Bovine Liver and 0.5 g Orchard Leaves were weighed and placed in separate 100 ml Pyrex beakers (glass), 25 ml of AristaR concentrated nitric acid added and each beaker covered. The mixture was allowed to stand for at least 2 hours. Then 5 ml each of

concentrated perchloric and sulphuric acids were added, plus a few glass spheres to minimise bumping. The beaker was placed on a hot-plate and gradually heated until the nitric acid boiled gently. When the volume was reduced to 25 ml, the temperature was raised to drive off nitric acid rapidly. The beaker was removed from the hot-plate when the volume had been reduced to about 15 ml, and allowed to cool for a few minutes. Then 3 ml of AristaR concentrated nitric acid were added. The digest was again heated until dense white fumes of perchloric acid appeared and the volume of solution was reduced to 5 ml. The solution was cooled and 5 ml of 10M hydrochloric acid added. The resultant mixture was then heated to about 100°C for 15 minutes, cooled and transferred into a 25 ml volumetric flask and diluted to the mark with DDW (31).

ii) Nitric acid/magnesium nitrate digestion

Concentrated nitric acid (10 ml) and 4 g of magnesium nitrate hexahydrate were added to either 0.25 g or 0.5 g of SRM (or sample) in a 100 ml Pyrex beaker. The mixture was allowed to stand at room temperature for at least 2 hours. The beaker was then put on a hot-plate and heated at 95°C for 3 hours, after which time the contents were very slowly evaporated to dryness overnight (on the hot-plate). The next morning, the temperature of the hot-plate was quickly raised and kept at its maximum temperature until volatilisation of nitrogen dioxide ceased. The beaker was then placed in a cold muffle furnace, heated up to 500°C and held at that temperature for 30 minutes. The beaker was cooled, 5 ml of 10M hydrochloric acid were added and the solution was heated at about 100°C for 15 minutes. The digest was allowed to cool and transferred into a 25 ml volumetric flask. Finally 5 ml of 40% w/v urea solution was added, followed by dilution to the mark with DDW (31,32).

3) Digestion of Plant Samples

Kumpulainen et al. (33) studied methods of digestion of selenium. They reported that in the digestion of selenium nutritional supplements, use of perchloric acid or sulphuric acid is not necessary, and recommended boiling samples overnight in concentrated nitric acid. Elsokkary (34) used nitric acid alone for digestion of seven different vegetable plant crops for determination of mercury. The method of digestion suggested by Holak et al. (35) involved using a Teflon digestion vessel with 5 ml concentrated nitric acid and heating at 150°C for 60 minutes in an oven.

In the present case, digestion of plant samples was carried out by using nitric acid in a closed system, with a Teflon vessel (or bomb) contained within a stainless steel sheath. In this case, a portion of the sample (0.1 to 1.0 g dependent on selenium content) was placed in the Teflon vessel and 5 ml of concentrated nitric acid added. The vessel was closed by tightening the screw-cap of the stainless steel sheath; the seal being made by the meeting of the upper lid of the Teflon vessel and a Teflon sealing disc. The whole vessel was placed in a preheated electric oven at 150°C for 60-75 minutes. The vessel was then removed from the oven and allowed to cool to room temperature. (If, after opening, the resultant solution was not clear, then the unit was resealed and the heating process repeated.)

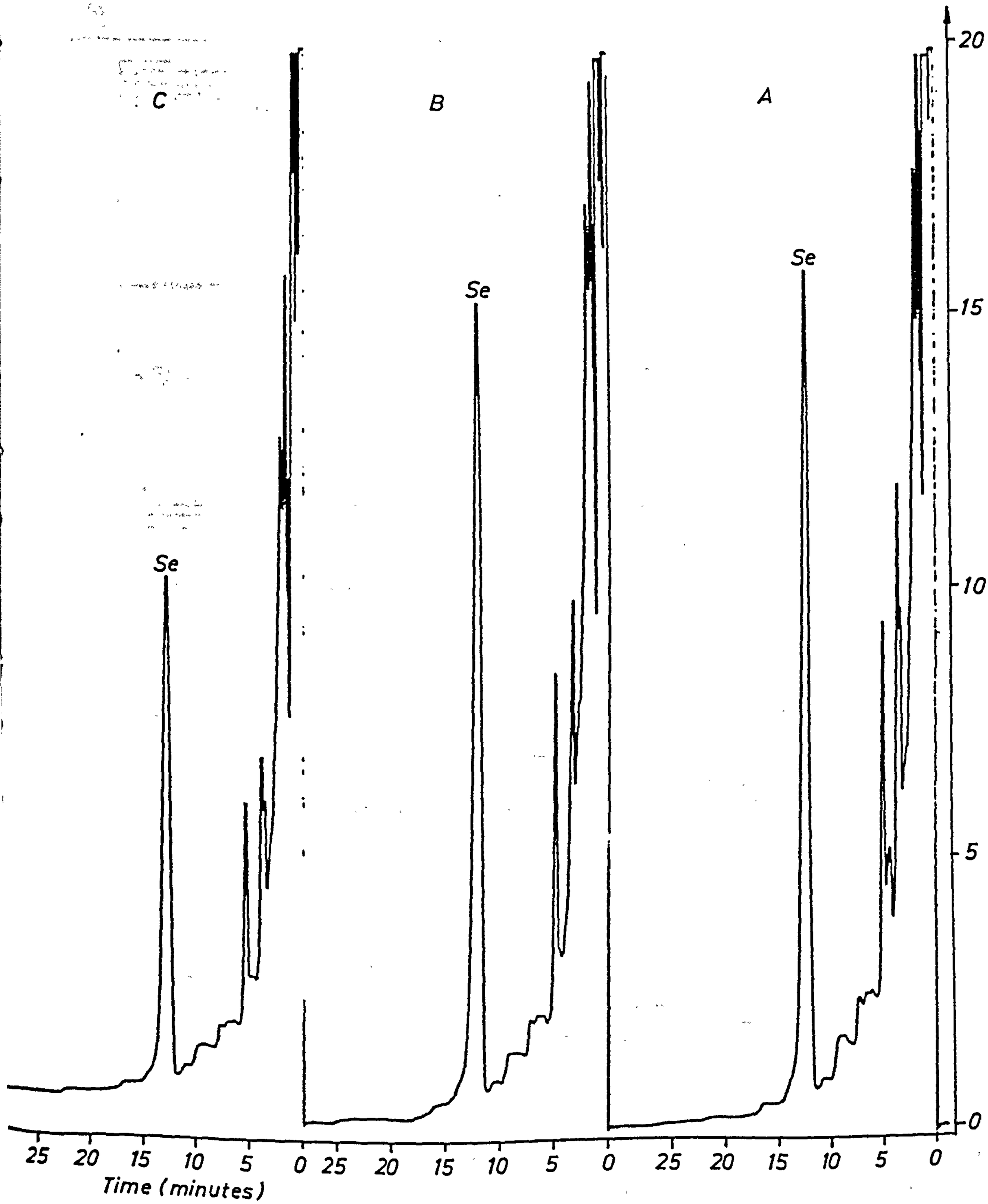
When the solution was clear, the digest was filtered through a Whatman No. 541 filter paper and the resultant solution transferred to a 25 ml volumetric flask and diluted to the mark.

c) Determination of Total Selenium in SRM and Sediment Samples

One millilitre of the SRM (Bovine Liver or Orchard Leaves) digest (by any method of digestion) or an aliquot of the conc. HF and conc. HNO_3 digested sediment solution (usually 1 ml) was placed into a 100 ml separating funnel and diluted with 10 ml of DDW. Next ligand solution (1 ml) of 0.1% w/v of 3,5-dibromo-1,2-diamino-benzene hydrochloride was added. The mixture was allowed to stand at least 2 hours at room temperature. Then 5 ml of toluene was added, the solution was vigorously shaken for 5 minutes and extracted. The extract was washed three times with 3 ml of 6M perchloric acid (1:1), and an aliquot of 2 ul or 3 ul of the toluene extract injected into the gas chromatograph, and the peak height of 4,6-dibromopiazselenol measured.

Figure 4.8 shows specimen GLC traces obtained for the total selenium in SRM of Bovine Liver and Orchard Leaves, while Figure 4.9 illustrates similar traces for total selenium in the six sediment samples when using 3,5-dibromo-1,2-diaminobenzene. Table 4.4 lists the comparison of total selenium in the SRM using the four different ligands. Table 4.5 lists the total selenium in sediment samples.

FIG. 4.8 TOTAL SELENIUM IN STANDARD REFERENCE MATERIAL



A and B = NBS Bovine Liver, SRM 1577

C = NBS Orchard Leaves, SRM 1571

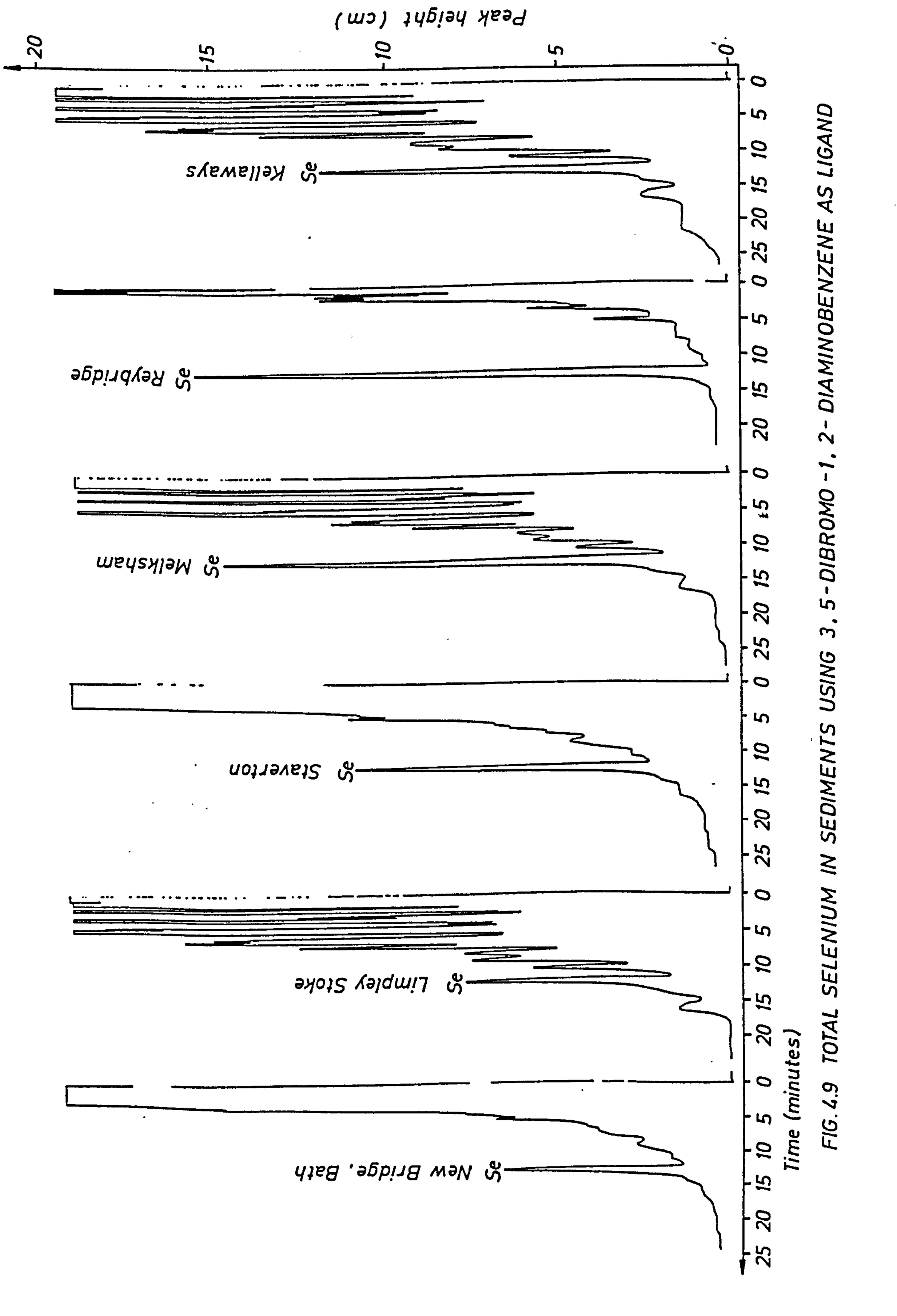


FIG. 4.9 TOTAL SELENIUM IN SEDIMENTS USING 3, 5-DIBROMO-1, 2-DIAMINOBENZENE AS LIGAND

Table 4.4: Analysis of Standard Reference Material for Total Selenium

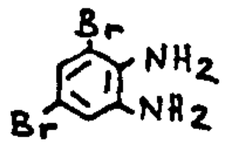
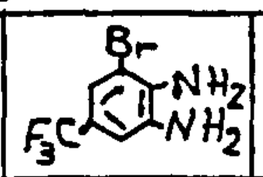
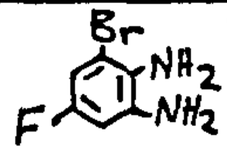
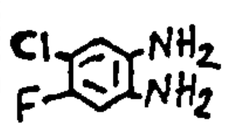
Sample	Number of Samples	Weight taken (g)	Certified Value (µg/g)	Experimental Value (µg/g)			
							
Bovine Liver (SRM 1577)	5	0.25	1.1 ±0.1	1.15 ±0.08	1.12 ±0.11	1.05 ±0.12	1.2 ±0.07
Orchard Leaves (SRM 1571)	5	0.5	0.08±0.01	0.075 ±0.012	0.085 ±0.009	0.073 ±0.01	0.083 ±0.01

Table 4.5: Total Selenium in Sediment Samples

Site	Total Selenium (µg/g)	Number of Samples
Kellaways	1.2 ± 0.1	3
Reybridge	2.25 ± 0.15	3
Melksham	1.57 ± 0.08	3
Staverton	1.1 ± 0.11	3
Limpley Stoke	0.95 ± 0.09	3
Newbridge, Bath	0.78 ± 0.07	3

iii) Plant Samples Determination and Results

In order to study the effect and uptake of selenium as sodium selenate ($Na_2SeO_4.10H_2O$) by the roots of plants with time, an experiment was carried out with Lolium perenne.

Lolium perenne (a ryegrass), which is a relatively hardy species of grass, was chosen to determine the critical level of selenium in plants. The grass is very quick-growing (especially in the summer) and is a typical grass, and hence ideal for this type of study.

Lolium perenne seeds were germinated in acid washed silver sand placed in a polyethylene potting tray which had been rinsed with DDW to eliminate any chance of metal or metalloid contamination of the seedlings. After 10 to 14 days of development in the sand, the seedlings were transferred to nutrient solutions (Hoagland's solution) which contained all essential elements for successful growth (36). The nutrient solution was prepared after the manner of Hoagland and described by Hewitt (37), and is shown in Table 4.6. The containers or pots used for the hydroponic culture of the plant were the bottom halves of a series of sawn-off Winchester acid or ether bottles and each held one litre of solution. The brown glass of these bottles excluded light from the roots of the plants. Square sheets of expanded polystyrene tiles were cut so that they completely covered the tops of the glass containers and five holes were cut in each polystyrene lid. The germinated seedlings were removed from the sand and their roots washed with DDW. Five seedlings were taken and small strips of sponge wrapped around them at the base of the shoot. Each hole in the polystyrene lid was carefully threaded with the above five seedlings, which were labelled and left to grow in the nutrient solution for two weeks. Then five seedlings were taken for the experiment. The roots

Table 4.6: Composition of Hoagland's Nutrient Solution (37) (Full Strength) used for Water Culture Experiments with Lolium perenne Seedlings

Compound		Concentration (g/L)
Name	Formula	
Potassium nitrate	KNO_3	0.656
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	0.944
Ammonium hydrogen phosphate	$NH_4H_2PO_4$	0.115
Magnesium sulphate	$MgSO_4.7H_2O$	0.49
Boric acid	H_3BO_3	0.00286
Manganous chloride	$MnCl_2.4H_2O$	0.00181
Copper sulphate	$CuSO_4.5H_2O$	0.00008
Zinc sulphate	$ZnSO_4.7H_2O$	0.00022
Molybdic acid	$H_2MoO_4=(MoO_3+H_2O)$	0.00009
Ferrous sulphate	$FeSO_4.7H_2O$	0.003
Tartaric acid	$CH(OH)COOH_2$	0.0036

of the seedlings were cut to 7 cm and the shoot to 10 cm and the fresh weight of each bunch (about 1.0 g) recorded. The experiment was carried out by adding selenium (VI) individually from 0.1 µg/ml to 30 µg/ml Se(VI) to the nutrient solutions and the plants being returned to grow for the next three weeks.

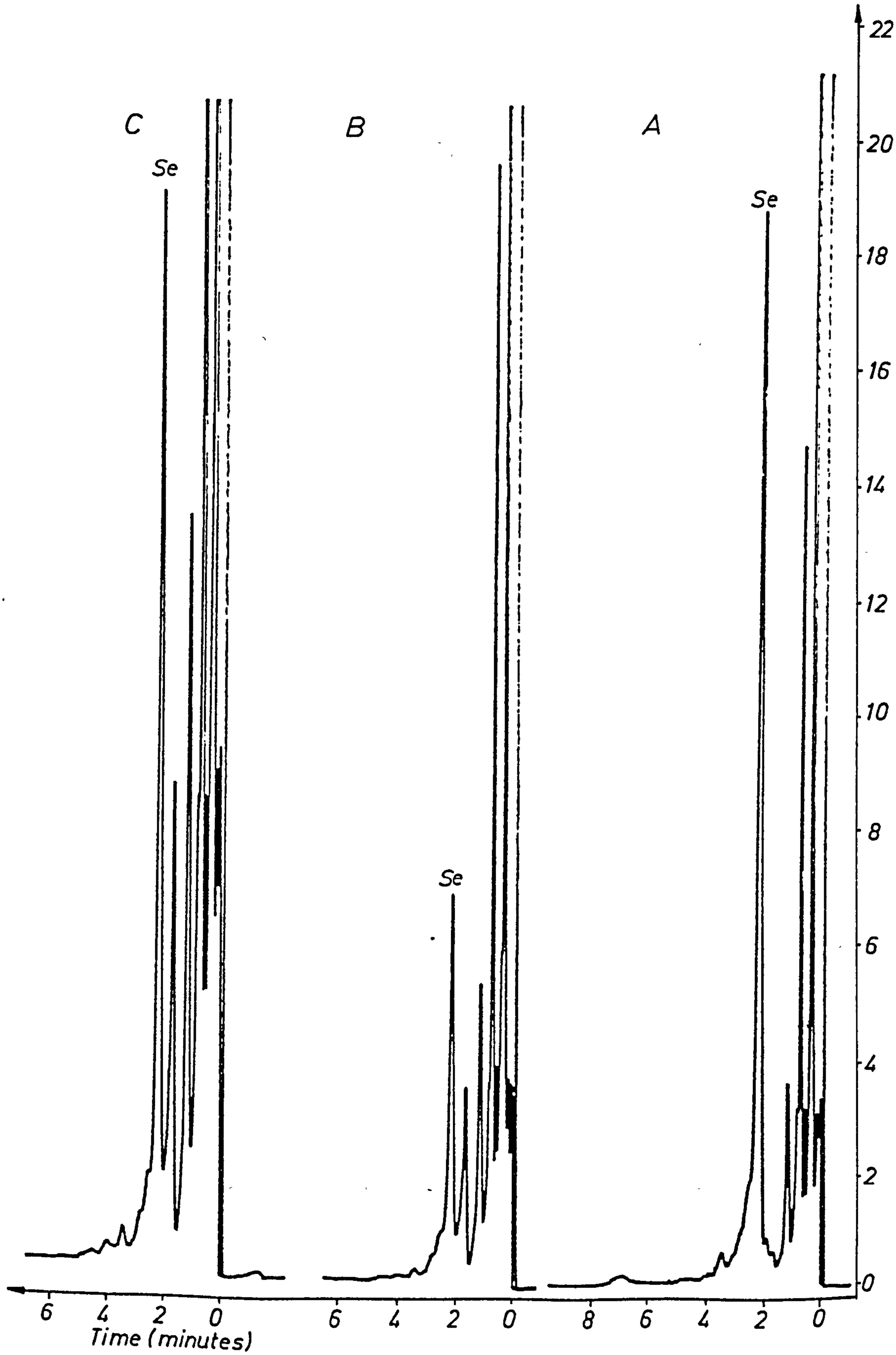
Each week, fresh nutrient solution containing the Se(VI) as spiking element was prepared and the old solution discarded. After the end of the third week, the seedlings were removed from the nutrient solution and each bunch dried between filter papers. The fresh weight,

root and shoot lengths were recorded. The roots and shoots of individual bunches were placed in beakers and left for 48 hours at 35°C in an oven. The next day, the dry weight of each sample was recorded.

Determination of the selenium content of the acid digested samples was carried out using a Varian AAS 775 with GTA-95. The results are listed in Tables 4.10 and 4.11.

However, the determination of selenium content in Experiment 1 (Se, Cd, Hg) (which will be discussed in detail in Chapter 6) of the acid digested samples was carried out using the GLC + ECD with 3-bromo-5-trifluoromethyl-1,2-diaminobenzene as ligand as well as by the Varian AAS 775 with GTA-95. Figure 4.10 illustrates the GLC traces for selenium in the shoot and root (plant samples), while Table 4.7 lists the results of selenium content in 20 shoot samples and 20 root samples.

FIG. 4.10 TOTAL SELENIUM IN PLANT SAMPLES USING 3-BROMO-5-TRIFLUOROMETHYL-1, 2-DIAMINOBENZENE AS LIGAND



A = Root sample

B & C = Shoot samples

Table 4.7: Total Selenium in Plant Samples using 3-Bromo-5-trifluoro-methyl-1,2-diaminobenzene as Ligand

Sample No.	Selenium Content	
	Roots Concentration µg/g	Shoots Concentration µg/g
1	5.20	1.35
2	17.85	28.24
3	4.32	1.34
4	4.73	1.64
5	18.36	28.18
6	22.40	29.06
7	4.48	1.45
8	17.91	30.14
9	75.22	369.50
10	2.93	4.97
11	5.15	6.10
12	1.48	2.15
13	3.10	3.76
14	2.95	2.67
15	5.27	3.68
16	5.40	4.15
17	7.05	4.70
18	5.30	4.30
19	6.11	3.95
20	6.25	4.10

4.2.4 Determination of Selenium by GFAAS in Sediment and Plant Samples

i) Apparatus and Instrumental Parameters

The determination of total selenium in sediments and plant samples was carried out by GFAAS using a Varian system AAS-775 atomic absorption spectrophotometer fitted with a Model GTA-95 graphite tube atomiser. The instrumental parameters and conditions are given in Table 4.8. The instrument was calibrated with standard solutions on concentration mode. The concentrations of selenium in the samples were compared with the concentration of standard solutions.

Table 4.8: Determination of Selenium by AAS-775 with GTA-95 Furnace Operating Parameters

Step No.	Temperature (°C)	Time (sec)	N_2 flow (L/min)	Read command
1	90	5	3	
2	120	60	3	
3	300	10	3	
4&5	500	5	3	
6	500	2	0.0	
7	2700	1.1	0.0	*
8	2700	2	0.0	*
9	2700	2	3	

Instrument Parameters (38):

Lamp current (HCL) = 5 mA

Spectral bandwidth = 1.0 nm

Wavelength = 196.0 nm

Background correction = ON

Standard or sample injected = 20 µl

* = Read signal

An aliquot of hydrofluoric/nitric acids digestion mixture was used to determine total selenium, with nickel (II) nitrate (100 µg/ml) in 1% v/v nitric acid solution being used as matrix modifier. The use of nickel nitrate extended the ashing temperature to beyond 1200°C, and so prevented pre-atomisation losses of selenium (17,20,23,33), so allowing a reasonable atomisation temperature of 2700°C to be used. The procedure was carried out using 10 µl of the standard or sample plus 10 µl of the modifier solution.

ii) Sediment and Plant Samples Results

a) Sediments

The same six sediment samples which had been examined using the GLC + ECD procedure (Results, see Table 4.5, p.262) for total selenium were also investigated with the GFAAS procedure.

The results obtained are given in Table 4.9.

Table 4.9: Total Selenium in Sediment Samples by GFAAS

Site sample	Total selenium (µg/g)	Number of samples
Kellaways	1.1 ± 0.08	2
Reybridge	2.35 ± 0.17	3
Melksham	1.75 ± 0.11	3
Staverton	1.0 ± 0.09	3
Limpley Stoke	0.80 ± 0.08	2
Newbridge, Bath	0.63 ± 0.075	2

b) Plants

An experiment to provide suitable biological tissue for total selenium analysis was carried out using Lolium perenne seedlings. Selenium (VI) as sodium selenate was added to nutrient solutions in which the seedlings were grown hydroponically. The seedlings were germinated, allowed to establish themselves on exposure of one week to pure nutrient solution, followed by a three week immersion in contaminated soslutions. The procedure has been discussed in detail on page 263.

An aliquot of the wet acid digestion of the dried tissue (using conc. HNO_3) in a Teflon vessel was used to determine the total selenium level with nickel (II) nitrate as matrix modifier. The AAS system used was a Varian A775 plus GTA-95 carbon furnace atomiser. The results are given in Tables 4.10 and 4.11.

Since the uptake of Se(IV) as sodium selenite by plants is not well documented, an attempt was made to use Se(IV) instead of Se(VI) in a similar experiment to that described above using Lolium perenne seedlings. Regretfully the experiment failed because of the precipitation of poorly soluble metal selenites (39). The divalent metals particularly Ca and Mg ions in the nutrient solution in the presence of Se(IV) were able to form low solubility selenites. As a consequence of this difficulty, the uptake of Se(IV) was not pursued.

Table 4.10: Selenium Concentration in Shoots of Lolium perenne

Concentration of Se(VI) µg/ml in nutrient solution	Average length (cm) of five plants	Dry weight (g) of plant	Concn. of Se µg/g in plant tissue	Log_{10} Concn. of Se in plant tissue
0.00 (Control)	21.0	0.394	N.D.	-
0.10	21.9	0.42	6.11	0.786
0.5	20.7	0.415	15.7	1.196
1.0	23.0	0.39	28.4	1.45
2.5	19.2	0.36	155.6	2.19
5.0	17.0	0.30	375.2	2.57
10.0	10.5	0.173	880.2	2.95
15.0	8.7	0.120	1092.7	3.038
20.0	4.6	0.080	1352.5	3.131
30.0	3.2	0.076	454.0	2.657

Table 4.11: Selenium Concentration in Roots of Lolium perenne

Concentration of Se(VI) µg/ml in nutrient solution	Average length (cm) of five plants	Dry weight (g) of plant	Concn. of Se µg/g in plant tissue	Log_{10} Conc. of Se in plant tissue
0.00 (Control)	17.0	0.117	N.D.	-
0.10	17.1	0.105	3.10	0.49
0.50	16.3	0.106	10.3	1.01
1.0	16.0	0.108	17.2	1.235
2.5	14.5	0.102	26.5	1.423
5.0	12.3	0.081	137.45	2.138
10.0	2.2	0.033	220.8	2.344
15.0	0.8	0.028	357.5	2.553
20.0	0.2	0.018	376.3	2.575
30.0	0.0	0.016	403.8	2.606

N.D. Not detectable

Table 4.12: Total Selenium in Plant Samples using:

i) 3-Bromo-5-trifluoromethyl-1,2-diaminobenzene with GLC

ii) GFAAS

Sample Trial No.	Root µg/g Se		Shoot µg/g Se	
	GLC	GFAAS	GLC	GFAAS
1	5.20	3.86	1.35	1.30
2	17.85	15.50	28.24	26.33
3	4.32	4.10	1.34	1.40
4	4.73	4.75	1.64	1.62
5	18.36	15.92	28.18	26.11
6	22.40	19.36	29.06	27.55
7	4.48	3.88	1.45	1.51
8	17.91	16.90	30.14	31.64
9	75.22	72.30	369.50	355.60
10	2.93	2.75	4.97	5.32
11	5.15	5.50	6.10	5.93
12	1.48	1.66	2.15	1.78
13	3.10	2.86	3.76	3.60
14	2.95	2.77	2.67	2.70
15	5.27	5.47	3.68	2.44
16	5.40	4.83	4.15	3.16
17	7.05	5.65	4.70	3.46
18	5.30	4.48	3.84	3.75
19	6.11	5.56	3.95	2.76
20	6.25	4.60	4.10	3.62

4.2.5 Determination of Mn, Fe, Cu and Zn in Plants

i) Apparatus and Instrumental Parameters

The determination of heavy metals such as Mn, Fe, Cu and Zn in plant samples was carried out by FAAS using an Instrumental Laboratory Inc. atomic absorption spectrophotometer model 151 fitted with background correction facilities.

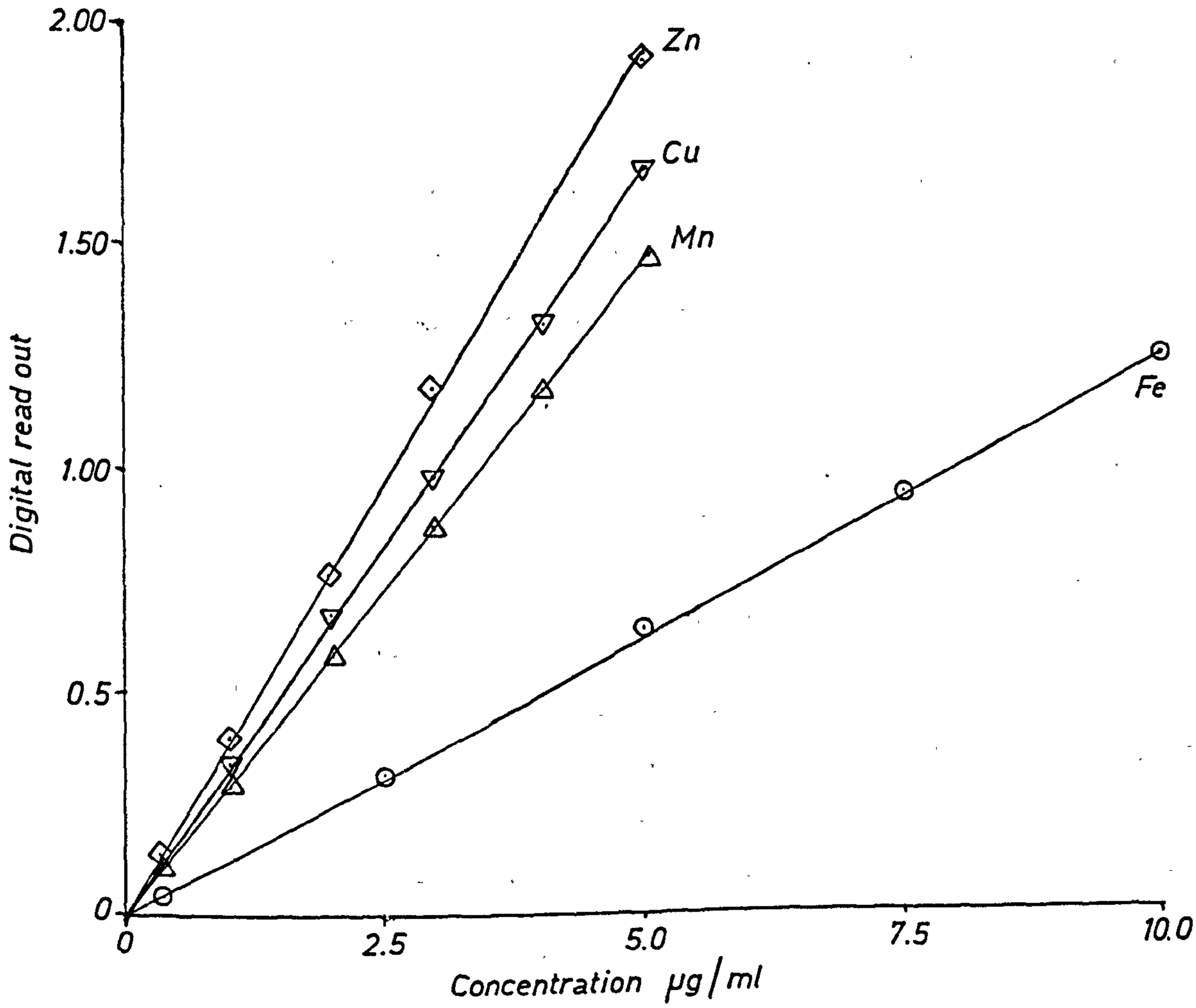
The analytical method involved the use of standard solutions for a direct comparison measurement to the unknown solution sample. The standards were prepared by dilution of the 1000 µg/ml stock solutions to give a range of standards suitable for the calibration plots. Since the working curve may change day to day due to small variations in lamp and flame conditions, the instrument had to be recalibrated for each batch, i.e. from 15 to 20 samples. Table 4.13 shows the instrumental conditions when using FAAS with a flame of air/ acetylene.

Table 4.13: Instrumental Conditions for FAAS

	Mn	Fe	Cu	Zn
Slit width (um)	80	80	320	320
Wavelength (nm)	279.5	248.3	324.7	213.9
Lamp current (mA) }	5	10	5	5
Scale Expand (usual) }				
Integration time (s)	1	1	1	1
Range of standards (ug/ml)	0.5 to 5	0.5 to 20	0.3 to 5	0.3 to 5

Calibration graphs are given in Figure 4.11.

Figure 4.11: Calibration Graphs for Mn, Fe, Cu and Zn by FAAS (ILL 151)



	Mn	Fe	Cu	Zn
Gradient	0.297	0.1056	0.3293	0.3633
Error on gradient	0.0013	0.0037	0.0024	0.0214
Intercept on Y axis	0.0258	0.1094	0.00654	0.1462
Maximum and minimum values of intercept	0.0291 & 0.0224	0.1380 & 0.0807	0.01208 & 0.00099	0.1861 & 0.100
Correlation	0.99994	0.9950	0.9998	0.9912

ii) Determination and Results

The determination of Mn, Fe, Cu and Zn in plant samples was carried out by digestion of each sample with concentrated nitric acid (10 ml) followed by heating for 10 to 15 minutes under an IR lamp. The sample, after cooling, was filtered through a Whatman No. 541 filter paper, each filtrate passing into a 25 ml volumetric flask and being diluted with DDW to the mark.

The results are listed in Tables 4.14 and 4.15.

Table 4.14: Tissue Concentrations (µg/g) of Se, Mn, Fe, Cu and Zn in Shoots of Lolium perenne seedlings

Concentration of Se(VI) µg/ml in nutrient solution	Length (cm)	Tissue Concentration (µg/g)					Dry wt. (g)
		Se	Mn	Fe	Cu	Zn	
0.00 (control)	21.0	N.D.	81.55	139.7	22.85	114.4	0.394
0.10	21.9	6.11	76.10	135.4	21.56	83.2	0.42
0.50	20.7	15.7	67.5	130.7	20.6	69.7	0.415
1.0	23.0	28.4	62.3	128.0	21.10	65.4	0.39
2.5	19.2	155.6	65.6	155.3	22.5	61.3	0.36
5.0	17.0	375.2	63.32	145.4	27.4	59.5	0.30
10.0	10.5	880.2	48.7	136.6	32.7	58.7	0.173
15.0	8.7	1092.7	51.36	160.4	36.6	78.2	0.120
20.0	4.6	1352.5	54.2	213.0	38.1	67.6	0.080
30.0	3.2	454.0	72.5	190.6	51.0	125.8	0.076

Table 4.15: Tissue Concentration (μg/g) of Se, Mn, Fe, Cu and Zn in Roots of Lolium perenne seedlings

Concentration of Se(VI) μg/ml in nutrient solution	Length (cm)	Tissue Concentration (μg/g)					Dry wt. (g)
		Se	Mn	Fe	Cu	Zn	
0.00 (control)	17.0	N.D.	178.5	4330	84.6	238.4	0.117
0.10	17.1	3.1	174.4	5519	77.8	242.2	0.105
0.50	16.3	10.3	165.6	5336	100.3	219.3	0.106
1.0	16.0	17.2	154.6	3841	110.1	203.8	0.108
2.5	14.5	26.5	147.8	3265	122.4	190.1	0.102
5.0	12.3	137.45	138.5	5335	135.6	177.6	0.081
10.0	2.2	220.8	115.0	7112	126.8	116.8	0.033
15.0	0.8	357.5	91.2	11530	130.4	97.0	0.028
20.0	0.2	376.3	91.8	15420	252.3	714.5	0.018
30.0	0.0	403.8	92.26	14771	242.7	750.2	0.016

4.3 DISCUSSION

i) Analytical Techniques and Methodology

a) Determination of Selenium in River Water

Hydroxides which have a relatively large molecular weight probably via polymerisation, e.g. iron (III) hydroxide, tend to be absorbed on the walls of glass or polyethylene containers. As selenium (IV) is readily coprecipitated with such iron (III) hydroxide, major losses of selenium in natural waters may occur on prolonged storage. So the selenium should be determined as soon as possible (26). Accordingly, the pH of water samples was adjusted to around pH = 2 with conc. HNO_3, followed by the addition of concentrated hydrochloric acid (1 ml/L) just after sampling to prevent the formation of hydroxides.

All 18 water samples collected from the River Avon were examined for Se(IV) and Se(VI). Both total selenium and selenium (IV) were determined by the recommended procedures (27), using 3,5-dibromo-1,2-diaminobenzene as the reactive ligand. The selenium was converted to 4,6-dibromopiazselenol and extracted into toluene for determination. Because of the inadequate sensitivity of the 5-chloropiazselenol method, selenium was unfortunately not detected in river water samples collected in 1983 (40).

For the 1984 samples very sensitive ligands were used for the examination of river waters, which were found to contain 15-828 ng/L of Se(IV) and 52-2958 ng/L as total (dissolved) selenium. The complete results are shown in Table 4.3. The percentage of Se(IV) in the waters varied from 11.27 to 53.55% of the total Se, which seemed to decrease as distance from the original point of injection increased, the conversion of Se(IV) to Se(VI) probably reflecting the oxidising nature of the river.

When two new ligands were used in a comparison against the dibromo ligand (an already established reagent), the results were very close, as shown in Table 4.2.

b) Recovery of Selenium in River Water

The recovery aspects of both Procedure 1 and Procedure 2 were evaluated. A six-fold replicate experiment was carried out in which known amounts of selenium (IV) were added in a Procedure 1 experiment, and in Procedure 2 known amounts of selenium (VI) were added to river water. The samples were then treated as described for both procedures respectively. Consequently, in all cases, theoretical recoveries were obtained to emphasise that the results were within experimental error, all results being shown in Table 4.16. Clearly, the recoveries for Se(VI) were lower than for Se(IV) reflecting the difficulty of reducing Se(VI) to Se(IV).

c) Determination of Selenium in Plants

Figure 4.10 depicts the type of GLC traces recorded when using the piazselenol procedure (3-bromo-5-trifluoromethyl-1,2-diamino-benzene as ligand). The root material offers a cleaner chromatogram than the shoot samples, compare Figure 4.10, A versus B and C. In the latter case, a faster eluting piazselenol would suffer very serious interference problems especially from those peaks eluting at around 1.5 minutes to 2.8 minutes. The reagent used here represents a good compromise between sensitivity, low retention time and adequate resolution from interference peaks.

As a further check on the validity of the selenium procedure using the piazselenol reaction, a comparison was made of the values found using similar Lolium perenne material with values determined using the GFAAS technique. Table 4.12 lists the results for both roots and shoots material.

Table 4.16: Recovery of Selenium added to 250 ml River Water using 3,5-Dibromo Ligand

Added Se(IV) determined by Procedure 1

Added Se(VI) determined by Procedure 2
River Water at Kellaways (28/6/84)

Se(IV) added (ng)	Se(VI) added (ng)	Se found (ng)	Se recovery (ng)	Recovery %
None	None	6.20		
10.0	None	15.80	9.6	96
10.0	None	16.30	10.1	101
10.0	None	16.45	10.25	102.5
10.0	None	16.00	9.8	98
10.0	None	16.38	10.18	101.8
	None	15.3		
	10.0	24.1	8.8	88
	10.0	24.9	9.6	96
	10.0	24.6	9.3	93
	10.0	24.44	9.14	91.4
	10.0	24.8	9.5	95

d) Comparison of the Results of Two Different Methods for Selenium Determination in Plants

Another way in which the results of analytical method may be tested is by comparing them with those obtained when using a second method (41), preferably based on a completely different principle. In this case, from Table 4.12 15 root samples and 15 shoot samples were taken for calculation. So the same number of determinations is carried out with both methods (GLC and GFAAS), i.e. $n_1 = n_2 = 15$.

Consequently a simple equation can be used for a comparison t-test as below (42):

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2 + S_2^2}{n-1}}} \qquad (4.1)$$

where $\bar{x}$ = sample mean = x/n

s = standard deviation = $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$

Table 4.17 lists the two methods for selenium determination.

From Table 4.17 and Equation (4.1), the t-test for roots:

$$t_{root} = \frac{4.648 - 4.1813}{\sqrt{\frac{2.201247 + 1.48644}{14}}} = \underline{0.9093367}$$

Similarly for shoots:

$$t_{shoot} = \frac{3.323 - 2.957}{\sqrt{\frac{2.1824153 + 1.929099}{14}}} = \underline{0.6753738}$$

In students' distribution tables we find $t = 2.78$ for $(1-\alpha) = 0.99$; $\alpha = 0.01$ and degrees of freedom in this case $\nu = 2n-2 = 28$.

Table 4.17: Comparison of the Results of Two Different Methods for Determination of Selenium

Sample Trial No.	Root µg Se/g		Shoot µg Se/g	
	GLC	GFAAS	GLC	GFAAS
	x_1	x_2	y_1	y_2
1	5.2	3.86	1.35	1.30
3	4.32	4.10	1.34	1.40
4	4.73	4.75	1.64	1.62
7	4.48	3.88	1.45	1.51
10	2.93	2.75	4.97	5.32
11	5.15	5.50	6.10	5.93
12	1.48	1.66	2.15	1.78
13	3.10	2.86	3.76	3.60
14	2.95	2.77	2.67	2.70
15	5.27	5.47	3.68	2.44
16	5.40	4.83	4.15	3.16
17	7.05	5.65	4.70	3.46
18	5.30	4.48	3.84	3.75
19	6.11	5.56	3.95	2.76
20	6.25	4.60	4.10	3.62
Σx	69.72	62.72	Σy 49.85	44.35
$\bar{x}$	4.648	4.1813	$\bar{y}$ 3.323	2.957
$\Sigma(x_1-x)^2$	30.817432	20.8101735	$\Sigma(y_1-y)^2$ 30.555	27.00735
s	1.48366	1.219197	s 1.4773	1.38892

The calculated values, $t_{root} = 0.909$, $t_{shoot} = 0.675$, are substantially less than the tabulated value which is 2.78. Although the determination of series 1 and 2 have been carried out under identical conditions with different matrices, there is no significant difference between the two sets of results. Thus, the GLC method appears to be a very suitable method for the determination of the selenium content of material such as plant tissue.

ii) Toxicity of Selenium to Plants

From the results listed in Tables 4.10 and 4.11, an attempt was made to determine the upper critical level of selenium in the roots and shoots of the Lolium perenne seedlings which had been grown in selenium contaminated hydroponic cultures. The yield curves are shown in Figures 4.12 and 4.13 respectively using the procedure of Beckett and Davis (43) which has been described in further detail by Morgan (44). Basically, the method consists of plotting the log tissue concentration of Se (μg/g of dry weight of material) versus the yield (g of dry weight of material) and attempting to find the best regression lines to fit the data which afford a point of intersection. Thus a best line to fit for points representing the unaffected weights of biological tissue and low Se values is made parallel to the abscissa. Similarly another line (making an obtuse angle to the abscissa) is obtained from the unaffected dry weight values, these also having relatively high levels of selenium content. Where the two lines intersect is termed the upper critical limit or T_C value. In this case Se is slightly more toxic to roots than to the shoots. The lethal Se concentration (T_L) is deemed to be that where the negative slope line cuts the abscissa at yield equal to zero. Notice, in this case, the T_L value for the shoots is very much higher than the T_L value for roots. Also, selenium appears to be a non-essential element for these seedlings because even at very low concentrations of Se in solution, the yield of biological material is not affected by a deficiency of selenium. The Se exhibits a classical non-essential element characteristic curve.

Figure 4.12: Yield Curve of Root Yield Plotted Against Log Tissue Concentration
(Selenium Upper Critical Level)

△ Control

Y_o = Yield unaffected by toxicity = 0.105 g

T_C = Upper critical level = 82.1 ug/g

T_L = Lethal concentration = 518 ug/g

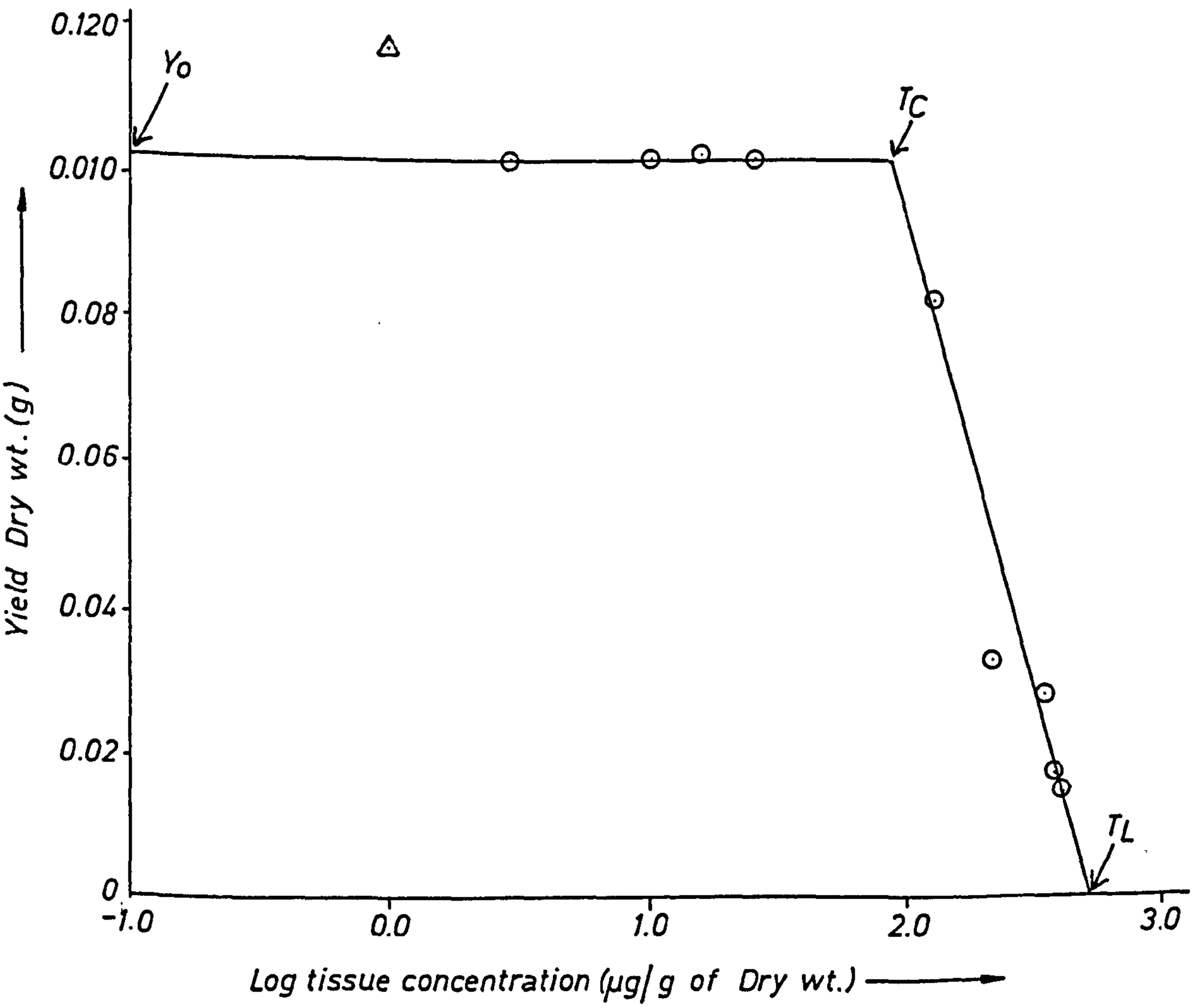


Figure 4.13: Yield Curve of Shoot Yield Plotted Against Log Tissue Concentration
(Selenium Upper Critical Level)

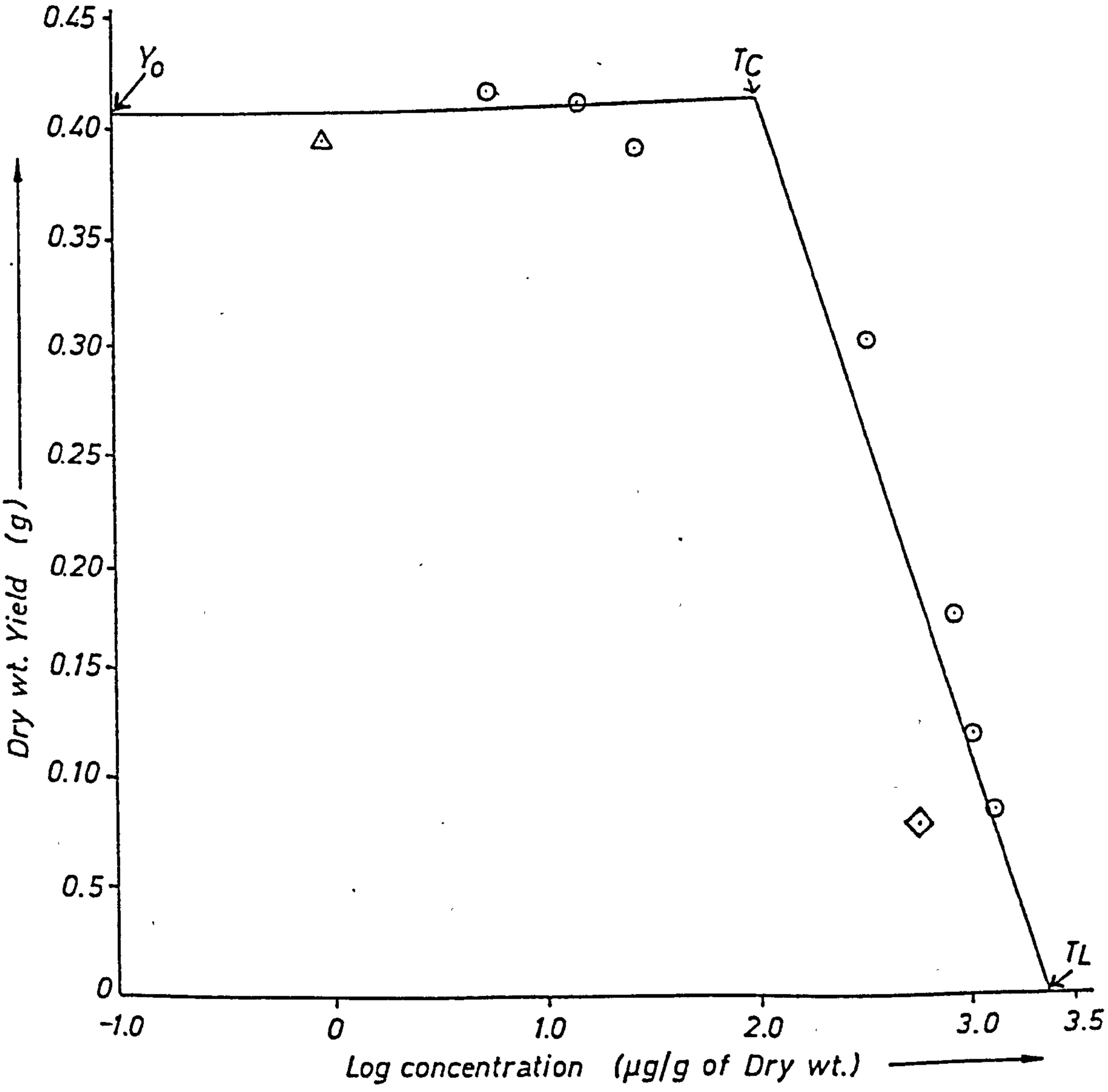
◇ This point was not used in the calculation as the plant was considered to be dead

△ Control

Y_o = Yield unaffected by toxicity = 0.41 g

T_C = Upper critical level = 116.6 ug/g

T_L = Lethal concentration = 2326.3 ug/g



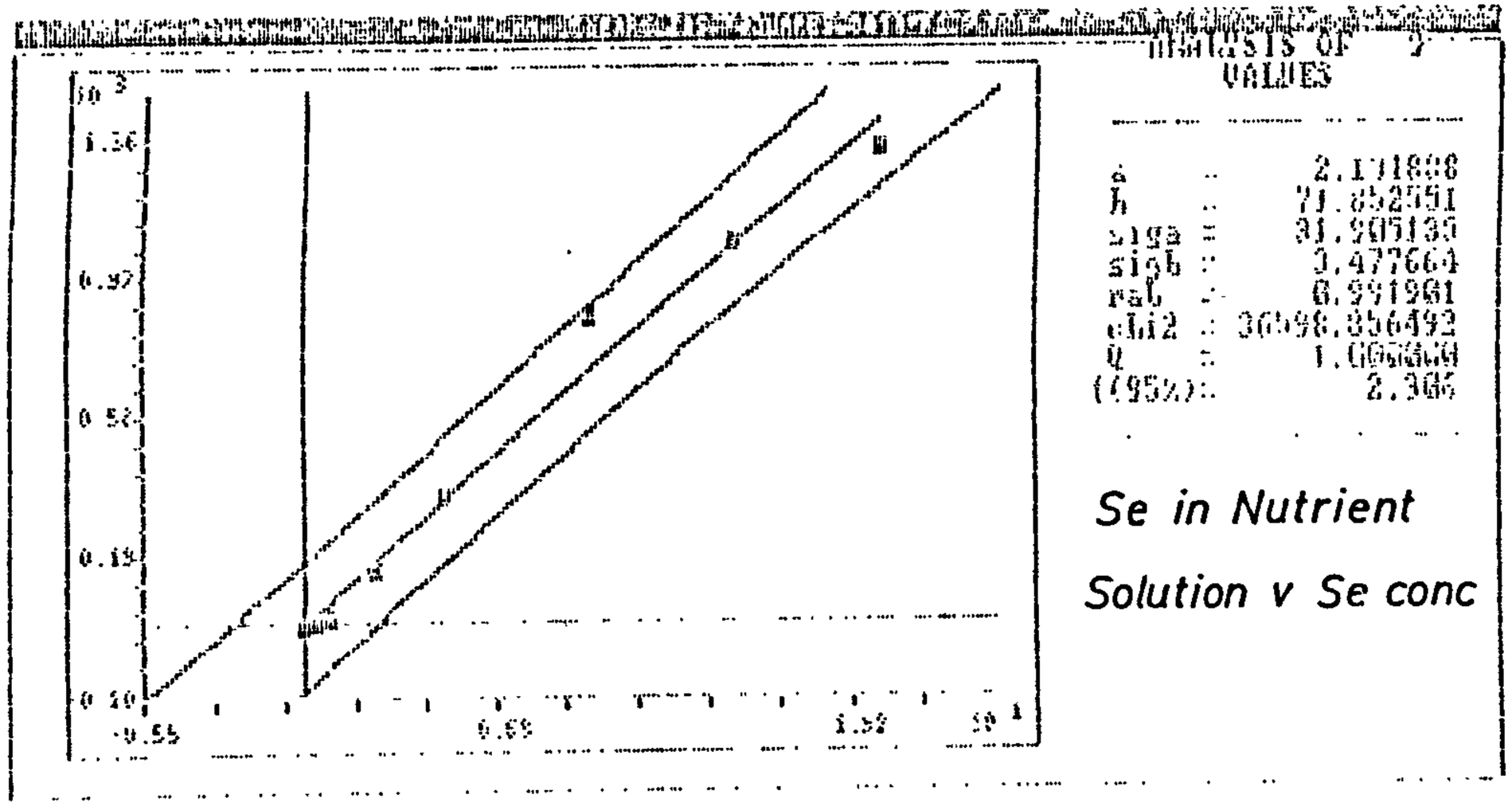
iii) Uptake of Selenium and Some Essential Metals (Mn, Fe, Cu and Zn) by Plant Samples

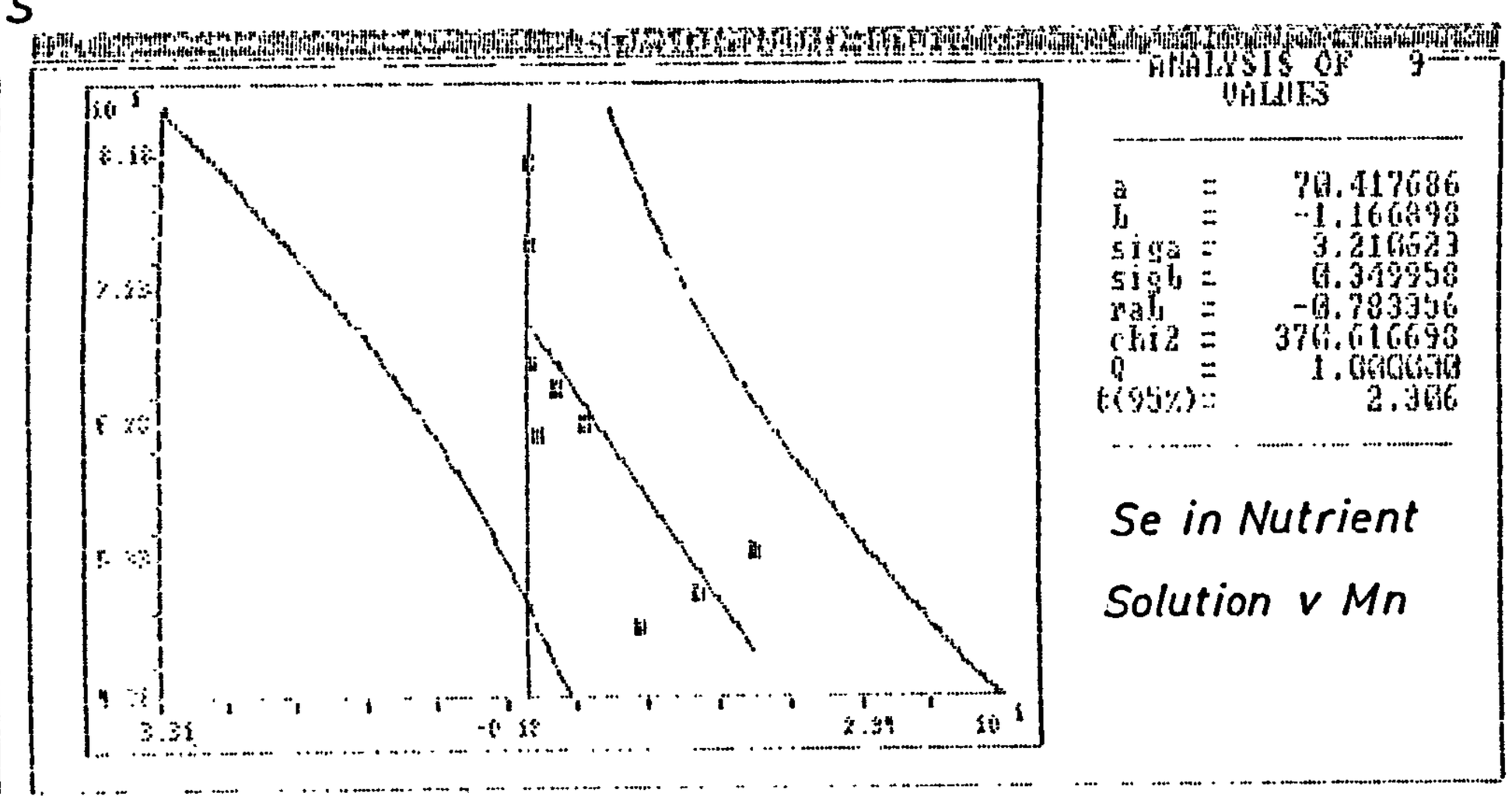
a) Shoots

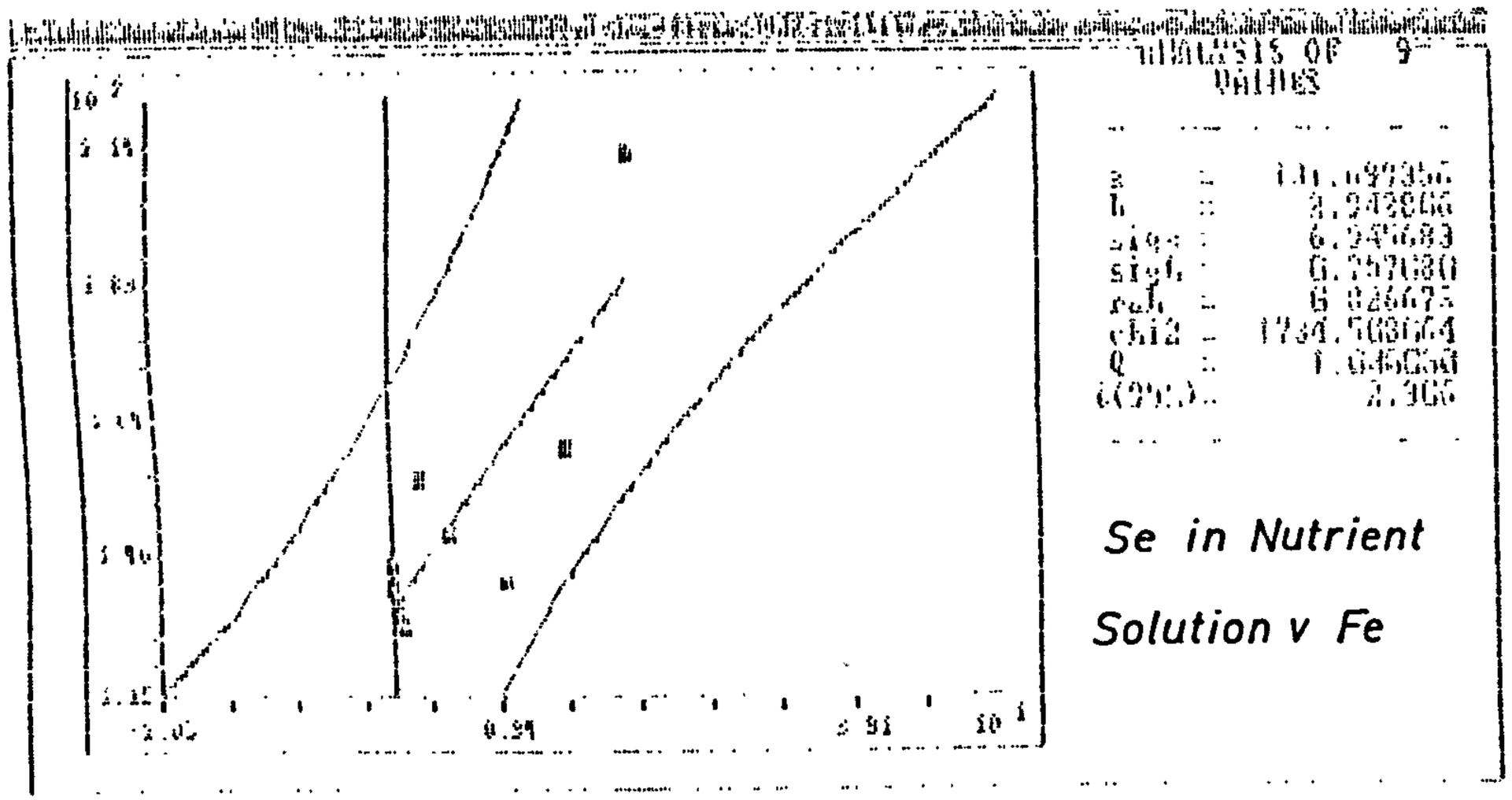
Table 4.14 illustrates the relationship between the selenium content in the nutrient solution and the uptake of Se, Mn, Cu, Fe and Zn. It is obvious that selenium uptake in the shoot increases with increase in selenium concentration in the nutrient solution, except at the 30 μg Se/ml level which decreases because the plant was completely dead. At up to the same concentration (30 μg Se/ml) the plant appeared to take up more of the essential metals like Mn, Fe, Cu and Zn, probably via a mechanism to overcome the selenium toxicity. Thus the Lolium perenne seedlings directly reflect the value of the selenium concentration fed to them, the species being neither an accumulator nor an excluder of Se. Figure 4.14 illustrates the relationship between selenium content in the nutrient solution and concentration of Se, Mn, Fe and Cu in the shoots.

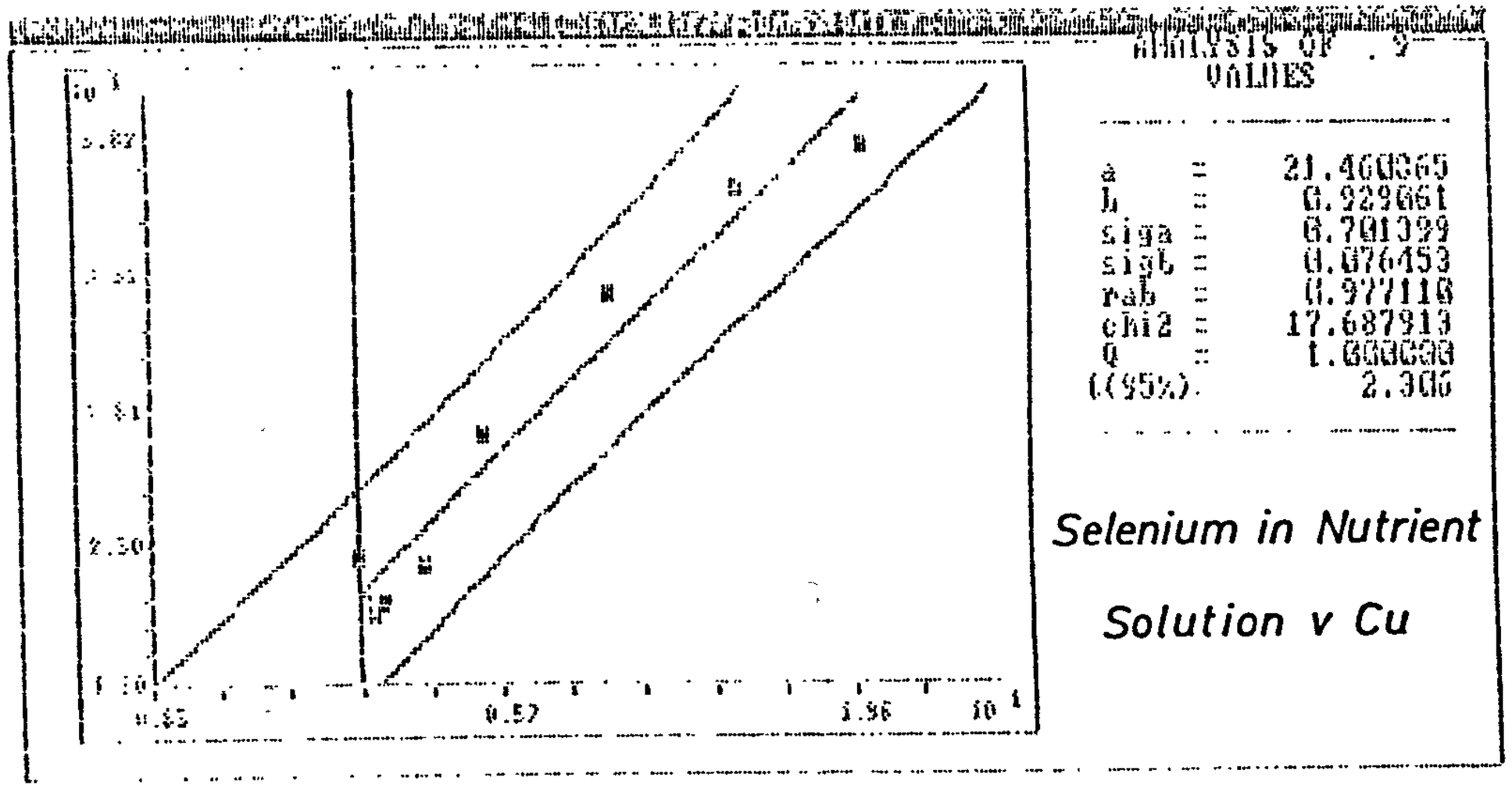
Generally, Mn uptake slightly decreases when selenium increases, but the uptake of Fe and Cu is more constant, until around 10 μg Se/ml; after this concentration, iron uptake increases. Obviously for Zn the uptake decreases when selenium increases. However, if the 30 μg/ml Se results are excluded because of the dead tissue, generally the Mn uptake was decreased slightly when the Se concentration in solution increased. The result is confirmed when a correlation plot is made with a negative slope and an r value of -0.78. But in the case of Cu and Fe, the uptake is almost constant until a level of 10 μg/ml of Se in solution is reached. At and after this concentration, the iron uptake increases in an approximately linear manner. For Cu the result is even more linear with an r-value of 0.977. However, for zinc the results are very difficult to interpret since the level of Zn appears to remain constant, when more than 0.1 μg/ml of Se is present in solution. If there is an

FIG. 4.14 RELATIONSHIP BETWEEN SELENIUM IN NUTRIENT SOLUTION AND SELENIUM, Mn, Fe and Cu [concentration (µg/g)] IN SHOOTS









interaction between Se and Zn it must be at levels below 0.1 μg/ml of Se in solution.

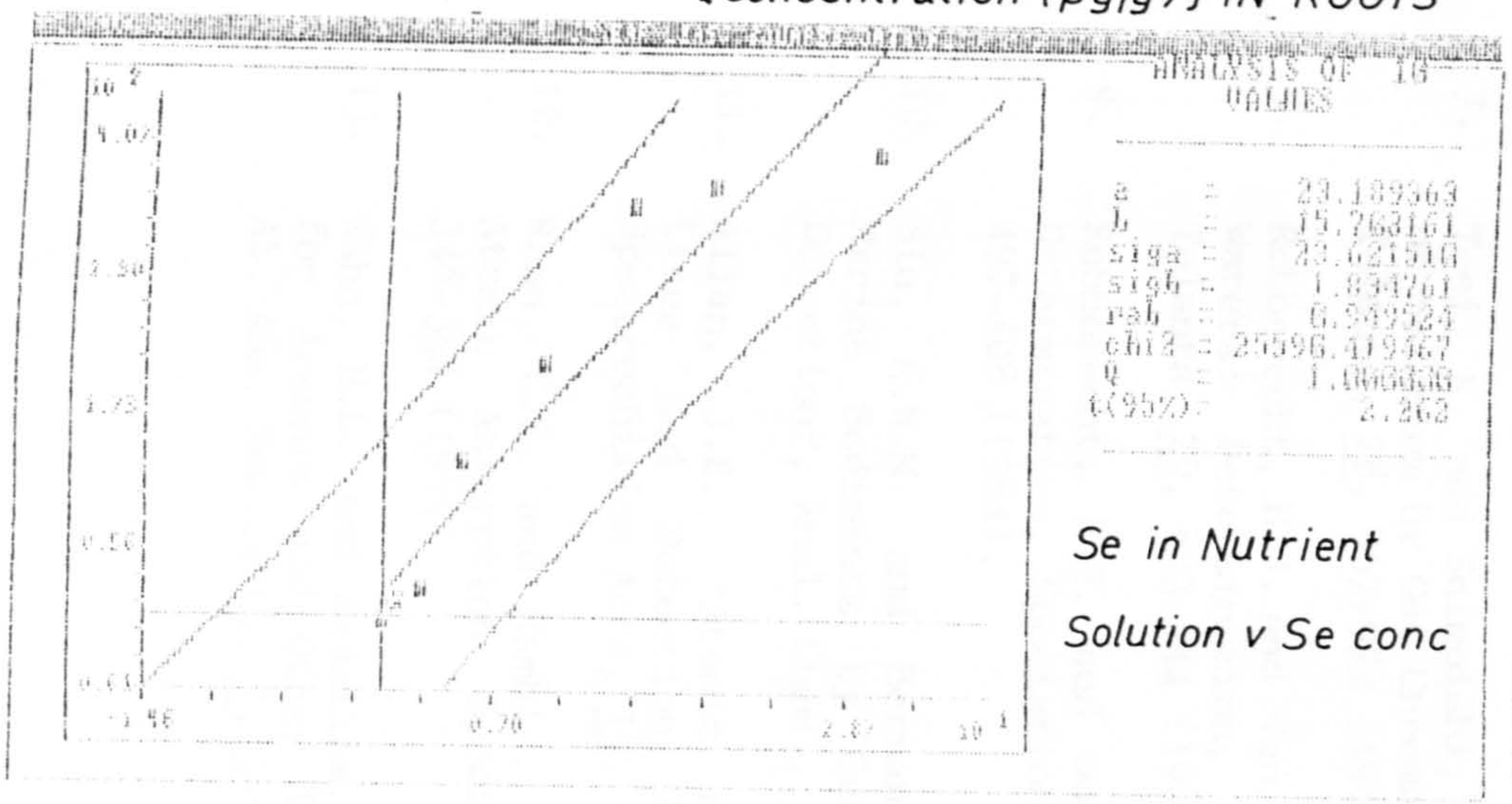
b) Roots

Table 4.15 shows the uptake of selenium and essential metals (Mn, Fe, Cu and Zn), while Figure 4.15 illustrates the relationship between selenium content in the nutrient solution and selenium concentration, and Mn, Fe and Cu in the roots.

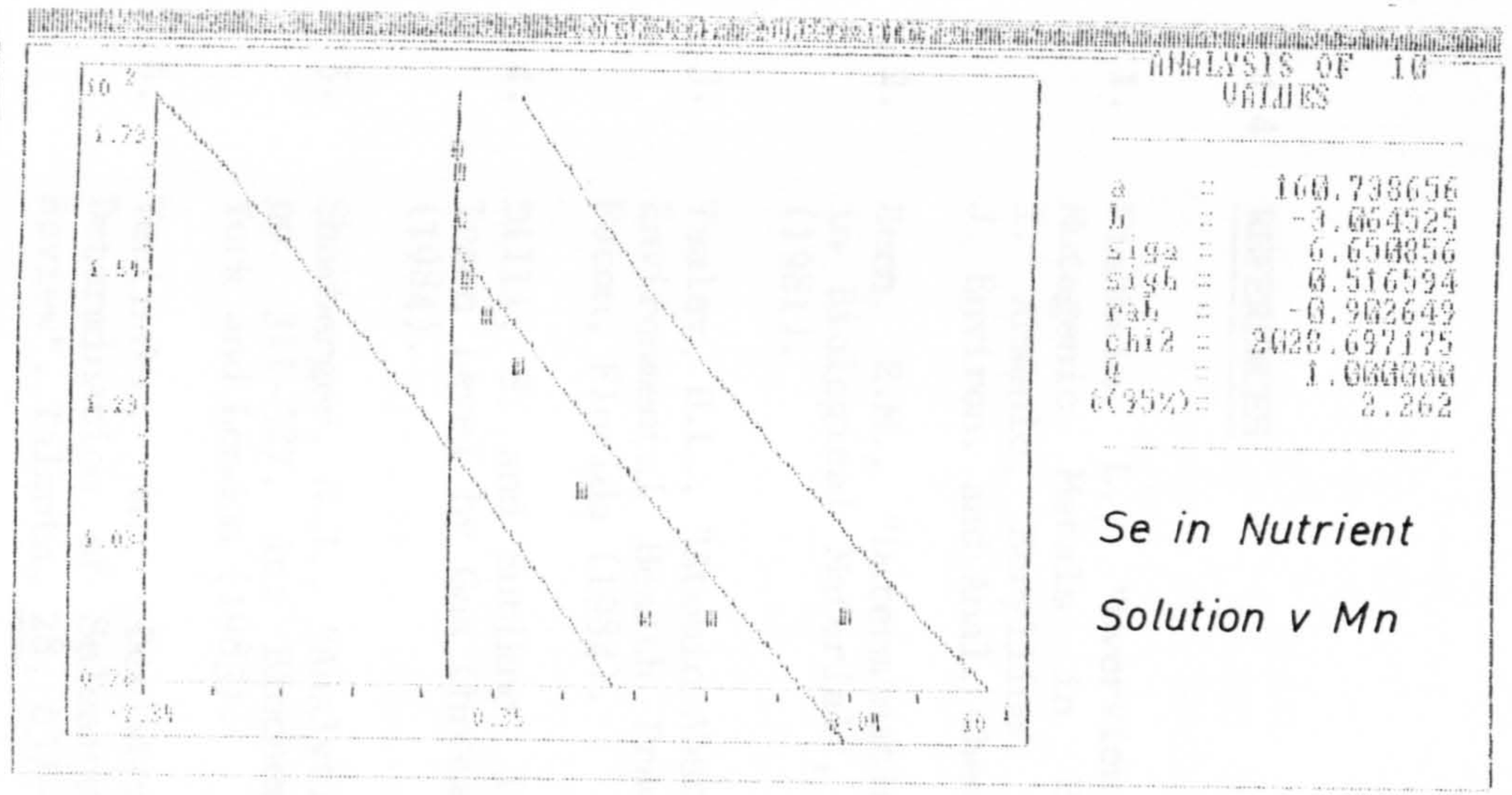
Not surprisingly the uptake by roots of selenium and the essential metals (Cu, Fe, Mn and Zn) were similar to the shoot uptake. The major difference was that the roots retained less selenium than did the shoots while the reverse was true for the four elements already mentioned. So there appears to be a linear relationship between selenium content in the nutrient and selenium concentration in the roots, and fairly linear relationships also between selenium nutrient content and the levels of Cu, Fe and Mn in roots. The zinc root-selenium nutrient relationship, whilst better (i.e. more linear) than the Zn shoot-Se nutrient level is still not a clear relationship. The intriguing relationship is the loss of Mn with increase in Se level both for roots and shoots whilst for Cu and Fe there is a marked increase for both elements. Perhaps there is a linkage between the role of Fe and Mn, in that there is some competitive action between these two elements. For example, does the increase in Fe content mean direct competition with Mn and hence loss of Mn. Again, the effects of levels of Se content below 0.1 μg/ml in the nutrient need to be investigated.

The uptake of selenium and other essential metals as well as certain toxic metals will be discussed in detail in Chapter 6.

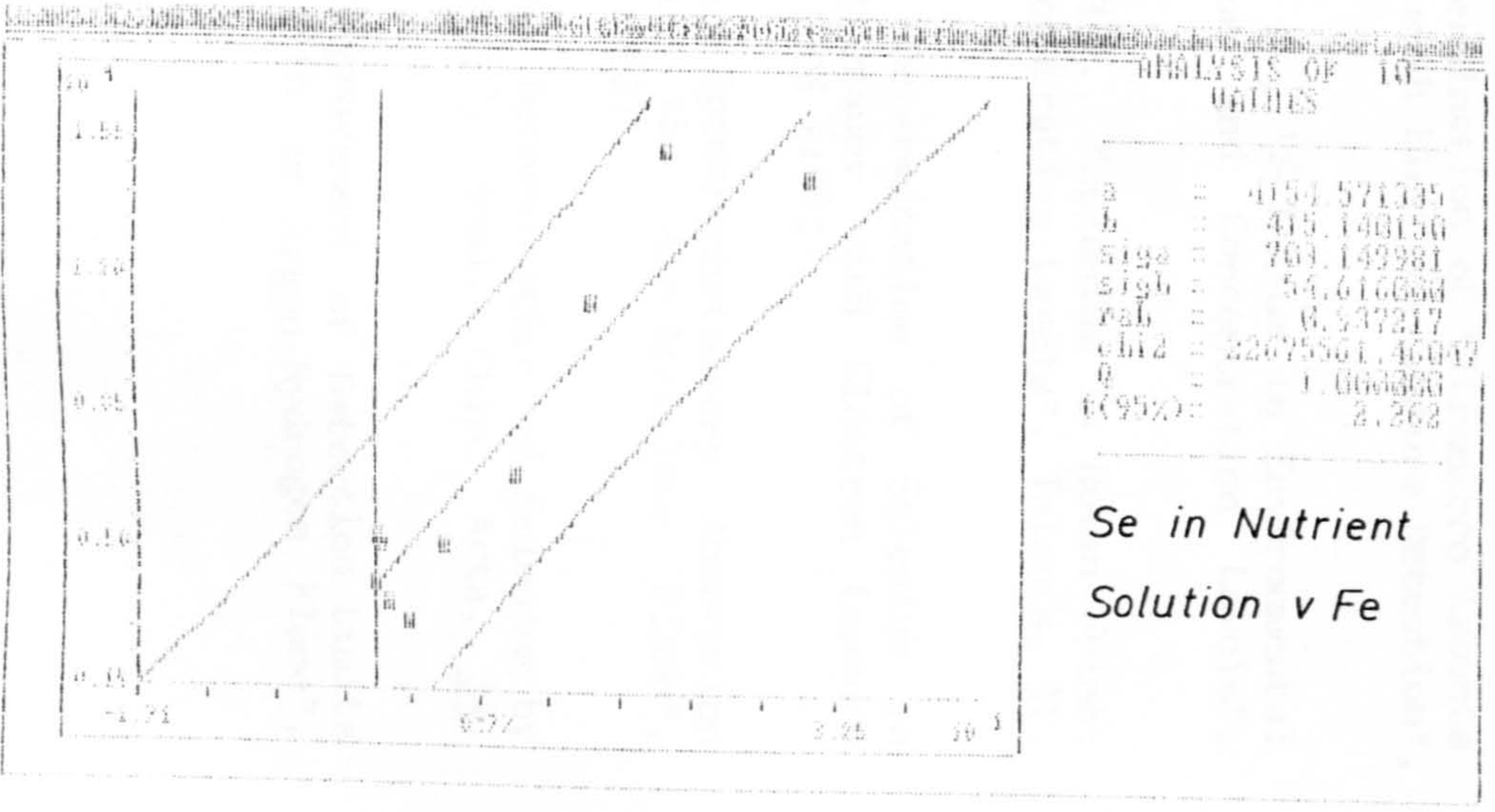
FIG. 4.15 RELATIONSHIP BETWEEN SELENIUM IN NUTRIENT SOLUTION AND SELENIUM, Mn, Fe and Cu [concentration (µg/g)] IN ROOTS



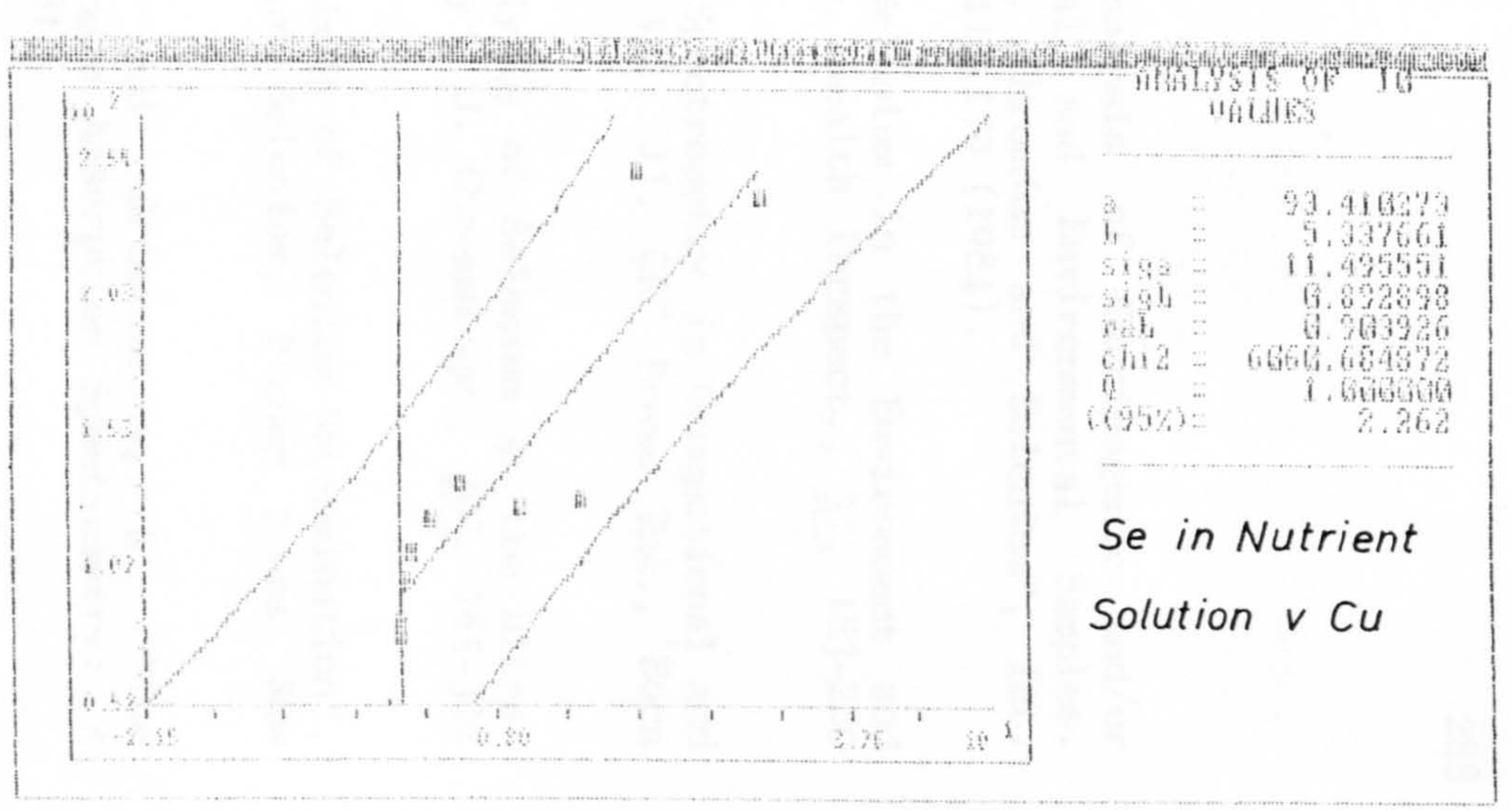
Se in Nutrient Solution v Se conc



Se in Nutrient Solution v Mn



Se in Nutrient Solution v Fe



Se in Nutrient Solution v Cu

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CHAPTER 5

CADMIUN , MERCURY , THALLIUM AND TELLURIUM DETERMINATION AND EFFECTS ON PLANTS

CONTENTS

5.1 INTRODUCTION

The interest arose in heavy metals because some of them are essential for maintenance of normal growth and development of many biological systems, while others are toxic, even highly so (1). Heavy metals can enter the food chain via plant uptake or contamination of fresh or marine waters (2).

The present chapter focuses on the determination of cadmium, mercury, thallium and tellurium. However, since tellurium was discussed in detail in Chapter 1, it is advantageous to offer a brief discussion of the other three toxic metals of interest in this dissertation, Cd, Hg and Tl.

i) Cadmium

Cadmium was discovered in 1817 by Stromeyer who isolated it from zinc carbonate (3).

Cadmium is a member of Group IIB (between zinc and mercury) of the Periodic Table (4); it is a strongly chalcophilic element, i.e. it is concentrated in sulphide minerals, in which characteristically it follows zinc and mercury and to a lesser extent lead and copper. Few cadmium minerals are known: chiefly greenockite (hexagonal CdS), the most common one; cadmoselite (CdSe). All other minerals are rare and are not commercial sources of the metal (5). The element is also encountered in zinc ores, zinc-bearing lead ores, or complex copper-lead-zinc ores. For this reason, Cd is almost invariably recovered as a by-product from the processing of such minerals (4).

The crustal abundance of Cd is somewhere between 0.1 and 0.5 μg Cd/g (4). The average concentration of Cd in igneous rocks is low (0.1-0.23 μg/g), while it tends to be enriched in shales (1.3 μg/g)

and oceanic sediments or phosphorites but depleted in red shales, sandstones and limestones relative to igneous rocks (5). However, Cd concentrations in most soils range between 0.5 to 1.0 μg/g, but concentrations of over 20 μg Cd/g occur naturally in some places in England (6).

Elemental Cd is used principally as an electroplated coating on fabricated steel for corrosion protection, whilst some is used in batteries, Cd being the negative electrode in rechargeable Ni-Cd and Ag-Cd batteries (4). Cadmium compounds are extensively used as pigments, plastic stabilisers, glass products, alloys, rectifiers, electrical and electronic industries (5,7).

Cadmium is not an essential trace element for organisms, but rather a typical contaminant. The concentration and distribution in organisms is influenced by the concentration in the environment (7). Cd is a potentially hazardous pollutant in the environment, and chronic human exposure to low concentrations of this element in the atmosphere, water or food may cause serious illness and possibly death (8). Deaths from acute Cd poisoning have resulted from inhalation of cadmium oxide fumes, and $CdCl_2$ aerosols (lethal dose = 6 mg Cd/m^3 over an 8h period) which cause severe lung damage in the form of pneumonitis accompanied by abdominal pain and nausea. Other results of chronic exposure include bone effects, anaemia, liver dysfunction and emphysema (4).

In Cd-contaminated environments, very high enrichment rates are found in cabbage, carrots, radishes, lettuce, potatoes and turnip roots. Obviously Cd is readily taken up by roots and distributed throughout the plant. Corn roots contained 2-4 times as much Cd as did shoots when grown in solution culture (7).

ii) Mercury

The commonest ore of mercury is the red sulphide, cinnabar (HgS), from which the element can be obtained by simple heating in air (9). The Phoenicians exploited cinnabar in Spain from the 8th century B.C. In the 5th century B.C. cinnabar was used as a pigment for cave and body decoration as well as for medical preparations. The principles of amalgamation were used by the ancient Egyptians, Greeks and Romans, but Aristotle is reputed to be the first in Europe who mentioned the metal itself, in the 4th century B.C., when it was used in religious ceremonies (10,11).

Throughout the Middle Ages (the Arab Period), mercury compounds were used for medicinal purposes by Arab physicians. Some kind of theoretical basis for the practical side of alchemy can be found in the "Jabirian corpus", a collection of writings in Arabic put together in the 9th century under the name of a certain Jabir ibn Hayyan, apparently an historical personage, though a shadowy one. According to "Jabir", all metals are made up of mercury, sulphur, and arsenic; to change a base metal into gold it was necessary to alter the proportions of mercury, sulphur and arsenic in the metal concerned (9). The endeavour to cure syphilis with mercury and its compounds persisted until the 19th century, although the toxic nature of mercury and the danger of mercury vapour had been demonstrated and reported in 1493 (10).

In nature, Hg occurs mainly in combination with sulphur to form more than a dozen different minerals. Commercially, the most important one is the red sulphide, cinnabar (HgS) (86.2% Hg) (11), and in lesser amount as the black sulphide (metacinnabar) (Hg,Zn,Fe)(S,Se) (10). Mercury is used in numerous and varied applications dependent mainly

on its physicochemical properties such as uniform volume expansion, electrical conductivity, battery, toxicity, and ability to alloy with other metals (11).

Before 1850, mercury was mined mostly in Spain, Yugoslavia and Peru. In the second half of the 19th century, mines in Italy and California became prominent, whereas more recently mines in the USSR, Algeria and China have produced significant quantities (11).

The average proportion of mercury in the earth's crust is about $5 \times 10^{-5}\%$ (0.5 ppm); it thus comes 62nd in order of abundance; for example the concentration in seawater is very low - about $3 \times 10^{-9}\%$ (0.03 ug/L) (12).

The major anthropogenic release of mercury into the environment began with the Industrial Revolution. In 1892 a new technique for the production of chlorine and caustic soda by electrolysis using a mercury cathode was introduced (the Castner-Kellner process). Moreover, in 1900, organo-mercury compounds were introduced as fungicides to treat seeds and from about 1950 as slimicides. The quantities discharged into the environment remained unnoticed and were disregarded until the serious hazards which occurred in the 1950s in Japan and Sweden brought the problem to light (10).

Mercury metal, its vapours, and most of its organic and inorganic compounds are protoplasmic poisons that can be fatal to humans, animals, and plants. The most toxic are the organic mercury compounds, such as the alkyl types (11).

Although the toxicity of mercury has long been known, the recent widespread concern regarding the pollution of the environment by mercury started with these incidents in the 1950s in Japan where many people were poisoned after consuming seafood which contained

between 27-102 ug/g (wet weight) of methyl mercury (13).

The most catastrophic outbreak of methyl mercury poisosning recorded took place in Iraq during the winter of 1971-1972. More than 6,000 patients were poisoned by methyl mercury when they consumed home-made bread prepared from wheat which had been treated with a methyl mercury fungicide. The wheat had originally been intended for sowing (14).

Factors that determine the effect of mercury poisoning on humans are:

i) the amount and rate of absorption,

ii) the physicochemical properties of the compounds, and

iii) individual susceptibility (11).

The incidents in Japan and Iraq were a result of almost direct poisoning attained as a result of man's ignorance of the movement and transformation reactions of mercury in the environment.

However, the uptake of mercury into vegetable crops grown in unpolluted and polluted soils has now been studied (15). Highly polluted soils contained higher levels of Hg (0.495 ppm) than those in the unpolluted soils (0.035 ppm). The topsoil was highly enriched with regard to Hg (than the subsoil) as the result of deposition of Hg particulates produced from an industrial area. Plants grown in topsoils near such industries contained high levels of Hg, up to 0.362 ppm. The amounts of Hg in plant species which were grown in winter were higher than those grown in summer (15).

Alkyl mercuric compounds, RHgX, are no longer manufactured in most of the world because of the long-lasting toxic hazards they present, and their destructive effect on the brain and central nervous system where they tend to accumulate. In general, they are white,

stable solids of appreciable volatility and are often prepared by a Grignard reaction in ethyl ether ($RMgX + HgX'_2 \longrightarrow RHgX' + MgXX'$) (11).

From a toxicological point of view it is necessary to distinguish between three classes of organomercurials: aryl, alkyl and alkoxyalkyl. Between them, these three groups span the entire toxicological spectrum from life-saving medicines to highly lethal poisons. Of the three, the alkyls are by far the most toxic (16).

Short chain alkyl mercury compounds are more soluble in lipids than are mercury (II) or elemental mercury. They are also about 100 times more soluble in lipids than in water, enabling CH_3Hg^+ to penetrate more readily into cells than inorganic forms (10).

iii) Thallium

Thallium was discovered in 1861 by Sir William Crookes, who, while searching (by spectroscopy) for tellurium in residues from a sulphuric acid plant, noticed a green line in the emission spectrum never seen before. He named it "thallium" or green branch (3).

The electronic configuration of thallium (Tl) is $4f^{14}\ 5d^{10}\ 6s^2\ 6p^1$, and so thallium can exist in two valence forms, the monovalent and trivalent states (17).

Thallium is not a particularly rare metal and its abundance in the earth's crust is 0.3 ppm. It occurs not only in oxide minerals but also as a chalcophilic element (sulphide). The metal commonly occurs in potash minerals and in a number of thallium-containing minerals. Of these, crookesite $(Cu,Tl,Ag)_2Se$ "17% Tl" and lorandite $TlAgS_2$ "59% Tl" are the most important (18).

Thallium has limited commercial applications because of its toxic nature (18). However, the metal or its salts have been used in certain alloys, optical lenses, jewellery, low-temperature thermometers, dyes and pigments and as a catalyst in scintillation counters. Thallium (I) sulphate is registered as a rodenticide and insecticide (19). Many thallium compounds have been used as reagents in organic syntheses (18).

Thallium concentration in seawater is 10-20 ng/L, and in freshwater 2-40 ng/L (17). Bowen gives the range of soil Tl concentrations as 0.1-0.8 µg Tl/g (20). Thallium also occurs in sediments; low concentrations of Tl occur in calcareous sediment (0.08 µg/g); but high concentrations of Tl in sediments containing abundant ferromanganese nodules (4.9 µg/g) (21).

Thallium in soil occurs in chemical forms that do not allow a rapid "wash out". Tl introduced from the atmosphere is generally available to the biosphere. In fact 75%-100% of the Tl contents of flue gas dust from the cement industry are soluble in water. However, this rate rapidly decreases with time after introduction into the soil (17).

The relative toxicities of Tl compounds depend on their solubilities and valence states. Souble monovalent Tl-compounds, e.g. the sulphate, acetate and carbonate, are especially toxic. They are rapidly and completely absorbed from the gastrointestinal tract, skin peritoneal cavity, the mucous membranes of the respiratory tract, mouth and lungs following inhalation of soluble thallium salts. Insoluble compounds, e.g. thallous sulphide and iodide, are poorly absorbed by any route and are much less toxic.

Thallium does not occur naturally in tissue and it is not essential to mammals, but it does accumulate in the human body. The lethal dose for man is not definitely known, but 1 g of adsorbed thallium is considered sufficient to kill an adult and 10 mg/kg body weight has been fatal to children (18).

Thallium and its compounds are highly toxic to biota. Plants take up Tl via both roots and foliage. The proportion between the Tl content in soils and the concentration in plants is relatively constant under otherwise equal conditions. The distribution of Tl in plants is similar to that of the nutrient ions, especially potassium (17). Tl inhibits chlorophyll formation and seed germination. Tl uptake via roots shows discolouration in the leaf which results from degradation of the chlorophyll (21).

5.2 EXPERIMENTAL AND RESULTS

5.2.1 General Aspects of Ecological and Aquatic Chemistry

Certain heavy metals have gained great significance in chemical and toxicological studies of the environment (22). The most hazardous and important heavy metals are Pb, Hg, Cd, As, Tl and Se. These metals have a history as occupational hazards and some of them have been linked to cancer and heart diseases (23). Also, they are generally toxic even at very low levels, but the potentially toxic metals (e.g. Cu, Zn, Ni) also have indispensable essential properties with different threshold levels for different types of plants and organisms, including man. In contrast to most of the numerous other environmentally important chemicals, metals have the particular property of not being biologically degradable. Instead, they are accumulated by plants and organisms and undergo a biogeochemical cycle in the environment during which they are transformed into various chemical species (complex and/or compounds). Of particular importance for the transfer of metals through the environment are all types of natural waters as well as the atmosphere (22).

5.2.2 Determination of Cadmium

The normal Cd concentrations in biological materials range broadly from subnanograms per millilitre to a few nanograms per millilitre in body fluids and up to micrograms per gram levels in some tissue specimens (hair, kidney, liver) (24). The most sensitive analytical techniques for the determination of nanogram and subnanogram amounts of Cd are GFAAS (25) (the method of choice nowadays), and ASV or DPASV (24).

Instrumental methods of determination are needed which combine high sensitivity with high accuracy, i.e. allowing a small risk of systematic error, with satisfactory precision. Reasonable rates of determination (especially simultaneous determinations), convenience in operation, compactness of instrumentation and reasonable costs are necessary for instrumentation and are really important features of any useful method (22).

The AAS determination of Cd is remarkably selective and sensitive. The characteristic concentration range in a lean air-acetylene flame is of the order of 9 to 25 ng/ml while the DLs are down to a level of 0.7 to 2 ng/ml. No major interferences should be expected from the background absorption, transport interferences, and non-specific bulk-matrix effect from an excess of total dissolved solids. All these effects are easily accounted for (24).

The GFAAS of Cd is extremely sensitive but prone to serious matrix effects. The characteristic concentration range is from 0.008 to 0.03 ng/ml while the DLs are down to 0.1 to 2 pg (26).

The DLs of Cd by ASV are as low as 0.001 to 0.01 ng/ml, this method is now the best alternative to GFAAS in ultratrace Cd assays. The apparatus is relatively inexpensive and a few more elements (Pb, Cu and Zn) can also be determined in the same digest (or matrix) (22,24,27,28).

i) Determination of Cadmium in Plant Samples by AAS

In order to study the effect and uptake of cadmium as cadmium acetate by the roots of plants with time, an experiment was carried out with Lolium perenne, similar to the uptake of selenium by plants (see Chapter 4, page 263).

Determination of the cadmium content of the acid digested (conc. HNO_3) samples was carried out using FAAS (ILL 151) and a Varian AAS 775 with GTA-95. The results are listed in Table 5.1.

However, the determination of cadmium content in Experiment 1 (Se, Cd, Hg) (which will be discussed in detailed in Chapter 6) of the acid digested samples was carried out using the Varian AAS 775 with GTA-95.

ii) Determination of Cadmium and Some Heavy Metals in Water Samples by GFAAS

Cadmium and other heavy metals (such as Cu, Cr, Ni, Pb and Zn) were determined in all the water samples collected from the River Avon (see Figure 4.1, page 243, for location sites).

While little sample treatment beyond filtration and acidification (using HNO_3) was required prior to the measurement of the major element composition of waters, the sensitivity of atomic spectrometric methods is rarely adequate to permit the direct determination of trace elements (such as Hg, Se, Te and Tl) in natural waters at the ng/L level, so preconcentration of the analyte and a degree of separation from the sample matrix are typically required prior to analysis (29), a process prone to contamination and one which is often tedious and lengthy.

Table 5: 1 . Tissue Concentrations of Cd, Mn, Fe, Cu and Zn (µg/g) in Shoots and Roots of Lolium perenne Seedlings.

Cd µg/ml in nutrient sol.	length* (cm)	Cd	Mn	Fe	Cu	Zn	Dry wt (g)
Control Shoot	28.5	ND	90.52	148.3	14.35	60.70	1.041
1×10^{-3}	28.7	0.848	85.36	143.00	15.40	87.90	0.936
5×10^{-3}	28.3	1.183	83.40	127.80	17.37	94.00	0.870
0.01	28.0	2.50	81.05	137.6	18.83	114.80	0.784
0.05	25.4	4.35	77.45	125.10	18.50	128.20	0.626
0.10	24.7	7.25	82.75	135.20	21.90	135.40	0.658
0.5	23.1	30.10	76.80	141.40	23.15	113.70	0.495
1.0	21.8	66.80	73.50	139.50	25.30	131.2	0.515
2.5	15.2	103.21	54.70	115.60	29.10	61.15	0.452
5.0	10.7	195.53	45.60	83.20	14.25	40.36	0.435
Control Root	17.2	ND	205.80	1872.3	47.80	229.6	0.229
1×10^{-3}	17.4	4.70	186.70	2760.6	50.10	217.30	0.180
5×10^{-3}	16.0	14.10	180.10	2951.3	52.60	201.80	0.177
0.01	15.3	17.87	174.20	3210.0	77.55	209.50	0.162
0.05	14.6	41.30	332.60	3471.0	85.30	163.7	0.155
0.10	12.5	85.70	168.40	3837.0	92.25	155.6	0.160
0.50	11.8	134.15	115.35	4152.0	100.75	138.2	0.138
1.0	10.5	307.80	60.20	4380.0	105.10	86.75	0.127
2.5	9.0	713.20	52.50	3617.0	81.15	71.44	0.115
5.0	5.8	1061.85	40.70	3705.0	73.60	68.25	0.089

* Average of five plants
Elongation from start of experiment

Several workers have utilised electrothermal atomisation (GFAAS) in the analysis of seawater for trace elements (29). Grobenski et al. (30) determined As, Cd, Mn, Mo and Ni directly in the seawater and claimed good agreement with certified values.

The investigation and optimisation of electrothermal atomisation parameters for the simultaneous multi-element determination of Co, Cr, Cu, Fe, Mn, Mo, Ni, V and Zn in acidified water standards has been reported (31).

In this case, the procedure suggested in (30,31) was applied to determine Cd and some other heavy metals directly in water samples using a Varian AAS-775 with GTA-95. The results obtained from GFAAS are shown in Table 5.2.

iii) Determination of Cd, Cu, Pb and Zn in Water Samples by ASV

Heavy metals, such as Cd, Cu, Pb and Zn, are frequently determined by differential-pulse anodic stripping voltammetry (DPASV) at a mercury electrode. If the hanging (HMDE) or stationary (SMDE) mercury drop electrode can be applied, these four metals may be determined simultaneously with the preconcentration process being achieved by applying a plating potential of -1.2 V vs. Ag/AgCl for several minutes with stirring of the solution (22).

a) Reagents

AnalaR and AristaR reagents were used whenever possible; these were:- hydrochloric acid, nitric acid, acetic acid, sodium acetate, cadmium (II) acetate, cadmium (II) nitrate, copper (II) nitrate, lead (II) nitrate and zinc (II) nitrate.

Table 5.2: Trace Metals in River Water Samples by GFAAS

Sample		Concentration (μg/L)					
Date of collection	Site	Cd	Cu	Cr	Pb	Ni	Zn
28/6/1984	Kellaways	0.73	6.10	1.37	2.55	65.0	416
	Reybridge	0.93	7.70	1.10	4.55	17.8	234
	Melksham	0.43	2.93	1.30	4.80	40.2	90
24/7/1984	Malmesbury	0.42	5.24	0.45	2.40	6.8	317
	Dauntsey	0.42	1.20	0.30	1.50	9.3	30
	Kellaways	0.77	1.40	1.15	1.15	10.1	94
	Reybridge	0.75	1.64	1.05	1.70	13.2	65
	Melksham	1.75	3.17	1.40	2.90	76.0	73
	Staverton	1.54	21.90	2.30	3.30	61.4	178
	Limpley Stoke	0.94	2.44	4.55	1.50	10.8	35
	Newbridge, Bath	0.55	1.40	2.45	1.40	10.5	20
	Keynsham	1.40	24.00	2.50	3.50	44.3	192
23/8/1984	Kellaways	4.10	7.00	1.15	1.20	6.9	35
	Reybridge	5.00	2.36	1.40	1.70	11.6	34
	Melksham	1.80	1.42	0.15	6.40	12.4	70
	Staverton	1.70	1.13	0.35	0.75	18.7	108
	Limpley Stoke	1.93	1.76	1.25	2.00	5.9	80
	Newbridge, Bath	1.30	24.20	1.60	4.50	8.7	22

b) Preparation of Solutions

Stock solutions containing 1000 µg of the appropriate metal per ml in HNO_3 (pH ~ 1) were prepared by dissolving the necessary weights of the metal nitrates in AristaR nitric acid ~5 ml and DDW, and diluted to a final volume of 500 ml. Acidification at pH 1.5 or less was necessary to ensure that the metals remained in solution (32,33).

The acetate buffer (containing 1M acetic acetate) was prepared by dissolving 34.02 g of sodium trihydrate in 150 ml DDW and 14 ml of glacial acetic acid (17.5M) and diluted to 250 ml with DDW and adjusting the pH to equal 5.

c) Apparatus and Instrumental Parameters

Anodic stripping voltammetry was carried out using a Princeton Applied Research (PAR) Model 174A Polarographic Analyser in conjunction with a PAR 315A Automated Electrolysis Controller, and a Hewlett Packard 7040A X-Y Recorder.

The working electrode was a Model 303 Static Mercury Drop Electrode (SMDE). A silver/silver chloride reference electrode and a platinum counter electrode were used to complete the three-electrode system.

A purge tube feeding purified nitrogen was also connected to the cell chamber in order to remove dissolved oxygen from the sample during the purge stage. The solution was stirred at a constant rate with a Model 305 stirrer and a magnetic bar.

Instrumental Conditions

i) Model 303:

Working electrode	HMDE (medium size)
Reference electrode	Ag/AgCl saturated with 3M KNO_3
Auxiliary electrode	Platinum wire

ii) Model 315A:

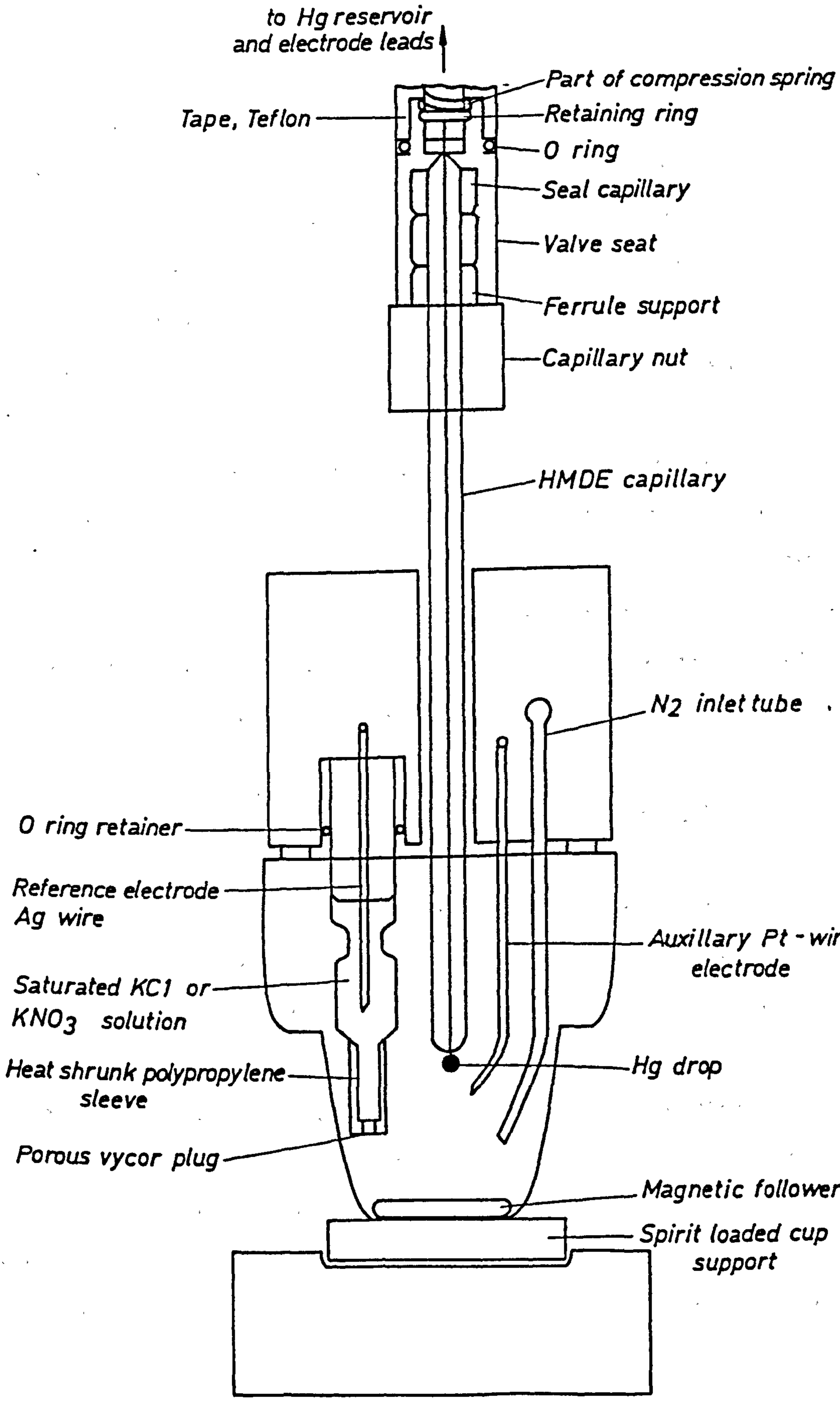
Initial Potential	-1.2V vs. Ag/AgCl
Final Potential	0.15V vs. Ag/AgCl
Conditioning Potential	0.15V vs. Ag/AgCl
Purge Time	10 minutes
Conditioning Time	0.0 min
Equilibration Time	30 seconds
Deposition time	120 seconds

iii) Model 174A:

Scan Rate	5 mV/s
Scan Direction	"+"
Modulation Amplitude	25 mV
Current Range	10 μA
Drop Time	0.5 second
Display Direction	" — "
Low Pass Filter	"OFF"
Mode	Differential Pulse
Initial Potential	0.0
Potential Scan Rate	1.5 V

A diagram of the Static Mercury Drop Electrode is given in Figure 5.1.

FIG. 5.1 MODEL 303 STATIC MERCURY DROP ELECTRODE



d) Procedure for Determination of Zn, Cd, Pb and Cu by DPASV

The DPASV analyses were performed on 5 ml aliquots of the prepared sample diluted with 5 ml of the electrolyte buffer (pH = 5) in a polarographic cell and deoxygenated by passing purified nitrogen (purge) for ten minutes. The voltammogram was then recorded. In all cases, calibration was carried out by the standard addition method, where each addition was of 20 µl of a new standard metal solution (10 µg/ml of Cd, Cu, Pb and Zn in the same flask), prepared by dilution of stock metal solutions. The metal content of the sample was obtained by extrapolation and then the concentration calculated.

The voltammograms in Figure 5.2 are of (a) DDW plus buffer pH = 5, (b) single distilled water (SDW), and (c) deionized water. Figure 5.2 illustrates the contamination in SDW and deionized water; while Figures 5.3 and 5.4 show the voltammograms of some sample waters collected from the River Avon (see Figure 4.1, page 243). The metal levels of Cd, Cu, Pb and Zn in µg/L are given in Table 5.3.

iv) Determination of Cadmium and Some Heavy Metals in Sediment Samples by AAS

Cadmium, copper, chromium, nickel, lead and zinc were determined in six sediment samples collected from the River Avon (see Figure 4.1, page 243, for location sites). Sample treatment has been discussed previously (page 256).

A 1.0 g sample was placed in a Teflon beaker, concentrated hydrofluoric acid (AristaR) (10 ml) was added and the mixture heated on a water bath at 70°C to dryness. Then 10 ml of concentrated nitric acid (AnalaR) was added and the mixture heated on a water bath to reduce the volume to about 2 ml. The solution was filtered through a Whatman

FIG.5.2 VOLTAMMOGRAMS DPASV + HMDE OF Zn, Cd, Pb AND Cu IN :—

(a) DDW + buffer pH5

(b) SDW + buffer pH5

(c) Deionized water + buffer pH5

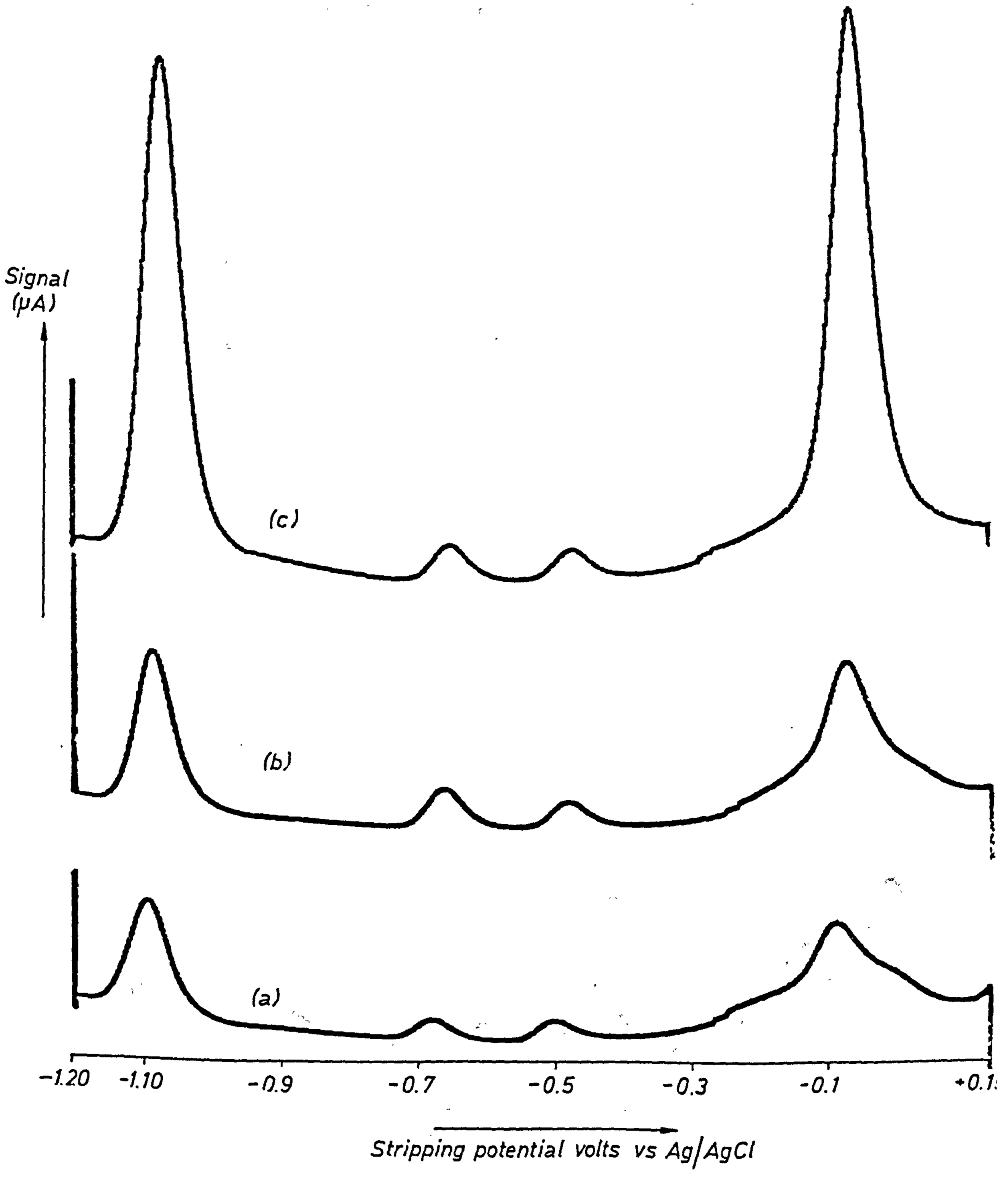


FIG.5.3 DIFFERENTIAL PULSE STRIPPING CURVE (HMDE) OF Zn, Cd, Pb AND Cu IN A KELLAWAYS WATER SAMPLE

(a) 5ml water sample + 5 ml buffer pH5
(b) a + 20ng/ml of (Zn, Cd, Pb and Cu)
(c) a + 40 ng/ml of (Zn, Cd, Pb and Cu)
(d) a + 60 ng/ml of (Zn, Cd, Pb and Cu)

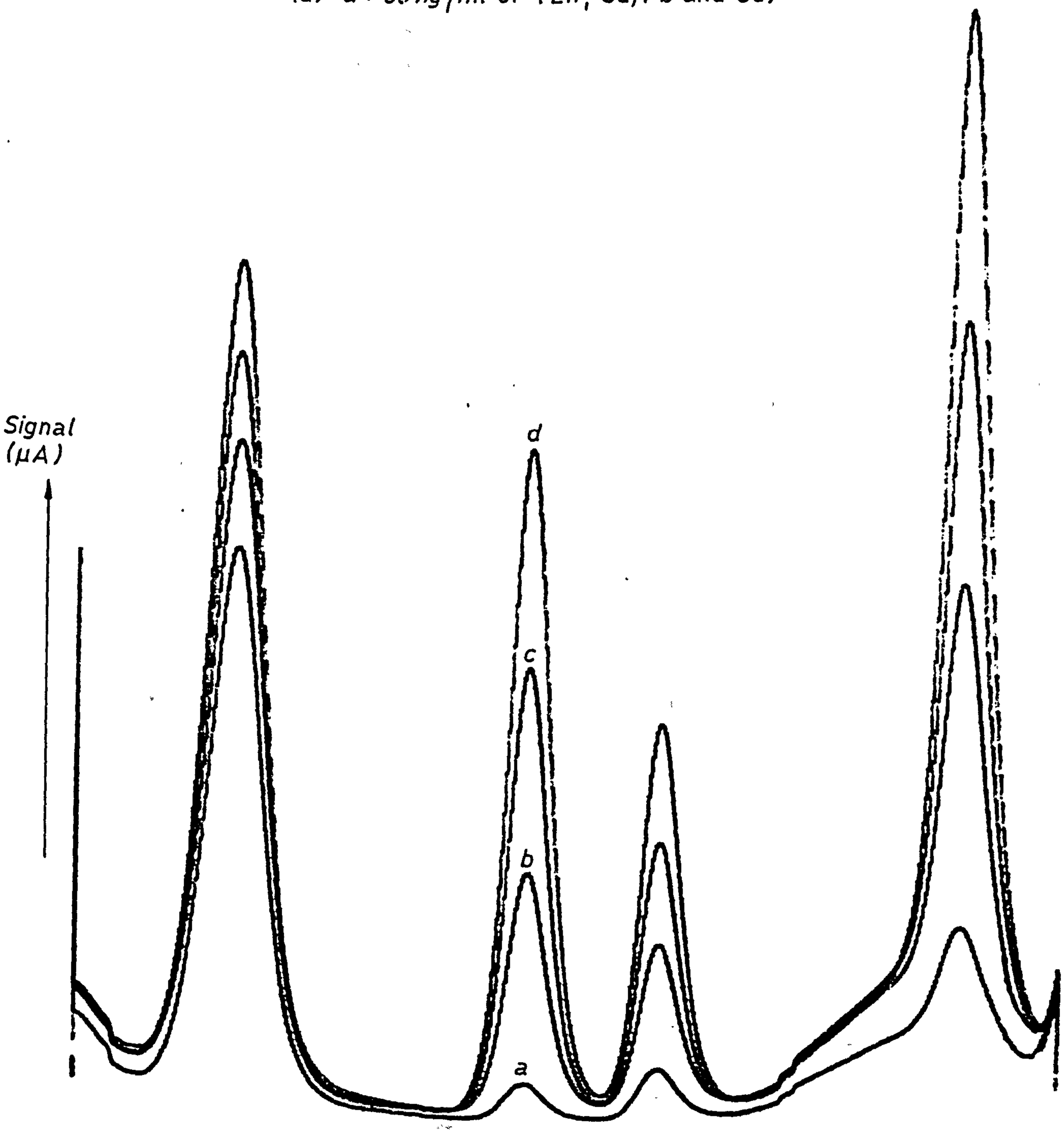


FIG.5.4 DIFFERENTIAL PULSE STRIPPING CURVE (HMDE) OF Zn, Cd, Pb AND Cu IN LIMPLEY STOKE WATER SAMPLE

(a) 5ml water sample + 5ml buffer pH5
(b) a + 20 ng/ml of (Zn, Cd, Pb and Cu)
(c) a + 40 ng/ml of (Zn, Cd, Pb and Cu)
(d) a + 60 ng/ml of (Zn, Cd, Pb and Cu)
(e) a + 80 ng/ml of (Zn, Cd, Pb and Cu)

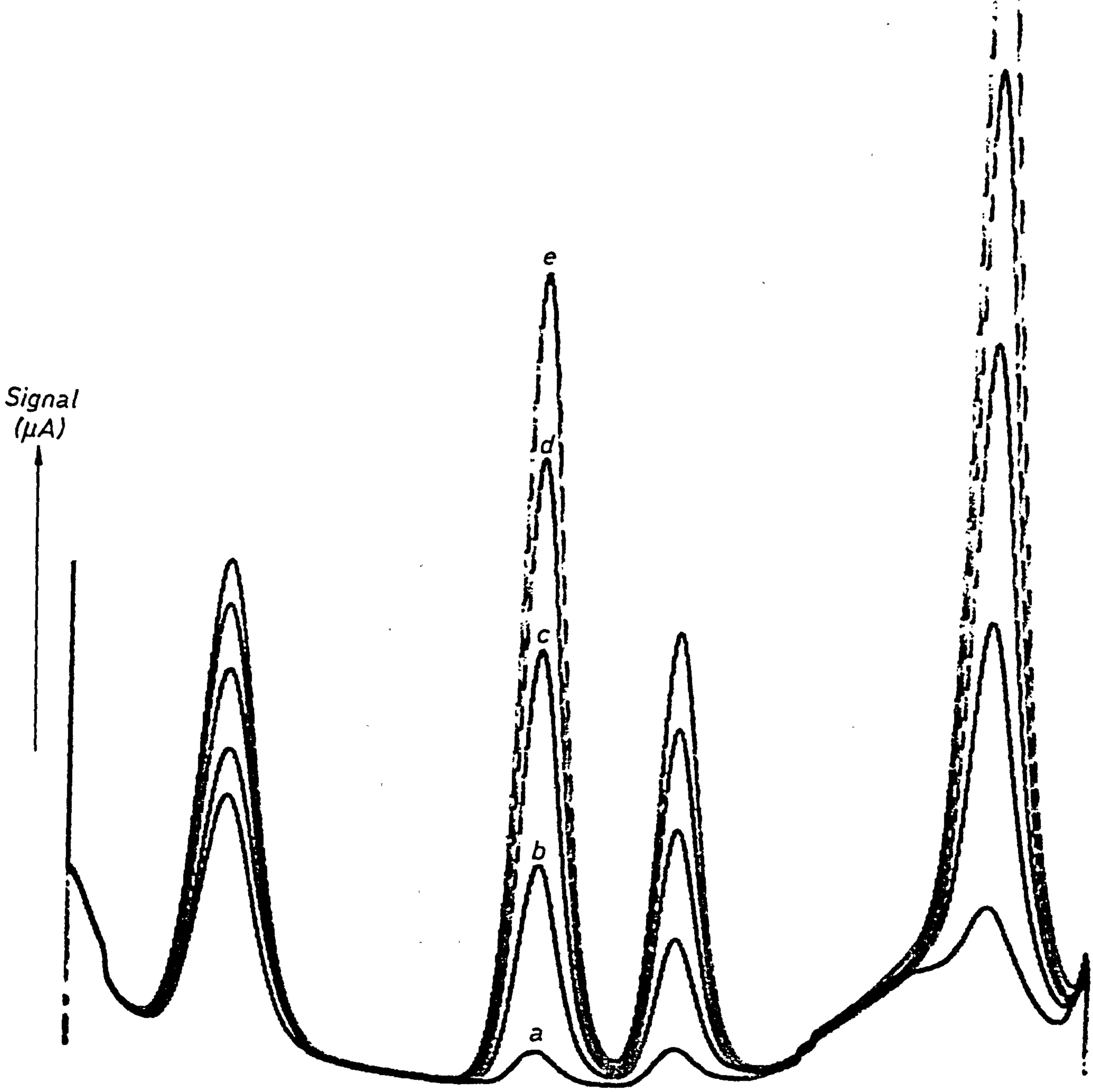


Table 5.3. Cadmium, Copper, Lead and Zinc in River Water Samples using DPASV

Sample		Concentration (μg/L)			
Date of collection	Site	Cd	Cu	Pb	Zn
28/6/1984	Kellaways	1.8	4.5	2.5	390
	Reybridge	2.5	8.6	4.0	250
	Melksham	1.5	4.0	6.5	88
24/7/1984	Malmesbury	2.3	5.0	3.4	330
	Dauntsey	1.5	N.D.	2.2	35
	Kellaways	1.2	2.5	2.0	100
	Reybridge	1.0	4.0	1.5	55
	Melksham	2.6	N.D.	4.2	68
	Staverton	2.0	25.8	5.1	160
	Limpley Stoke	1.2	2.5	2.0	40
	Newbridge, Bath	2.5	2.0	N.D.	24
	Keynsham	3.0	23.5	4.8	185
23/8/1984	Kellaways	6.4	5.0	2.1	28
	Reybridge	7.0	4.8	3.5	17
	Melksham	2.1	2.0	5.2	55
	Staverton	3.2	2.5	N.D.	83
	Limpley Stoke	2.8	1.5	2.2	75
	Newbridge, Bath	2.5	25.5	6.8	26

No. 541 ashless filter paper; the clear solution being transferred to a 25 ml volumetric flask and diluted to volume with DDW.

An aliquot of this conc. HF/HNO_3 solution was used for determination of heavy metals. The analyses were carried out on duplicate subsamples of the same material and blanks were run to control any contamination.

Determination of the cadmium, lead and nickel content of the acid digested samples was carried out using a Varian AAS 775 with GTA-95, while the copper, chromium and zinc content of the acid digested samples was carried out by FAA using an Instrumental Laboratory Inc. AAS model 151 fitted with background correction facilities. The results are listed in Table 5.4.

Table 5.4. Some Heavy Metals in Sediment Samples

Site Sample	Concentration (µg/g)					
	Cd	Cu	Cr	Ni	Pb	Zn
Kellaways	0.48	16.38	20.9	33.1	1.03	115
Reybridge	0.065	23.3	15.0	36.0	2.40	170
Melksham	0.37	53.2	15.2	29.1	2.92	211
Staverton	1.01	23.40	20.6	27.0	1.90	163
Limpley Stoke	0.64	21.50	13.4	30.0	2.70	125
Newbridge, Bath	0.40	75.60	57.8	41.8	3.26	214

5.2.3 Determination of Mercury in Plant Samples by GFAAS and Cold Vapour AAS (CVAAS)

In recent years, the number and variety of quantitative or qualitative methods and instruments used to determine trace quantities of inorganic and organic mercury occurring in natural or synthetic substances has increased greatly. This proliferation came about after a series of mercury poisonings alerted the world to the dangers of mercury from industrial discharges, mercury-containing products, and wastes to the environment (11).

Mercury levels in biomaterials range broadly within several orders of magnitude: from subnanograms to above micrograms per gram. The cold-vapour technique of AAS (CVAAS) is the best routine method: it is easily accessible, sufficiently sensitive, and relatively simple and fast. GFAAS is only of limited use, due to the extreme volatility of the analyte and background problems (24); however, using a matrix modification procedure it is possible to overcome the volatilisation problem for determination of mercury by GFAAS (34).

a) Determination of Hg by GFAAS

In order to study the effect and uptake of mercury as mercury (II) acetate by the roots of plants with time, an experiment was carried out with Lolium perenne.

Lolium perenne seeds were germinated in acid washed silver sand and then transferred to nutrient solution and finally fed with Hg(II) in a similar experiment to that for Se discussed previously (see page 263).

Plant samples were digested in a Teflon digestion vessel with 5 ml conc. HNO_3 and heated at 150°C for 60 minutes in an oven, similar to the selenium digestion procedure (page 258).

Determination of the mercury content of the acid digested samples was carried out using a Varian AAS 775 with GTA-95. The instrumental parameters and conditions are given in Table 5.5.

Table 5.5. Determination of Mercury by AAS 775 with GTA-95
Furnace Operating Parameters

Step No.	Temperature (°C)	Time (sec)	N_2 flow (L/min)	Read command
1	90	5	3	
2	120	60	3	
3	120	30	3	
4	120	2	3	
5	120	2	0.0	
6	2000	1	0.0	*
7	2000	1.1	0.0	*
8	2400	2	3	

Instrumental Parameters (35):

Lamp current (HCL) = 5mA
Spectral Bandwidth = 0.5 nm
Wavelength = 253.7 nm
Background Correction = OFF

Chemical Modifier (34):

0.05% $K_2Cr_2O_7$ (w/v) in 1% HNO_3 (v/v) which extends the maximum possible ashing temperature to 250°C for mercury.

Performance Data:

Standard solution: 1.0 µg Hg^{++}/ml+ 0.05% $K_2Cr_2O_7$ in 1% HNO_3 (AristaR)

Working solutions:

Solution No. 1 = 8 µl of 1 µg Hg/ml + 12 µl Blank = 0.4 µg Hg/ml
Solution No. 2 = 12 µl of 1 µg Hg/ml + 8 µl Blank = 0.6 µg Hg/ml
Solution No. 3 = 20 µl of 1 µg Hg/ml = 1.0 µg Hg/ml

The instrument was calibrated with standard solutions using the concentration mode. The concentrations of mercury in the plant samples were compared with the concentration of standard solutions. The results are listed in Table 5.6. However, the determination of mercury content in Experiment 1 (Se, Cd, Hg), which will be discussed in detail in Chapter 6, of the acid digested samples was carried out using the cold-vapour AAS as well as by the Varian AAS with GTA-95. Table 5.7 lists the results of mercury content in 20 shoot samples and 20 root samples.

Table 5.6. Tissue Concentration of Mercury (µg/g) in Shoots and Roots of Lolium perenne seedlings by GFAAS and CVAAS

Hg µg/ml in nutrient solution	Roots		Shoots	
	GFAAS	CVAAS	GFAAS	CVAAS
Control	N.D.	N.D.	N.D.	N.D.
1×10^{-4}	1.55	1.81	0.94	0.84
5×10^{-4}	3.05	3.20	1.08	1.05
1×10^{-3}	3.87	4.00	1.14	1.16
5×10^{-3}	8.60	11.65	1.16	1.30
0.01	17.80	18.28	1.69	1.83
0.05	100.14	101.35	2.20	2.25
0.10	131.75	133.18	2.30	2.60
0.50	622.35	649.33	14.00	15.52
1.0	1545.0	1574.8	28.20	30.70
5.0	11406.90	11810.70	330.5	328.30

Table 5.7. Mercury in Plant Samples by:

a) GFAAS

b) CVAAS

Trials No.	µg Hg/g Roots		µg Hg/g Shoots	
	GFAAS	CVAAS	GFAAS	CVAAS
1	7.00	8.51	0.99	1.04
2	4.72	7.10	1.20	1.25
3	6.10	7.77	1.37	0.97
4	127.80	136.73	2.12	2.59
5	5.27	5.69	1.34	1.28
6	187.72	194.94	1.82	1.95
7	183.20	197.24	2.00	2.21
8	231.20	249.05	3.18	3.61
9	13.95	15.12	1.46	1.61
10	19.50	28.66	0.44	0.67
11	25.53	26.34	2.20	2.69
12	28.12	32.27	0.35	0.37
13	1505.72	1532.77	29.60	33.34
14	3.35	4.34	0.64	0.58
15	12.11	11.83	1.34	1.31
16	12.33	9.36	1.07	1.05
17	8.34	8.87	1.20	1.20
18	12.75	8.75	0.92	1.15
19	9.17	9.28	0.85	0.90
20	12.90	11.87	0.95	1.11

b) Determination of Mercury in Plant Samples by Cold-Vapour AAS (CVAAS)

A cold-vapour atomic absorption spectrometry (CVAAS) method was used for total mercury determination.

The principle of CVAAS is simple; mercury is reduced to its elemental state (Hg°) by adding a reductant to the sample solution and sweeping the Hg° out of the solution via a purge gas (N_2). The purge gas carrying Hg° passes through drying agents, next it enters an absorption cell (gas cuvette) placed along the optical pass of the spectrophotometer, where the atomic absorption of Hg° vapour is measured (24), and finally into an amalgamation attachment to prevent release of Hg to the atmosphere.

The development of this technique was discussed in detail in Chapter 2 (page 126). The analysis of Hg was carried out with the IL 151 AAS. The apparatus is shown schematically in Figure 2.3, page 126A, and photographically in Figure 2.4 (page 126B). The apparatus as shown in Figures 2.3 and 2.4 consisted of two bubblers which act as the reduction vessels; these can be used separately or simultaneously enabling large sample volumes to be analysed. Simpson and Nickless (36) reported that a 50 ml bubbler is the most efficient and also that if it is enlarged at the top, this reduces the foaming and sample carry over encountered with the straight-sided bubblers. In the apparatus used by Simpson and Nickless (36), the tops of the bubblers were closed with stoppers, when a sample was introduced into the bubbler the stopper had to be removed and then quickly replaced after the injection. This could thus lead to a small amount of sample loss, and therefore the stoppers were replaced by rubbers caps (subaseal), through which the sample could be injected by means of a syringe.

The reduction solution was a 20% w/v suspension of tin (II)

chloride in 1M sulphuric acid. Standards containing 0.01, 0.1 and 1.0 μg Hg(II)/ml in a mixture of 5% v/v nitric acid and 0.1% w/v potassium dichromate solution were obtained by dilution of 1000 μg Hg(II)/ml stock solution. The recommended procedure was used (36).

The reduction solution was injected into the bubbler before the start of the analysis, to ensure against losses of mercury in the bubbler. Nitrogen was bubbled through the reducing solution and residual mercury flushed through the absorption cell. A steady baseline on the recorder indicated the absence of mercury in the system. The sample was injected into the aerated stannous chloride solution and the liberated mercury swept into the cell for measurement when the mercury peak returned to the baseline, either:

i) another sample was injected and the reducing solution re-used,

or ii) the solution in the bubbler was blown to waste and the whole sequence repeated with another sample.

The sampling time for the flow of purge gas was one minute. A nitrogen flow rate of 500 ml/min was employed in the analysis of standard and sample solutions.

Linear calibrations were obtained for a number of ranges between 5 ng Hg(II)/ml to 100 ng Hg(II)/ml, the most convenient range for examining samples was 10 ng to 75 ng/ml. The calibration value was determined from an average of three injections for each of 5, 10, 25, 50, 75 and 100 ng of mercury. The traces recorded and the calibration graph plus detection limits obtained are shown in Figures 5.5 and 5.6 respectively. The results of mercury determination in plant samples by CVAAS are listed in Table 5.6 while Experiment 1 (Se, Cd, Hg) will be discussed in detail in Chapter 6. The levels of mercury content in plant samples determined by CVAAS are listed in Table 5.7.

FIG. 5.5 RESPONSES OF Hg BY CVAAS
Chart speed 0.2 cm/min,
Recorder voltage 10mV

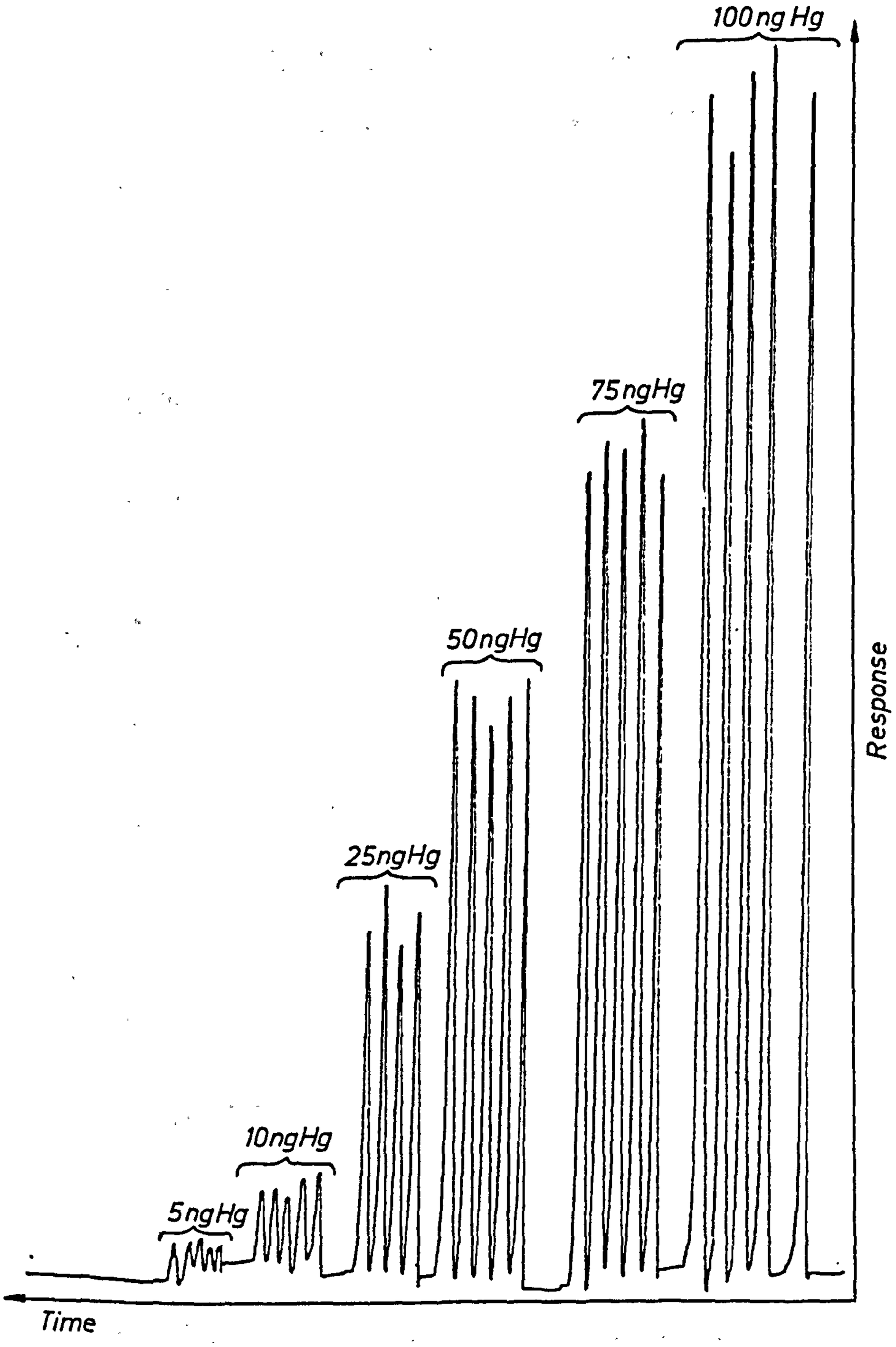
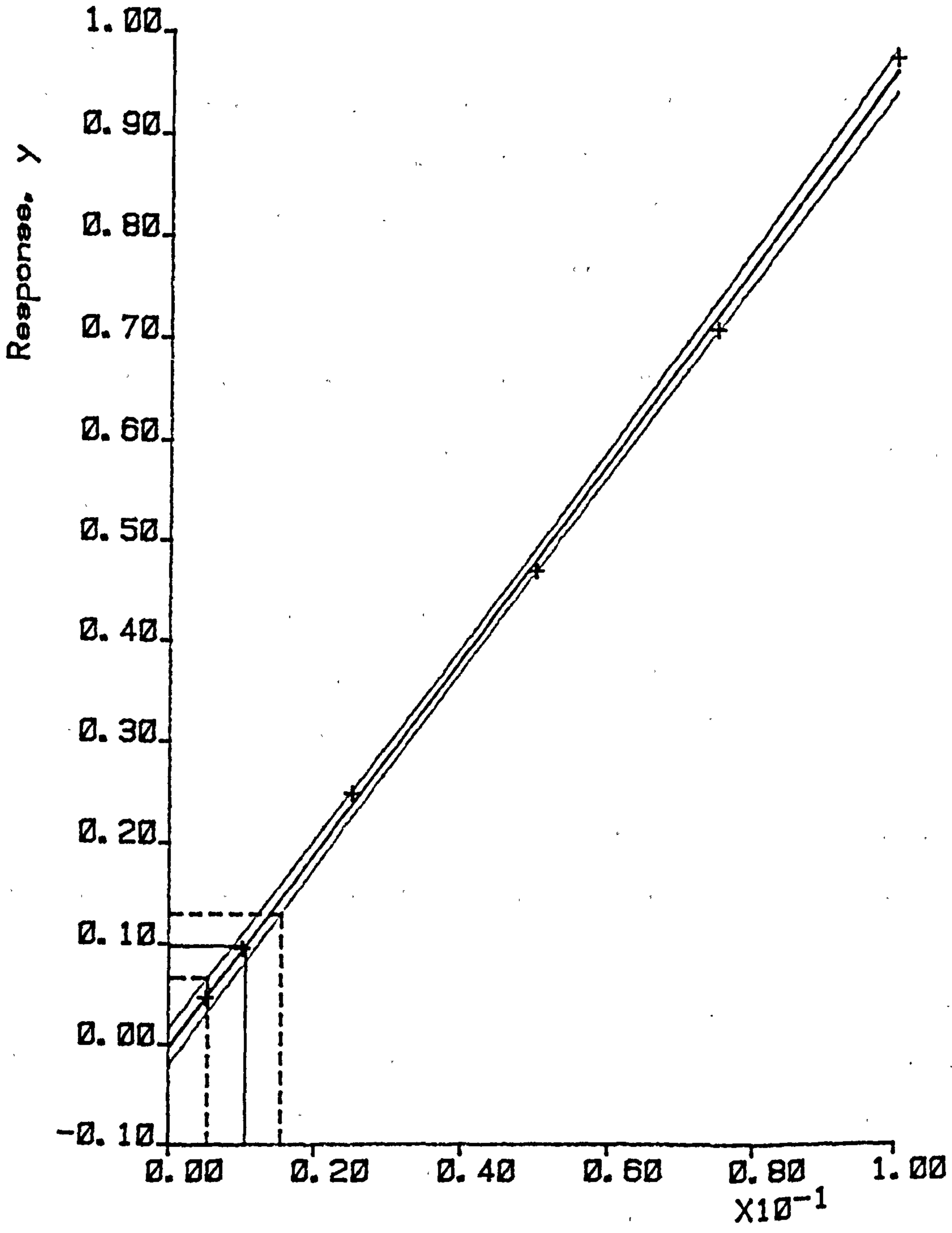


FIGURE 5.6 DETECTION LIMIT FOR MERCURY BY CVAAS

$y = -0.00137 + 9.56 * conc$

95.0% Confidence Limits

Detection Limit = 0.0104 µg Hg/ml



5.2.4 Determination of Thallium in Plant Samples and Sediments

The normal levels of Tl are very low: ⩽1 ng/ml in biological liquids and < 10 ng/g in most other biological tissue.

DPASV and GFAAS are the most sensitive and convenient techniques for the determination of nano- and subnanogram amounts of Tl; the DLs are of both techniques are below 0.1 ng/ml and they also suitable for routine work (24).

Both techniques appear to be suitable because they provide a high degree of sensitivity and reliability while being rapid, easy to handle and relatively cheap. There are, however, some decisive differences between the two methods with respect to thallium (37).

i) Determination of Thallium in Plant Samples by GFAAS

GFAAS has the necessary sensitivity for Tl determination in environmental samples but the interference problems with this technique are high (38). Also in GFAAS the analysis of real samples is marred by many anions, e.g. hydrochloric and perchloric acids (39), as well as metals, e.g. Fe, Co, Ni, Zn even in the µg/ml range (37).

For example, Tl(I) nitrate decomposes at 450°C and will be lost during the ashing step while Tl(III) nitrate is converted to Tl(I) nitrate at 145°C and so will also be lost at 450°C, i.e. the nitrate form should not be used in GFAAS. In fact, this is a most important consideration since nitric acid is a most popular and useful agent as a sample matrix for most flameless work (38). To avoid this problem, a small volume of 1% (v/v) sulphuric acid has been added as chemical modifier to prevent loss of thallium during the ashing step and also to reduce interferences in the presence of HCl, $HClO_4$ and NaCl (35,39).

Determination of thallium was carried out by GFAAS using a Varian 775 instrument and a GTA-95. The parameters and conditions are given in Table 5.8. The instrument was calibrated with standard solutions on concentration mode. The concentrations of thallium in the samples were compared with the concentrations of standard solutions.

Table 5.8. Determination of Thallium by AAS-775 with GTA-95

Step No.	Temperature (°C)	Time (sec)	N_2 flow (L/min)	Read
1	75	5.0	3.0	
2	90	60.0	3.0	
3	120	10.0	3.0	
4	200	2.0	3.0	
5	250	5.0	0.0	
6	2200	1.1	0.0	*
7	2200	2.0	0.0	*
8	2400	1.0	3.0	

Lamp Current = 10 mA

Spectral Bandwidth = 0.5 nm

Wavelength = 276.8 nm

Background Correction = ON

Sample Injected = 20 ul

In order to study the effect and uptake of thallium as thallium (I) nitrate by the roots of plants with time, an experiment was carried out with Lolium perenne seedlings, similar to that described for the uptake of selenium by plants (see Chapter 4, page 263).

Determination of the thallium content was made on the acid digest (conc. HNO_3) using a Teflon vessel, and heated at 150°C for 60-75 minutes; the full details of this procedure were described on page 258. Measurement was carried out using a Varian AAS 775 with GTA-95. The results are listed in Table 5.9.

Also, the determination of thallium content in Experiment 2 (Se, Te, Tl) (which will be discussed in detail in Chapter 6) on the acid digested samples was also carried out using the same equipment.

ii) Determination of Thallium in Sediments and Plant Samples by DPASV at HMDE

Differential pulse anodic stripping voltammetry (DPASV) with HMDE compares favourably with GFAAS because it can be applied to a wider measurement range in concentration terms. DPASV is less prone to the effects of high salt concentrations (e.g. halides) and larger sample aliquots can be used. Besides these advantages, the apparatus costs are much lower (37).

However, metal ion interferences from, for example, Cd, Sn, Sb, Pb, are encountered in the determination of thallium, because the potential for the reversible redox system Tl^+/Tl is very close to the potentials of these elements. Variation of the electrolysis potential helps to avoid some of these problems (37). In the presence of tartarate and polyethylene glycol (M.Wt. 4000), thallium can be determined along with Pb and Cd at the HMDE (40).

Table 5: 9 .Tissue Concentration of Tl^{+}, Mn, Fe, Cu and Zn (µg/g) in Shoots and Roots of Lolium perenne Seedlings.

Tl^{+} µg/ml in nutrient sol.	length* (cm)	Tl^{+}	Mn^{++}	Fe^{3+}	Cu^{++}	Zn^{++}	Dry wt (g)
Control Shoot	27.4	ND	58.80	131.75	11.70	51.6	0.918
1×10^{-4}	27.0	ND	46.72	133.0	10.04	46.8	0.937
5×10^{-4}	30.0	0.075	40.93	125.8	9.85	45.75	0.928
1×10^{-3}	27.45	0.160	42.57	118.5	9.20	41.8	0.907
5×10^{-3}	26.5	0.48	56.04	79.7	10.40	42.5	0.930
0.01	27.2	1.050	68.9	73.4	10.20	77.9	0.915
0.05	27.2	1.34	50.78	70.0	8.60	68.4	0.777
0.10	25.0	2.65	56.4	68.7	7.85	81.0	0.743
0.50	12.60	26.30	83.42	145.2	18.75	78.4	0.380
1.0	9.0	61.85	83.74	90.0	14.30	56.7	0.216
2.5	7.1	144.05	115.11	95.7	16.24	57.8	0.125
Control Root	17.6	ND	262.10	1512.0	55.6	174.0	0.216
1×10^{-4}	17.2	0.42	256.4	1191.0	48.3	168.4	0.213
5×10^{-4}	17.44	0.77	262.8	1123.0	47.4	154.5	0.230
1×10^{-3}	17.8	1.45	191.35	1170.0	43.5	134.8	0.223
5×10^{-3}	16.5	2.3	154.25	1146.0	41.60	126.0	0.218
0.01	15.8	7.1	187.55	1195.0	36.70	90.7	0.215
0.05	15.7	12.9	164.0	1258.0	40.0	95.3	0.200
0.10	15.1	33.25	134.5	1383.0	65.4	120.0	0.198
0.50	13.1	68.5	73.52	3222.0	83.8	167.0	0.093
1.0	0.20	369.1	93.74	6034.0	105.10	255.6	0.048
2.5	0.0	576.0	94.04	6580.0	157.0	68.3	0.040

* Average of five plants
Elongation from start of experiment.

For accurate determination of Tl at extremely low levels, it should be separated from complex materials (41). A separation often involves a preconcentration step, so that very low detection limits can be achieved. Thus, for example, as little as 3.7 ng Tl/L can be determined in water by using an ion-exchange resin for that step (42).

A number of interferences by metal ions can be reduced or eliminated using the disodium salt of EDTA. Complex formation is strongly pH-dependent. As an example, the lead peak overlaps exactly with the thallium peak at pH $\leqslant$ 5.5 with a deposition potential of -0.6 V vs. SCE while cadmium appears at -0.5 V when their concentrations are above certain well-defined concentrations. A drawback to the use of EDTA lies in a shift of the peak potentials of the complexes of, for example, Cu, Mo, Sb and Bi to more negative values which accentuates possible interferences (37).

In order to avoid precipitation of EDTA on addition to acidic decomposition solutions, buffer substances are needed, e.g. acetate, tartarate or citrate. Thus EDTA precipitates in acidic solutions containing only 0.1M citrate, but not in solutions that are 1M in citrate which have been buffered. Moreover, the presence of citrate during the neutralisation of decomposition solutions with ammonia avoids the precipitation of metal hydroxides which redissolve only very slowly (37,43). Thus Zitko et al. (44) selected a 2M acetate buffer containing 0.2M EDTA for distinguishing between and allowing determination of thallium and lead in natural waters by ASV; however Batley and Florence (42) used 1M acetate with 0.2M EDTA buffer (pH 4.6) for the determination of thallium in natural waters.

A supporting electrolyte consisting of 0.01M EDTA and acetate

buffer (0.2M sodium acetate and 0.2M acetic acid) has a sufficient buffer and complexing capacity to avoid these difficulties and was used throughout the investigations.

a) Apparatus and Instrumental Parameters

ASV was carried out using PAR Model 174A Polarographic Analyser in conjunction with a PAR 315A Automated Electrolysis Controller and a Hewlett Packard 7040A X-Y Recorder (see page 306).

Instrumental Conditions for Determination of Thallium by DPASV with HMDE

i) Model 303:

Working Electrode	HMDE (medium size)
Reference Electrode	Ag/AgCl saturated with 3M KNO_3
Auxiliary Electrode	Platinum Wire

ii) Model 315A:

Initial Potential	-1.2V vs. Ag/AgCl
Final Potential	-0.2V vs. Ag/AgCl
Conditioning Potential	+0.2V vs. Ag/AgCl
Purge Time	10 minutes
Conditioning Time	0.0 sec
Equilibration Time	30 sec
Deposition Time	200 sec

iii) Model 174A:

Scan Rate	5 mV/sec
Scan Direction	"+"
Current Range	5 μA
Modulation Amplitude	25 mV
Potential Range	3.0 V
Drop Time	0.5 sec
Display Direction	" - "
Mode	Differential Pulse
Initial Potential	0.0
Low Pass Filter	"OFF"

b) DPASV Curve with HMDE

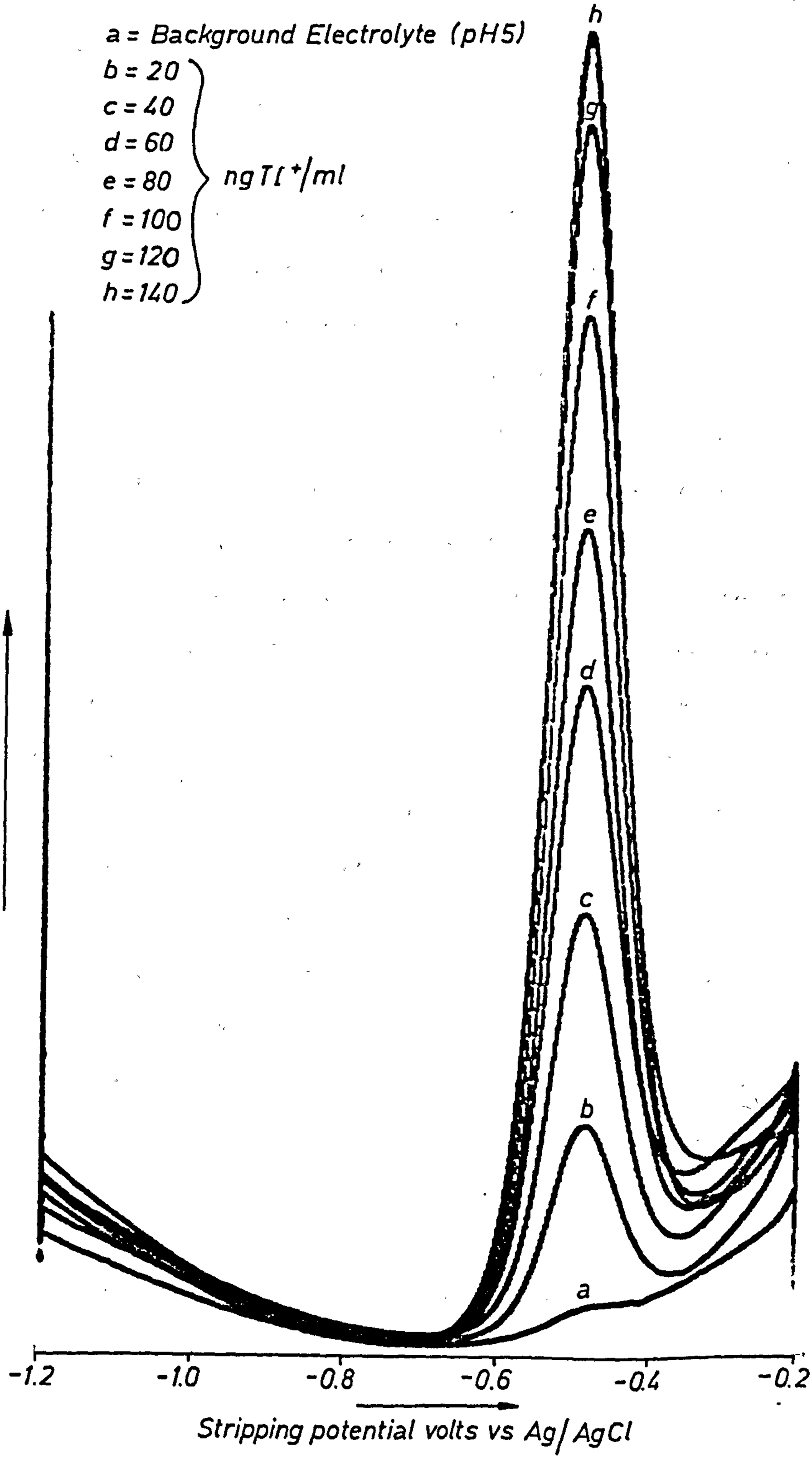
The background electrolyte (0.2M sodium acetate + 0.2M acetic acid + 0.01M EDTA), 10 ml, was placed in a voltammetric cell and deoxygenated by passing purified nitrogen for 5 minutes. The voltammogram was then recorded. Seven separate additions of 20 μl thallium (10 μg/ml) were made and a voltammogram was recorded following each addition. Voltammograms typical of those obtained are shown in Figure 5.7.

c) Determination of Thallium in Sediment Samples by DPASV

The same six sediment samples collected from the River Avon (see Figure 4.1, page 243) which had been examined for determination of selenium and some heavy metals were also investigated using DPASV for thallium content.

In this case, the digestion procedure employed was different from that used for selenium or heavy metals.

FIG.5.7 VOLTAMMOGRAM OF THALLIUM BY DPASV WITH HMDE



Digestion Method (45):

Sample (1.00 g) was placed in a 150 ml PTFE beaker, together with 5 ml of concentrated HF (sp.gr. 1.15) and 5 ml of concentrated HNO_3. The mixture was placed on low heat on a hot plate and allowed to evaporate slowly to dryness. The residue was dissolved in 10 ml of a hydrobromic acid/bromine mixture, the whole being warmed gently until most of the bromine was expelled. Next 25 ml of hot distilled water was added, the mixture being covered and warmed until the residue had dissolved. Next the mixture was allowed to cool to room temperature, after which it was quantitatively transferred to a 100 ml separating funnel, marked at 50 ml capacity. The solution was diluted to that mark with double distilled water and 15 ml of diethyl ether added. The solutions and funnel were shaken for two minutes.

The process of extraction with diethyl ether was repeated three times with fresh organic solvent. The organic layers were removed to another separating funnel, combined and 25 ml of 1M HBr solution added; the mixture being shaken for one minute. The lower acid layer was removed and discarded. The upper ether layer was transferred to a 150 ml glass beaker and evaporated to dryness. This residue was dissolved in 2 ml of conc. HNO_3 plus 2 ml of aqueous sulphuric acid (1 + 3) and again the mixture was evaporated to dryness. Finally the acid residue was dissolved in hot base electrolyte (acetate buffer), cooled to room temperature and 2.5M sodium hydroxide solution added until the solution reached a pH of 5.0 ± 0.5. The resultant solution was transferred to a 50 ml graduated flask and diluted to volume with DDW.

For the electrochemical determinations, 10 ml of digest solution were transferred to a voltammetric cell containing a small magnetic

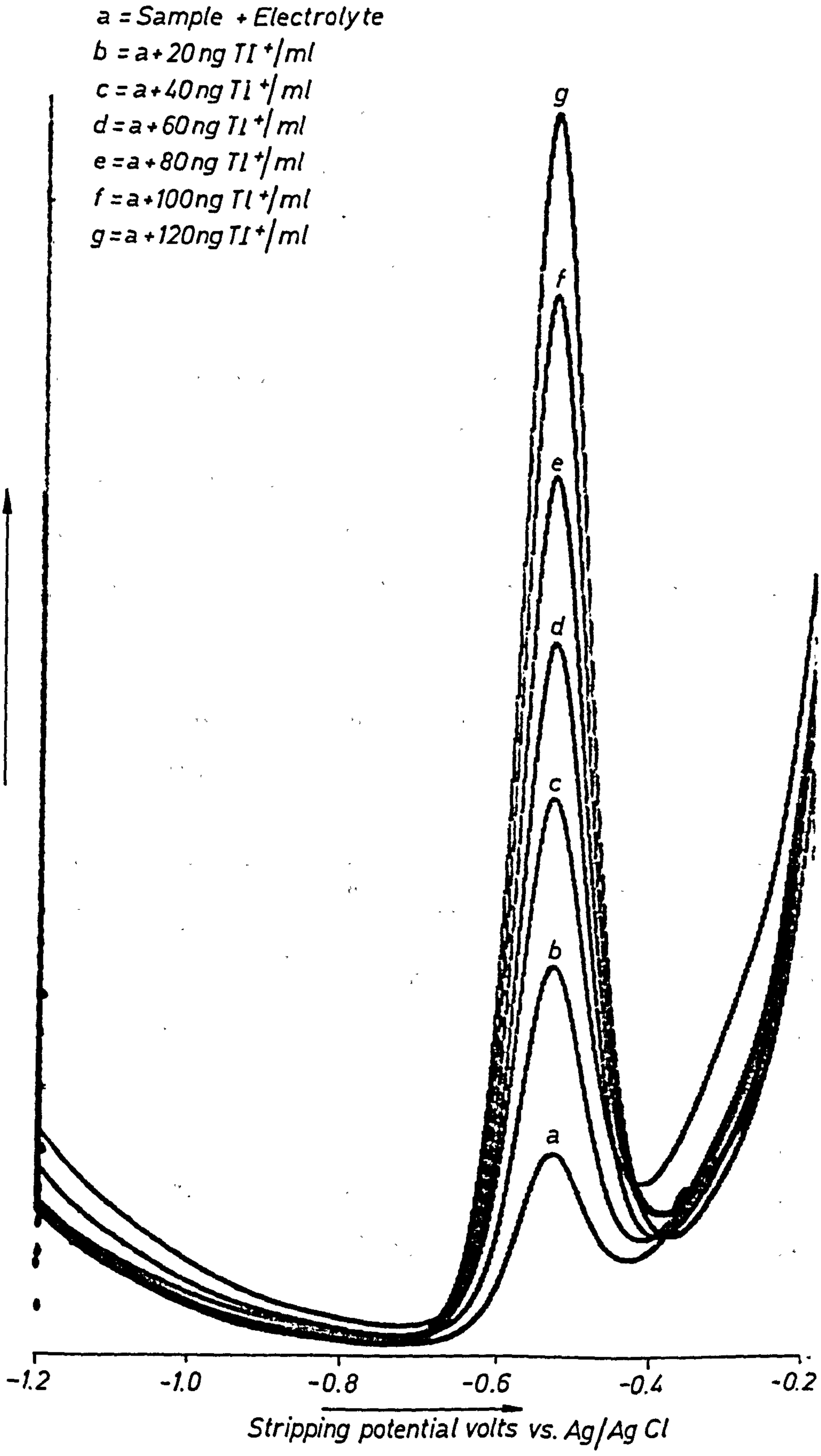
follower and then was purged with purified nitrogen for 10 minutes. The voltammogram was then recorded. Several sequential additions of 20 µl thallium containing solution (10 µg/ml) were made to this solution and a voltammogram recorded following each addition. Then, the thallium content in each sample was determined using the standard addition procedure.

A blank was run for each determination in an attempt to ascertain the level of contamination; fortunately for each blank the level of thallium was not enough to be detected by DPASV at the HMDE. Figure 5.8 illustrates the voltammograms of thallium in Limpley Stoke sediment samples and the levels of thallium in the sediments are presented in Table 5.10.

Table 5.10. Determination of Thallium in Sediment Samples by DPASV at HMDE

Sample Site	Concentration ng/g Thallium
Kellaways	30.0
Reybridge	33.0
Melksham	17.5
Staverton	22.5
Limpley Stoke	42.5
Newbridge, Bath	25.0
Blank	< 5.0

FIG. 5.8 VOLTAMMOGRAM OF THALLIUM IN LIMPLEY STOKE SEDIMENT SAMPLE



d) Determination of Thallium in Plant Samples by DPASV at HMDE

Determination of the thallium content was made on the acid digest [(conc. HNO_3), using a Teflon vessel, and heated at 150°C for 60-75 min, see page 258]. An aliquot of the digest solution (usually 2.0 ml) was added to 8 ml of buffer [0.2M sodium acetate plus 0.2M acetic acid + 0.01M EDTA (diNa)], in a voltammetric cell, the solution was then purged with purified nitrogen for 10 minutes. The voltammogram was then recorded, measurement being carried out on an HMDE using DPASV, using the method of standard additions.

The results are listed in Table 5.11. Figure 5.9 illustrates the voltammograms of thallium in a root sample, while Figure 5.10 shows the voltammograms for the thallium content in shoot material.

Table 5.11. Thallium in Plant Samples Using:

i) GFAAS

ii) DPASV

Sample Trial No.	Roots µg Tl/g		Shoot µg Tl/g	
	GFAAS	DPASV	GFAAS	DPASV
1	5.81	7.30	1.31	2.10
2	10.56	13.02	2.74	4.05
4	76.20	81.50	39.84	44.90
6	93.75	105.25	42.97	47.60
7	92.86	103.20	26.70	30.08
8	82.42	91.03	28.18	31.30
13	401.36	421.50	204.02	215.60

FIG. 5.9 VOLTAMMOGRAM OF THALLIUM IN PLANT SAMPLE (ROOTS)

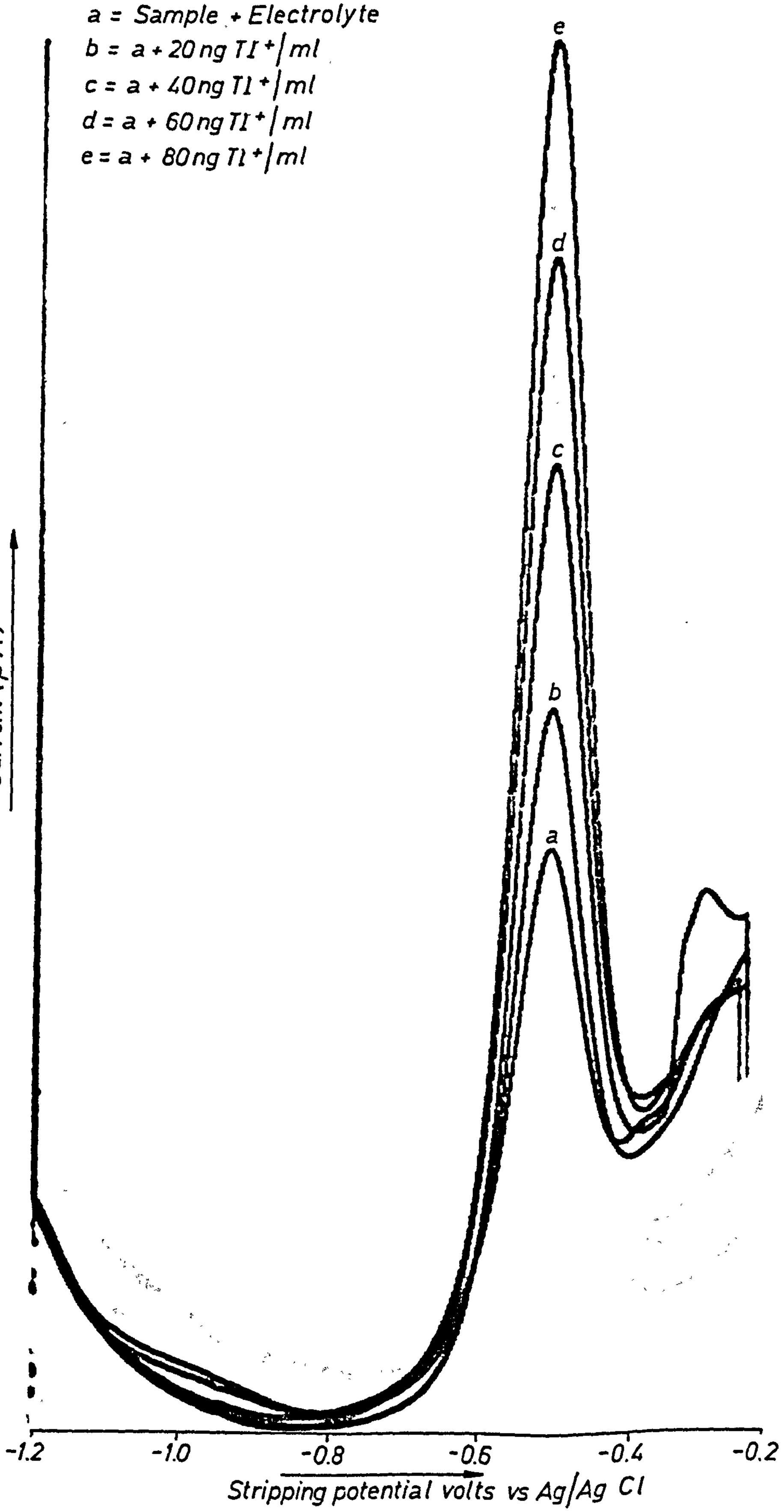
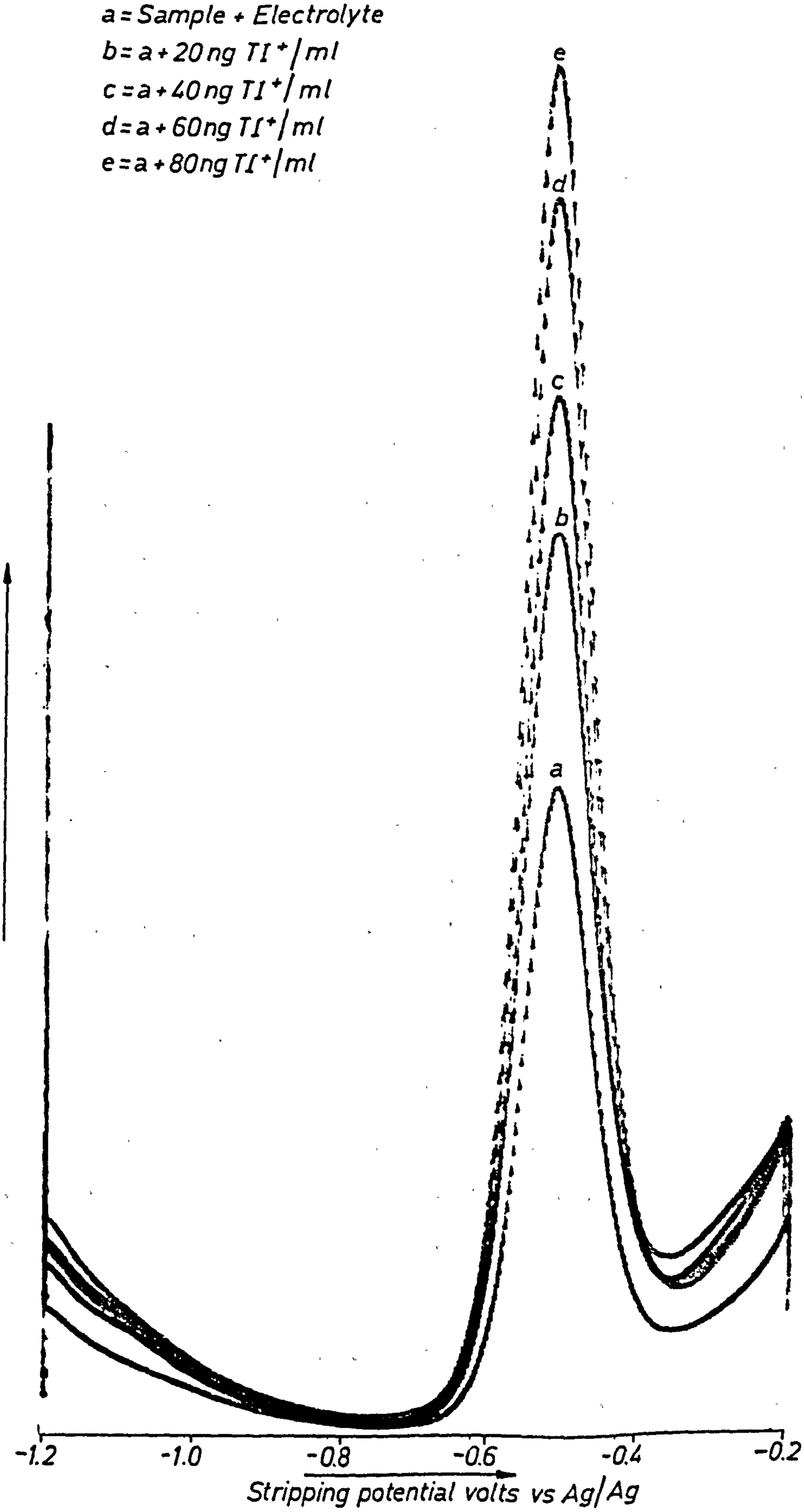


FIG. 5.10 VOLTAMMOGRAM OF THALLIUM IN PLANT SAMPLE (SHOOTS)



5.2.5 Determination of Tellurium

Determination of tellurium in trace amounts has received increasing attention in connection with the wide application of its compounds and/or environmental pollution. Although tellurium is a comparatively rare element, it is widely used in the electronics industry and is a potentially toxic environmental pollutant. Occupational exposure to tellurium causes a sour garlic stench in the breath (46,47).

Recent data suggests that the tellurium levels in biological liquids should normally be $\leqslant$ 1 ng/ml; concentrations in the lower nanograms/gram range should be expected for most other biomaterials (24).

Hence, sensitive methods of determination are required for monitoring tellurium concentrations in the environment and to allow the biochemical role and toxicological effects of tellurium to be assessed (47).

Although many techniques are available for the determination of tellurium (48-53), few analytical techniques have the sensitivity to permit the study of tellurium in biological and other environmental materials at naturally occurring levels (47). The reported DLs of some procedures seem to have well above the normal tellurium levels in natural samples, for example by direct GFAAS, DL 3 ng/ml; by neutron activation analysis (NAA), DL 2 ng/ml (54). Tellurium contents of food (55), water (56,57) and atmospheric aerosols (52) have also been suggested to be below the DLs of the respective procedures. Therefore, normal tellurium concentrations can be determined only after some pre-concentration step.

Generally, GFAAS is potentially applicable, and the most popular technique to apply to nanogram tellurium levels in slightly contaminated natural samples (24).

i) Determination of Tellurium in Plant Samples by GFAAS

In order to study the effect and uptake of tellurium as Te(VI) by the roots of plants with time, an experiment was carried out with Lolium perenne.

Lolium perenne seeds were germinated in acid washed silver sand and then transferred to nutrient solution and finally fed with Te(VI) in a similar manner to that discussed previously for Se (see page 263).

Plant samples were digested in a Teflon digestion vessel with 5 ml conc. HNO_3 and heated at 150°C for 60 minutes in an oven, similar to the selenium digestion procedure (page 258).

Determination of the tellurium content of the acid digested samples was carried out using a Varian AAS 775 with GTA-95. The instrumental parameters and conditions are given in Table 5.12.

The instrument was calibrated with standard solutions using the concentration mode. The concentrations of tellurium in the plant samples were compared with the concentration of standard solutions. The results are listed in Table 5.13. However, the determination of tellurium content in Experiment 2 (Se, Te, Tl) of the acid digested samples, which will be discussed in detail in Chapter 6, was also carried out using the Varian AAS 775 with GTA-95. Table 6.6 lists the results of tellurium content in 20 shoot samples while Table 6.7 illustrates the results of tellurium content in 20 root samples.

Table 5.12. Determination of Tellurium by AAS 775 with GTA-95.

Furnace Operating Parameters

Step. No.	Temperature (°C)	Time (sec)	N_2 flow (L/min)	Read command
1	90	5.0	3	
2	120	60.0	3	
3	350	10.0	3	
4	350	1.0	0.0	
5	1900	0.9	0.0	*
6	1900	2.0	0.0	*
7	2400	2.0	3	

Instrumental Parameters:

Lamp Current (HCL) = 5 mA

Spectral Bandwidth = 0.5 nm

Wavelength = 214.3 nm

Background Correction = ON

Performance Data:

Standard solution: Telluric acid standard solution "SpectrosoL" for atomic absorption (from BDH, Poole, England), 1000 µg/ml.

Working Solutions:

Solution No. 1 = 20 ng Te/ml

Solution No. 2 = 80 ng Te/ml

Solution No. 3 = 140 ng Te/ml

Table 5:13. Tissue Concentrations of Te, Mn, Fe, Cu and Zn (µg/g) in Shoots and Roots of Lolium perenne Seedlings.

Te µg/ml in nutrient sol.	Length* (cm)	Te	Mn	Fe	Cu	Zn	Dry wt. (g)
Control Shoot	27.4	ND	76.2	155.3	29.50	118.9	0.501
0.1	25.4	7.55	75.10	146.7	23.70	109.6	0.497
0.5	25.0	18.75	66.5	165.0	21.40	98.4	0.432
1.0	23.5	44.30	62.8	128.4	19.10	85.7	0.417
2.5	21.1	96.25	63.10	141.5	21.35	73.355	0.310
5.0	16.8	267.80	60.4	120.3	26.50	124.15	0.262
10.0	12.3	780.40	56.65	181.4	20.70	92.75	0.113
15.0	8.0	1158.5	69.60	257.3	38.60	87.36	0.050
20.0	7.6	1435.9	134.30	475.0	45.85	75.10	0.039
30.0	5.5	618.2	128.62	701.0	36.2	83.30	0.032
40.0	2.1	245.0	103.30	360.5	24.3	69.44	0.0292
50.0	0.9	153.6	108.0	216.2	23.2	80.65	0.025
Control Root	18.9	ND	173.6	4150.6	193.50	242.3	0.1156
0.1	17.1	4.13	162.8	4252.0	175.60	231.5	0.1152
0.5	16.7	9.60	147.3	3935.3	161.20	160.0	0.1102
1.0	12.9	33.40	126.4	4466.3	125.6	128.9	0.1070
2.5	12.2	59.32	108.90	4070.3	95.9	98.78	0.073
5.0	11.0	98.20	89.4	6052.1	115.7	85.75	0.065
10.0	7.6	157.5	82.6	6256.7	125.8	100.3	0.033
15.0	1.9	201.7	66.65	13016.5	131.3	115.8	0.017
20.0	0.0	310.8	59.30	16044.8	143.8	277.5	0.012
30.0	0.0	758.3	40.80	17310.0	211.2	94.0	0.095
40.0	0.0	418.2	24.30	16430.0	228.4	76.30	0.0087
50.0	0.0	365.1	18.55	14217.0	209.5	120.6	0.0085

* Average of five plants
Elongation from start of experiment

Table 5:14. Tissue Concentration of Hg, Mn, Fe, Cu and Zn (µg/g) in Shoots and Roots of Lolium perenne Seedlings.

Hg µg/ml in nutrient sol.	length* (cm)	Hg	Mn^{++}	Fe^{3+}	Cu^{++}	Zn^{++}	Dry wt (g)
Control Shoot	27.7	ND	85.1	72.85	11.85	53.71	0.945
1×10^{-4}	29.0	0.84	76.6	64.0	12.90	56.75	0.800
5×10^{-4}	28.4	1.05	77.0	61.72	15.20	56.4	0.720
1×10^{-3}	27.0	1.16	75.0	58.6	12.4	43.22	0.680
5×10^{-3}	28.1	1.30	79.3	56.0	13.05	49.50	0.620
0.01	26.5	1.83	60.0	54.1	11.23	51.73	0.613
0.05	25.8	2.25	55.0	45.1	10.00	41.0	0.630
0.10	22.0	2.60	66.6	49.0	11.62	56.23	0.590
0.50	18.9	15.52	54.0	65.2	11.00	37.8	0.580
1.0	14.6	30.70	51.6	57.7	12.06	32.0	0.354
5.0	6.4	328.30	64.0	41.3	18.6	30.34	0.140
Control Root	17.4	ND	291.0	1717.1	43.30	35.0	0.255
1×10^{-4}	16.8	1.81	300.0	2340.0	48.02	35.20	0.214
5×10^{-4}	17.0	3.20	305.13	2539.0	54.64	34.54	0.180
1×10^{-3}	17.10	4.00	381.5	2770.0	49.75	41.20	0.175
5×10^{-3}	16.9	11.65	463.65	2826.1	70.30	40.90	0.155
0.01	17.0	18.28	357.0	2319.0	72.0	38.43	0.140
0.05	16.1	101.35	290.7	2133.2	54.0	34.35	0.132
0.10	16.0	133.18	300.4	2032.0	45.4	31.33	0.161
0.50	14.8	649.33	270.00	2263.0	48.0	37.41	0.141
1.0	8.6	1574.8	58.85	3242.0	58.30	27.40	0.064
5.0	0.20	11810.70	28.0	7200.0	107.70	113.20	0.030

* Average of five plants
Elongation from start of experiment

5.3 DISCUSSION

5.3.1 Water Analysis

In principle a large number of instrumental methods exist for the determination of heavy metals in natural waters (58). However, only AAS, and particularly GFAAS, with hydride generation for As and Se and cold vapour for Hg modes are the most common instrumental methods for laboratories engaged in tasks in aquatic trace metal chemistry; while voltammetry, mainly with DPASV, offers an alternative instrumental approach (58). Most other methods fail either through lack of sensitivity (e.g. colorimetry), problems of interferences (arc emission spectroscopy), or the need for a major investment in equipment (e.g. neutron activation or ICP-emission or mass spectroscopy).

However, the higher sample throughput often claimed for AAS remains largely fictitious in practice, because AAS is conventionally a single-element technique. In aquatic systems, several heavy metals (e.g. Cu, Pb, Cd, Zn, or Ni and Co or Hg and Cu, etc.) usually have to be determined. The potential of voltammetry for simultaneous determinations thus balances the faster measurement rate of AAS for a single element; moreover if comparable precision is to be achieved, AAS needs more test measurements than voltammetry (22). Also, disadvantageous is the rather restricted dynamic range of GFAAS and the contamination sensitivity resulting from the use of very small analyte volumes (5-50 µl) per firing (58).

For most problems in the trace metal chemistry of natural waters, voltammetric determinations of the toxic heavy metals require prior preconcentration for low levels of concentration, in the same way as other instrumental methods. This is where the electrochemical approach

provides its greatest inherent advantage, because the preconcentration can be done electrochemically in the same cell as the final measurement. Thus, in contrast to common chemical preconcentration procedures there is little risk of additional contamination (22). Tables 5.2 (page 305) and 5.3 (page 313) indicate that the levels of Cd, Cu, Pb and Zn were detectable; fortunately all metals were present in low concentrations, except for zinc which was present at much higher levels in all 18 samples (range 17 to 390 µg/L). In spite of the low flow rate in the river, because of sampling during summer time, the levels of these metals do not represent a major problem; which is satisfactory because in high flow conditions, e.g. in winter or after rain, the levels of metals would be expected to be much lower. Higher metal levels were to be expected at Reybridge, Staverton and Keynsham (all near industrial sites or sewage works).

The results demonstrate and emphasise the importance of weather conditions on pollution levels. Randall (6) reported on some samples collected from the River Avon after heavy rainfall which were found to contain metal levels similar to those of the background electrolytes.

The general trend of metals level given in Table 5.2 is Cd < Cr < Pb < Cu < Ni < Zn, that order being maintained for all 18 samples. The zinc concentration was well above the median concentration of 15 µg/L usually associated with fresh waters (20). A similar situation arises for Ni which was present in samples in the range (5.9-7 6 µg/L) which is also higher than the range reported for fresh water (0.02-27 µg/L).

With these two exceptions, all other metal levels were as expected, thus Cr levels of 0.15 to 4.55 µg/L were within the recommended range (0.1-6 µg/L) (20). Similarly, the levels of Cd, Cu and Pb were within

those expected for fresh waters. The analytical results obtained for Cd seem to differ for the two techniques employed, with the DPASV result being higher than for GFAAS, probably because of volatilisation problems mentioned earlier for Cd when using GFAAS. At the same time, however, both methods demonstrate an increased level of Cd at Reybridge, Kellaways and Keynsham.

However, for Cu and Pb the levels reported by both techniques are very close together. Cu was present in all samples but over a wide concentration range, being the opposite of the Pb levels which appear to occupy a rather narrow range of concentrations. As expected, Staverton, Newbridge (Bath) and Keynsham exhibited higher levels of copper, each being downstream of an urban area, in particular sewage works.

5.3.2 Sediment Sample Analysis

The results in Table 5.15 collect the results listed in Table 4.9 for selenium, Table 5.4 for Cd, Cu, Cr, Ni and Zn, and Table 5.10 for Tl. The selenium content in the sediments (measured by GFAAS and GLC) was discussed in detail in Chapter 4, while the Tl content in sediment was measured by DPASV only, with the Cd, Cu, Cr, Ni and Zn being determined by GFAAS.

The mean level of Cd expected in such sediments is 0.17 μg/g (20) and examination of Table 5.15 shows that for all sites except Reybridge this level is exceeded. The level at Staverton was particularly high, reflecting general contamination of the river sediments but without cause for major concern.

In the case of copper, only two results were higher than those reported for the mean sediment value (33 μg/g), which reflects contamination, especially at Newbridge (Bath), being downstream of a major urban area. However, the chromium content in the sediments was lower than reported for the mean sediment value [72 μg/g (20)], but again the Bath sample had the highest Cr content. A similar situation holds for Ni which was present at a lower level than reported for the mean sediment i.e. nickel does not appear to be a major contaminant. However, for Pb it must be emphasised that no contamination of these samples appears since all levels of Pb were very much lower than that suggested for the mean sediment value of 19 μg/g (20)). Turning to the Zn content in sediments, in each case it was present at levels above the mean concentration [95 μg/g for sediment (20)], being similar to the Zn content of the water samples. Finally the thallium content in all samples of sediment was less than 50 ng/g Tl, which indicates no contamination problem from the thallium content

in sediments. Such a result was expected because the geological nature of the river basin is mainly limestones, which usually do not contain thallium, and few industries along the Avon are expected to use the element.

Finally the selenium content in sediment was present in the range 0.63-2.35 μg/g, with the reported mean value in sediment being 0.42 μg/g (20). The results indicate that the area (Reybridge and downstream) is contaminated with selenium, reflecting the history of Se being used in the Chippenham area.

In general the metals and selenium listed in Table 5.15 follow the order:

$$Zn > Cu > Cr > Ni > Pb > Se > Cd > Tl$$

This sequence indicates that zinc is a very prevalent metal while thallium is a very rare one, and that above average levels exist for Zn, Cu, Cd and Se but not so for Cr, Ni and especially Pb.

Table 5.15. Selenium and Heavy Metals in Sediments

Site Sample	Concentration (μg/g)							
	Cd	Cu	Cr	Ni	Pb	Se*	Tl**	Zn
Kellaways	0.48	16.4	20.9	33.1	1.03	1.1	0.030	115
Reybridge	0.065	23.3	15.0	36.0	2.40	2.35	0.033	170
Melksham	0.37	53.2	15.2	29.1	2.92	1.75	0.0175	211
Staverton	1.01	23.4	20.6	27.0	1.90	1.0	0.0225	163
Limpley Stoke	0.64	21.5	13.4	30.0	2.70	0.80	0.0425	125
Newbridge, Bath	0.40	75.6	57.8	41.8	3.26	0.63	0.025	214

* Selenium determined by GFAAS and GLC

** Thallium measured by DPASV

Cd, Pb and Ni determined by GFAAS

Cr, Cu and Zn measured by FAAS

5.3.3 Digestion of Plant Materials

Particularly in the field of trace analysis, decomposition processes should be controlled to prevent falsification of analytical results due to sample contamination in laboratories, through dirty equipment or because of losses caused by volatilisation, adsorption, etc. Systematic errors of this kind increase as the concentration of the element to be determined in the sample decreases. It is, therefore, essential to avoid such sources of errors not only by controlling the individual analytical steps after sample preparation, but also during sampling and sample storage (59).

In the last few years, the method of "wet" sample preparation within closed containers has become widely applied (59).

Since 1955 numerous modified pressure systems have been described involving, as an inner chamber, a vessel made of polytetrafluoroethylene (PTFE) (60-62).

Pressure decomposition procedures have as their advantages the following (59):

a) Increased reactivity - faster reaction;

b) Decrease in blank values;

c) Avoidance of trace element losses such as As, Be, Cd, Cr, Hg, Pb and Se.

In Chapter 4, pp. 256-258, different methods for digestion including digestion with a Teflon vessel contained within a stainless steel sheath were used. The recommended method for digestion was carried out on plant materials to determine Se, Cd, Hg, Tl and Te by using a Teflon vessel containing 5 ml concentrated nitric acid and heating at 150°C for 60 minutes in an electric oven.

Many workers have recommended acid pressure decomposition in

trace element analysis. Thus for the determination of thallium in plant materials (21,63), a method reported by Williams (64) was employed, which uses a pressure container. A similar procedure was suggested by Elsokary (15) for the determination of mercury in plant material, with satisfactory results. While Kumpulainen et al. (65) studied several methods of digestion of selenium, they reported that in the determination of selenium in nutritional supplements, use of perchloric acid or sulphuric acid is not necessary, and recommended boiling samples in nitric acid.

However, for the determination of Mn, Fe, Cu and Zn in plant materials a simple digestion method, involving nitric acid in a glass beaker under an IR lamp for 30-60 minutes before evaporation to dryness and filtration through a Whatman No. 541 filter paper was used.

5.3.4 Plant Analysis

i) Determination of Mercury

Mercury was determined in plant materials by two techniques, GFAAS and CVAAS. Tables 5.6 and 5.7 illustrate the results for Hg by the two different techniques. Generally the level of mercury in plants determined by CVAAS was found to be higher than by GFAAS for the same sample. Possibly there is loss of mercury during the drying and ashing stages of the GFAAS procedure, since Hg is a very volatile element which is easily produced through reduction. However, before a more definitive opinion can be expressed a detailed examination of the GFAAS method must be made. CVAAS afforded good precision, and sensitivity, and is a very fast technique.

ii) Determination of Thallium

Thallium was measured in plant materials by GFAAS and DPASV. The results in Tables 5.9 and 5.10 indicate the level of thallium in plant and sediment determined by (one technique only) GFAAS and DPASV respectively, while Table 5.11 demonstrates the level of thallium in some plant samples determined by both techniques. It was found that the level of thallium determined by DPASV with HMDE was higher than in GFAAS for the same sample. The inhomogeneity of the sample and different matrices used with each technique may be responsible for the difference in level of thallium in each sample. It is thought unlikely that there was loss of Tl through volatilisation prior to the GFAAS atomisation step. The GFAAS method was fast and required a more rigorous dissolution procedure. The thallium voltammograms obtained by DPASV with HMDE are shown in Figures 5.8, 5.9 and 5.10, and demonstrate the thallium content of a sediment, root and shoot respectively.

iii) Determination of Cadmium and Tellurium

Cadmium and tellurium in plant materials were determined by GFAAS only. For cadmium, it is a typical metal for estimation using GFAAS. The AAS determination of Cd is remarkably selective and sensitive, while DLs are down to 0.1 to 2 pg (26), with no major interferences. Table 5.1 indicates the result of Cd in plants.

Fortunately, tellurium was determinable by GFAAS, because it was difficult to find another technique available in the laboratory to estimate the tellurium levels in plant materials. Another advantage for this determination of Te was that it used the same digested acid sample as when measuring Se, Tl and Te. The results of the Te level in plants were expected to be similar to the Se level in plants, because of their similarity from a chemical point of view. Table 5.13 indicates the Te level in plants while Tables 6.6 and 6.7 (see Chapter 6) show the Te level in Experiment 2.

5.3.5 Toxicity of Cadmium, Mercury, Thallium and Tellurium to Plants

From the results listed in Tables 5.16-5.19, an attempt was made to determine the upper critical level of Cd, Hg, Tl and Te in the roots and shoots of Lolium perenne seedlings which had been grown in Cd, Hg, Tl and Te individually contaminated hydroponic cultures. The yield curves are shown in Figures 5.11 to 5.14 respectively using a similar procedure as for selenium (see page 283). Basically, the method consists of plotting the Log tissue concentration of (Cd, Hg, Tl, Te) μg/g of dry weight of material versus the yield in grams of dry weight of material and attempting to find the best regression lines to fit the data which afford a point of intersection. Hence one can estimate the upper critical (T_c) value and the lethal metal

Table 5.16. Cadmium Concentration in Shoots and Roots of Lolium perenne

Concentration of Cd (µg/ml) in nutrient solution	Conc. of Cd (µg/g) in plant tissue	Log_{10} conc. of ^{10}Cd in plant tissue	Dry weight (g) of plant
Control Shoot	N.D.	-	1.041
1×10^{-3}	0.848	-0.072	0.936
5×10^{-3}	1.183	0.073	0.870
0.01	2.50	0.398	0.784
0.05	4.35	0.638	0.626
0.10	7.25	0.860	0.658
0.50	30.10	1.478	0.495
1.0	66.80	1.825	0.515
2.5	103.21	2.014	0.452
5.0	195.53	2.291	0.435
Control Root	N.D.	-	0.229
1×10^{-3}	4.70	0.672	0.180
5×10^{-3}	14.10	1.149	0.177
0.01	17.87	1.252	0.162
0.05	41.30	1.616	0.155
0.10	85.70	1.933	0.160
0.50	134.15	2.127	0.138
1.0	307.80	2.488	0.127
2.5	713.20	2.853	0.115
5.0	1061.85	3.026	0.089

FIG. 5.11 CADMIUM UPPER CRITICAL LEVEL: YIELD CURVE FOR SHOOTS AND ROOTS PLOTTED AGAINST LOG TISSUE CONCENTRATION

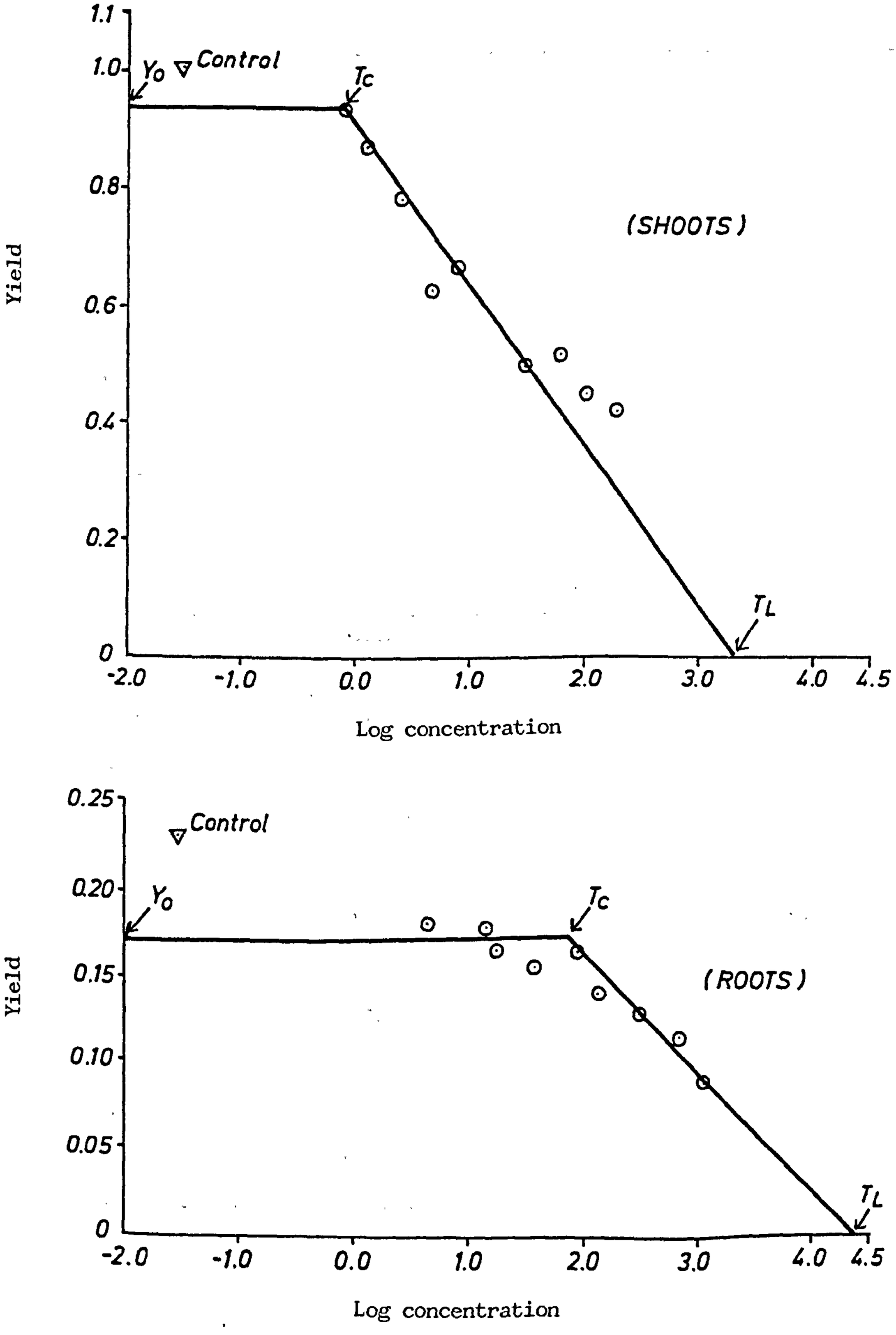


Table 5.17. Mercury Concentration in Shoots and Roots of Lolium perenne

Concentration of Hg (µg/ml) in nutrient solution	Conc. of Hg (µg/g) in plant tissue	Log_{10} conc. of Hg in plant tissue	Dry weight (g) of plant
Control Shoot	N.D.	-	0.945
1×10^{-4}	0.84	-0.076	0.800
5×10^{-4}	1.05	0.021	0.720
1×10^{-3}	1.16	0.064	0.680
5×10^{-3}	1.30	0.114	0.620
0.01	1.83	0.262	0.613
0.05	2.25	0.352	0.630
0.10	2.60	0.415	0.590
0.50	15.52	1.191	0.580
1.0	30.70	1.487	0.354
5.0	328.30	2.516	0.140
Control Root	N.D.	-	0.255
1×10^{-4}	1.81	0.257	0.214
5×10^{-4}	3.20	0.505	0.180
1×10^{-3}	4.00	0.602	0.175
5×10^{-3}	11.65	1.066	0.155
0.01	18.28	1.262	0.140
0.05	101.35	2.006	0.132
0.10	133.18	2.124	0.161
0.50	649.33	2.812	0.141
1.0	1574.8	3.197	0.064
5.0	11810.7	4.072	0.030

FIG. 5.12 MERCURY UPPER CRITICAL LEVEL : YEILD CURVE FOR SHOOTS AND ROOTS PLOTTED AGAINST LOG TISSUE CONCENTRATION

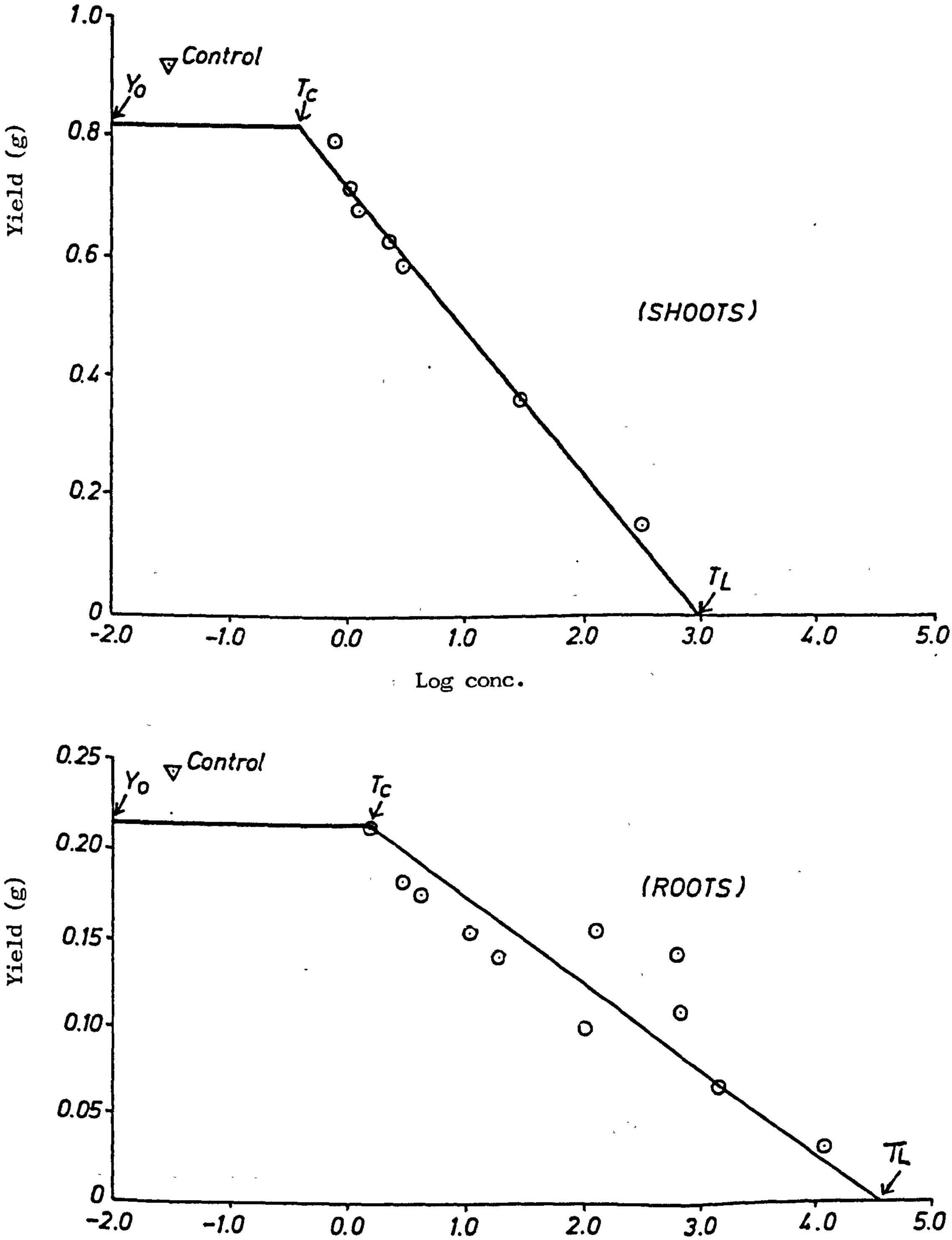


Table 5.18. Thallium Concentration in Shoots and Roots of Lolium perenne

Concentration of Tl (µg/ml) in nutrient solution	Conc. of Tl (µg/g) in plant tissue	Log_{10} conc. of Tl in plant tissue	Dry weight (g) of plant
Control Shoot	N.D.	-	0.918
1×10^{-4}	N.D.	-	0.937
5×10^{-4}	0.075	-1.125	0.928
1×10^{-3}	0.160	-0.796	0.907
5×10^{-3}	0.48	-0.318	0.930
0.01	1.05	0.021	0.915
0.05	1.34	0.127	0.777
0.10	2.65	0.423	0.743
0.50	26.30	1.420	0.380
1.0	61.85	1.791	0.216
2.5	144.05	2.158	0.125
Control Root	N.D.	-	0.216
1×10^{-4}	0.42	-0.377	0.213
5×10^{-4}	0.77	-0.113	0.230
1×10^{-3}	1.45	0.161	0.223
5×10^{-3}	2.3	0.362	0.218
0.01	7.1	0.851	0.215
0.05	12.9	1.11	0.200
0.10	33.25	1.522	0.198
0.50	68.5	1.836	0.093
1.0	369.1	2.567	0.048
2.5	576.0	2.76	0.040

FIG. 5.13 THALLIUM UPPER CRITICAL LEVEL: YIELD CURVE FOR SHOOTS AND ROOTS. YIELD PLOTTED AGAINST LOG TISSUE CONCENTRATION

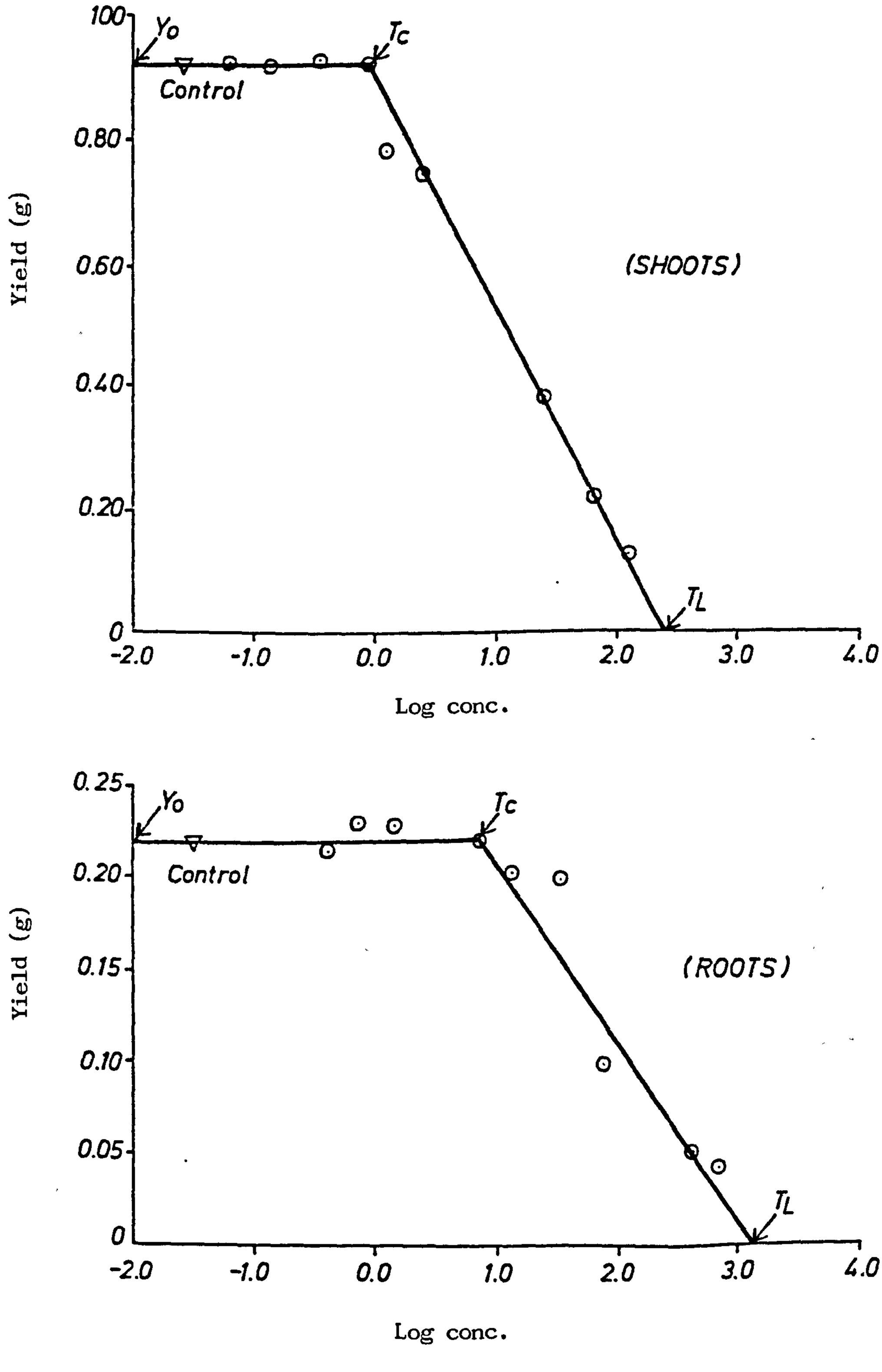
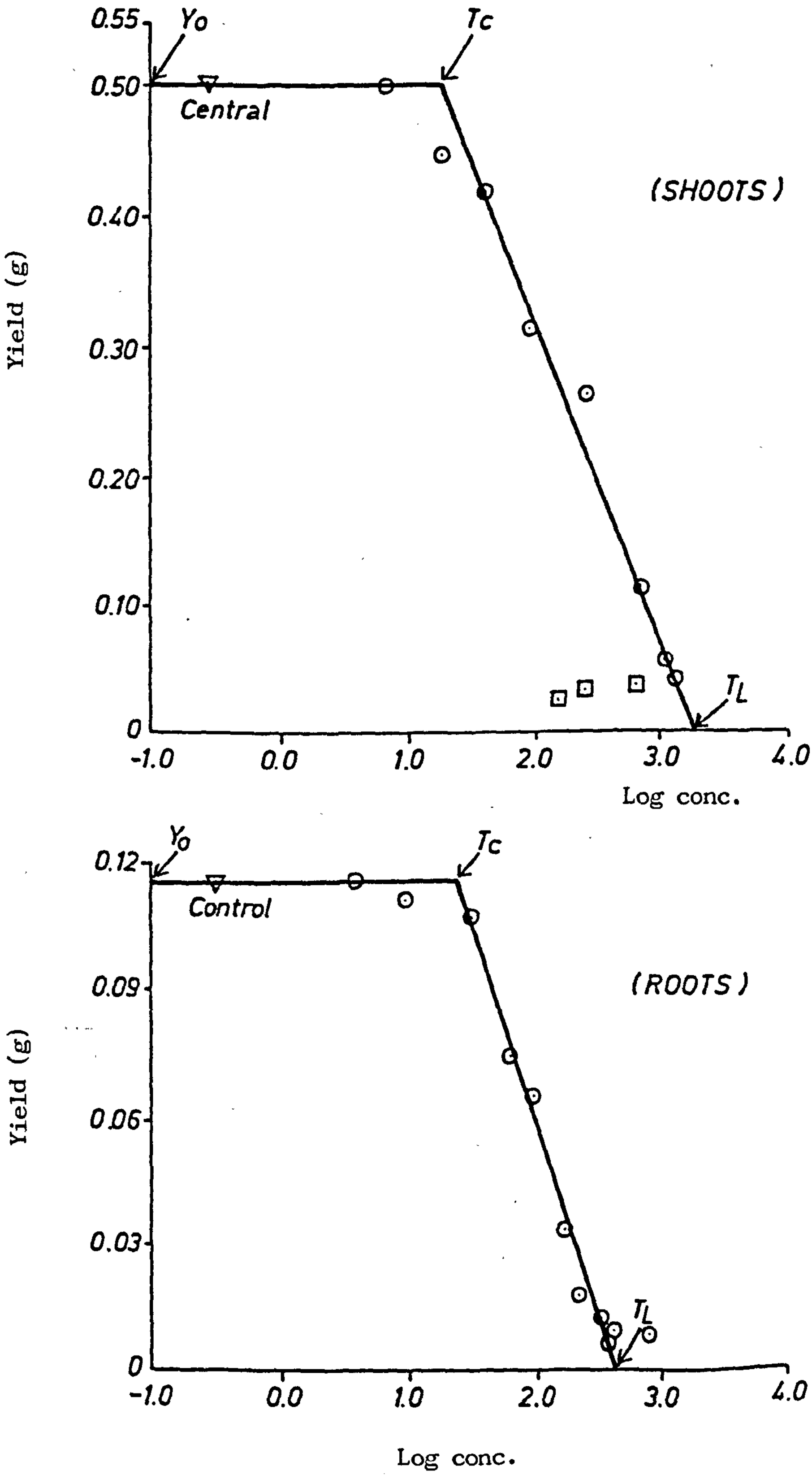


Table 5.19. Tellurium Concentration in Shoots and Roots of Lolium perenne

Concentration of Te (μg/ml) in nutrient solution	Conc. of Te (μg/g) in plant tissue	Log_{10} conc. of Te in plant tissue	Dry weight (g) of plant
Control Shoot	N.D.	-	0.501
0.1	7.55	0.878	0.497
0.5	18.75	1.273	0.432
1.0	44.30	1.646	0.417
2.5	96.25	1.983	0.310
5.0	267.8	2.428	0.262
10.0	780.4	2.892	0.113
15.0	1158.5	3.064	0.050
20.0	1435.9	3.157	0.039
30.0	618.2	2.79	0.032
40.0	245.0	2.389	0.029
50.0	153.6	2.186	0.025
Control Root	N.D.	-	0.1156
0.1	4.13	0.616	0.1152
0.5	9.60	0.982	0.1102
1.0	33.40	1.524	0.1070
2.5	59.32	1.773	0.073
5.0	98.20	1.992	0.065
10.0	157.5	2.197	0.033
15.0	201.7	2.305	0.017
20.0	310.8	2.492	0.012
30.0	758.3	2.88	0.0095
40.0	418.2	2.621	0.0087
50.0	365.1	2.562	0.0085

FIG. 5.14 TELLURIUM UPPER CRITICAL LEVEL: YIELD CURVE FOR SHOOTS AND ROOTS. YIELD PLOTTED AGAINST LOG TISSUE CONCENTRATION



concentration T_L for roots and shoots as listed in Table 5.20.

If Tl is taken as an example, the critical and lethal concentrations for roots were 7.08 and 1122 µg/g and for shoots 1.58 and 251.2 µg/g respectively. In fact, these results may not be sufficiently precise and from a practical point of view the critical level T_C may be lower than reported here. The levels for the other elements are similar in value.

The results in Table 5.20 indicate that the toxicity of these metals decreases in the following order:

Shoots Hg > Cd > Tl > Te

Roots Hg > Tl > Te > Cd

However, the estimation of an unaffected yield Y_o was not accurate because the experiments were run in sequence, so the growth conditions varied over the duration of the experiment.

Table 5.20. Critical Concentration of Cd, Hg, Tl and Te in Shoots and Roots of Lolium perenne seedlings

Plant material	Critical concentration	Cd	Hg	Tl	Te
Shoot	Y_o (g)	0.94	0.82	0.82	0.50
	T_C (µg/g)	1.0	0.4	1.58	22.4
	T_L (µg/g)	3162	1000	251.2	1585
Root	Y_o (g)	0.17	0.217	0.22	0.115
	T_C (µg/g)	63.1	1.78	7.08	25.1
	T_L (µg/g)	25118	3548	1122	398

5.3.6 Uptake of the Spiked Metal and Some Essential Metals (Mn, Fe, Cu and Zn) by Plant Samples

For each experiment the level of the spiked metal (namely Cd, Hg, Tl or Te respectively) in either the dried roots or shoots was determined together with the corresponding levels of four essential elements (Mn, Fe, Cu and Zn).

The determination of Mn, Fe, Cu and Zn in plant samples was carried out by digestion of each sample with concentrated nitric acid (10 ml) followed by heating under an IR lamp, and carried out using FAAS Model IL 151 with the parameters as discussed in detail earlier (see page 273). The results of these essential metals are listed in Tables 5.1, 5.9, 5.13 and 5.14 for Cd, Tl, Te and Hg respectively.

Table 5.1 illustrates the relationship between the cadmium content in the nutrient solution and the uptake of Cd, Mn, Fe, Cu and Zn. It is obvious that Cd uptake in shoots and roots increases with increase in cadmium concentration in the nutrient solution. Thus the Lolium perenne seedlings directly reflect the value of the cadmium concentration fed to them. The result is confirmed when correlation plots were made and r values of 0.9913 and 0.9879 for shoots and roots were obtained respectively; the result of all correlations are listed in Table 5.21.

The Mn uptake decreases with increase in Cd concentration both for the shoots and roots, although the former correlation is much better at -0.9515 than the latter (-0.690). However, for iron there appears to be little correlation between the level of iron in the roots with increasing concentration of added Cd. But for the shoots a similar result to that for Mn in shoots is obtained with a negative correlation with added Cd of -0.90. There seems to be no correlation

between the uptake of Cu by these plant tissues and the level of Cd in solution for this particular species; the correlation coefficients being around 0.1 or less. Thus no competition between Cu and Cd is expected. Although the data appears scattered in terms of precision, as expected there is a negative correlation between the level of zinc in both roots and shoots and increasing Cd concentration - an expected result in view of the chemical similarity of these two elements and one which has been observed for Holcus lanatus by Coughtrey and Martin. What is disappointing is that the quality of the data does not allow a higher correlation coefficient than approximately -0.7 to be reached (see Table 5.21).

Next considering mercury, there are the expected correlations between Hg-levels in both roots and shoots and the level in the nutrient solution, with extremely high correlation coefficients being reported. The relationship between Hg-level in solution and essential element concentration in the roots and shoots is not so clear cut as for Cd. There appears to be no relationship between level of Mn in either roots or shoots and Hg-content, which is to be compared to the Cd case. However, this cannot be said for the Fe case, here a strong positive correlation exists between Fe-levels in roots and Hg-level in the nutrient solution, but with a negative correlation between Fe-levels in shoots and Hg in solution. For the case of Cu both correlations are positive, increase in Hg-toxicant concentration bringing about an increase in Cu concentration in the biological tissue. Finally for zinc, the situation is similar to that for iron, there is a positive correlation in the case of root concentration and a negative one for shoot levels. The roots appear to take in more Fe, Cu and Zn but less Mn with rise in mercury concentration and transmit to the

shoots decreasing levels of Mn, Fe and Zn, while Cu also increases in concentration.

In a similar manner the level of Tl in either the roots or shoots is a direct reflection of the level of that element in the nutrient solution. Two very high correlation coefficients testify to this suggestion. The only correlations of merit are those between Tl level in solution and the Fe- and Cu-contents of the roots (+0.920 and +0.968 respectively), and Mn-content of the shoots (+0.904). Thus Tl appears to behave differently to both Cd and Hg and presumably has a different mode of toxicity. Presumably it is probably interfering with the K^+-ion transport rather than being a general toxic agent. Zinc levels in both roots and shoots appear independent of Tl concentration.

Finally, Table 5.13 illustrates the Te-level in roots and shoots, along with the concentration of Mn, Fe, Cu and Zn in some plant tissue. Although a range of concentrations from 0 to 50 µg/ml of Te(VI) in nutrient solution was , correlations in the range 0 to 30 µg/ml will be used because at the 40 and 50 µg/ml concentrations of Te(VI) the plants died.

Like Se(VI), there is a reasonable correlation between the level present in roots or shoots and that in nutrient solution, with the added similarity that the level in shoots is greater than in roots. The reverse is always true for the heavy metals studied. For both roots and shoots there are high positive correlations with levels of Fe in the tissue with the level of Te(VI) in solution. Mn shows a positive correlation for shoots but a negative one for roots, which is similar to that found for Se. For Zn, moreover, there appears to be no relation between uptake for either roots or shoots and toxicant

presence. Copper tends to show the same behaviour as Fe but the trends are not so clear cut, probably reflecting the lower overall range of levels of Cu when compared to Fe. Certainly, the plants appear to take in increasing concentrations of iron in response to increasing levels of Te(VI). Now work should be carried out in order to ascertain the control mechanism which is being disturbed.

Table 5.21. The Correlation Values of Toxic Metals Against Essential Metals (Mn, Fe, Cu, Zn)

			Relationship between metal "spike" v essential metals			
Plant material	Toxic metal in nutrient solution	Metal in nutrient v metal conc.	Mn	Fe	Cu	Zn
Shoot	Cd	Cd v Cd 0.9913	-0.9515	-0.9003	0.0364	-0.6527
	Hg	Hg v Hg 0.9932	-0.243	-0.539	0.7835	-0.6606
	Tl	Tl v Tl 0.9991	0.9042	-0.0687	0.6625	0.0629
	Te	Te v Te 0.7532	0.7855	0.9387	0.7484	-0.484
Root	Cd	Cd v Cd 0.9879	0.692	0.3245	0.1267	-0.7836
	Hg	Hg v Hg 0.9971	-0.7653	0.970	0.844	0.9419
	Tl	Tl v Tl 0.9733	-0.5995	0.9199	0.9681	-0.1523
	Te	Te v Te 0.9555	-0.8828	0.9643	0.3068	-0.1356

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CHAPTER 6

SELENIUM AND ITS INTERACTIONS (VIA RESPONSE SURFACE METHODOLOGY)

CONTENTS

6:1 INTRODUCTION

Response surface methodology (RSM) was developed and described by Box and his colleagues (1).

(Box and Wilson 1951, Box 1954, Box and Youle 1955) (2-4). RSM exploits the concepts of simple empirical models such as low-degree polynomials in order to approximate the relationship between a response variable and a set of input variables (independent variables) over a current region of interest (1). Thus RSM now consists of a group of techniques used in the empirical study of relationships between one or more measured responses on the one hand and a number of independent variables on the other (5,6).

Since its introduction in the early 1950s, RSM has developed into an accepted and widely used set of concepts and techniques (1).

There are several monographs which contain detailed accounts of RSM. Davies (7; Chapter 11), Cochran and Cox (8; Chapter 8A). Other examples are John (9; Chapter 10) and the book by Myers (6) including both the design of response surface experiments and the estimation and interpretation of the fitted surface. An introduction to the subject at a more elementary level is given in Box, Hunter and Hunter (5; Chapter 15).

RSM for more than two factors is difficult to visualise but it can be approached by plotting two factors at a time while holding the other factors constant (10,11).

Therefore, the response surface may be represented in three dimensions showing the relationship between the response and the levels of two factors (12,13).

6:1:1 Linear Models

A common misconception involves the meaning of the words "linear model" to mean (and to be limited to) straight-line relationships of the form

$$y = a + bx \tag{6.1}$$

where y is generally considered to be the dependent variable, x is the independent variable, and a and b are the parameters of the model (intercept and slope, respectively). Although it is true that equation (6.1) is a linear model, the reason for this is not that its graph is a straight line. Instead, it is a linear model because it is constructed of additive terms, each of which contains one and only one multiplicative parameter (5,13). That is, the model is first-order or linear in the parameters. This definition of "linear model" includes models that are not first-order in the independent variables. The model

$$y = a + bx + c(10^x) + d \log (x) + ex^2 \tag{6.2}$$

is a linear model by the above definition.

The model

$$y = a [\exp(-bx)] \tag{6.3}$$

however, is a nonlinear model because it contains more than one parameter in a single term. For some nonlinear models it is possible to make transformations on the dependent variable, on the independent variable, or on both, to "linearise" the model. Taking the natural logarithm of both sides of equation (6.3), for example, gives a model that is linear in the parameters a' and b :

$$\log_e(y) = a' - bx, \text{ where } a' = \log_e(a) \quad (13).$$

6:1:2 Response Functions and Fitting Model to Data

The relationship between response R and two factors x_1 and x_2 or more may be represented by a surface called the response surface:

$$R = \phi\ (x_1, x_2, \ldots\ x_k) \tag{6.4}$$

This function ϕ is called the response function.

It is convenient to visualise the relation between response and the factor levels geometrically.

A model of such a surface, in which x_1 may be the reaction time, x_2 the concentration of one of the reactants, and R the yield is shown in Figure (6.1). The possible form of true function ϕ is a polynomial which satisfies the relationship between the response R and the K important variables x_1, x_2, ... x_K (7).

The common polynomials are:

i) first order polynomials

$$R = B_o + B_1x_1 + B_2x_2 + \ldots\ B_Kx_K \tag{6.5}$$

ii) second order polynomial

$$R = B_o + B_1x_1 + B_2x_2 + \ldots\ B_Kx_K + B_{12}x_1x_2 + \ldots\ B_{K-1}x_{K-1}x_K + B_{11}x_1^2 + B_{22}x_2^2 \ldots\ + B_{KK}x_K^2 \tag{6.6}$$

iii) third order polynomial

$$R = B_o + B_1x_1 + B_2x_2 + B_{11}x_1^2 + B_{22}x_2^2 + B_{12}x_1x_2 + B_{111}x_1^3 + B_{222}x_2^3 + B_{112}x_1^2x_2 + B_{122}x_1x_2^2 \tag{6.7}$$

The coefficients B_o, B_1, B_2, B_{11}, B_{22}, B_{12}, B_{111}, B_{222}, B_{112} and B_{122} are parameters to be estimated from the data. Table 6:1 gives the number of coefficients in different degrees of polynomial (3).

Figure 6:1: RESPONSE SURFACE FOR TWO FACTORS

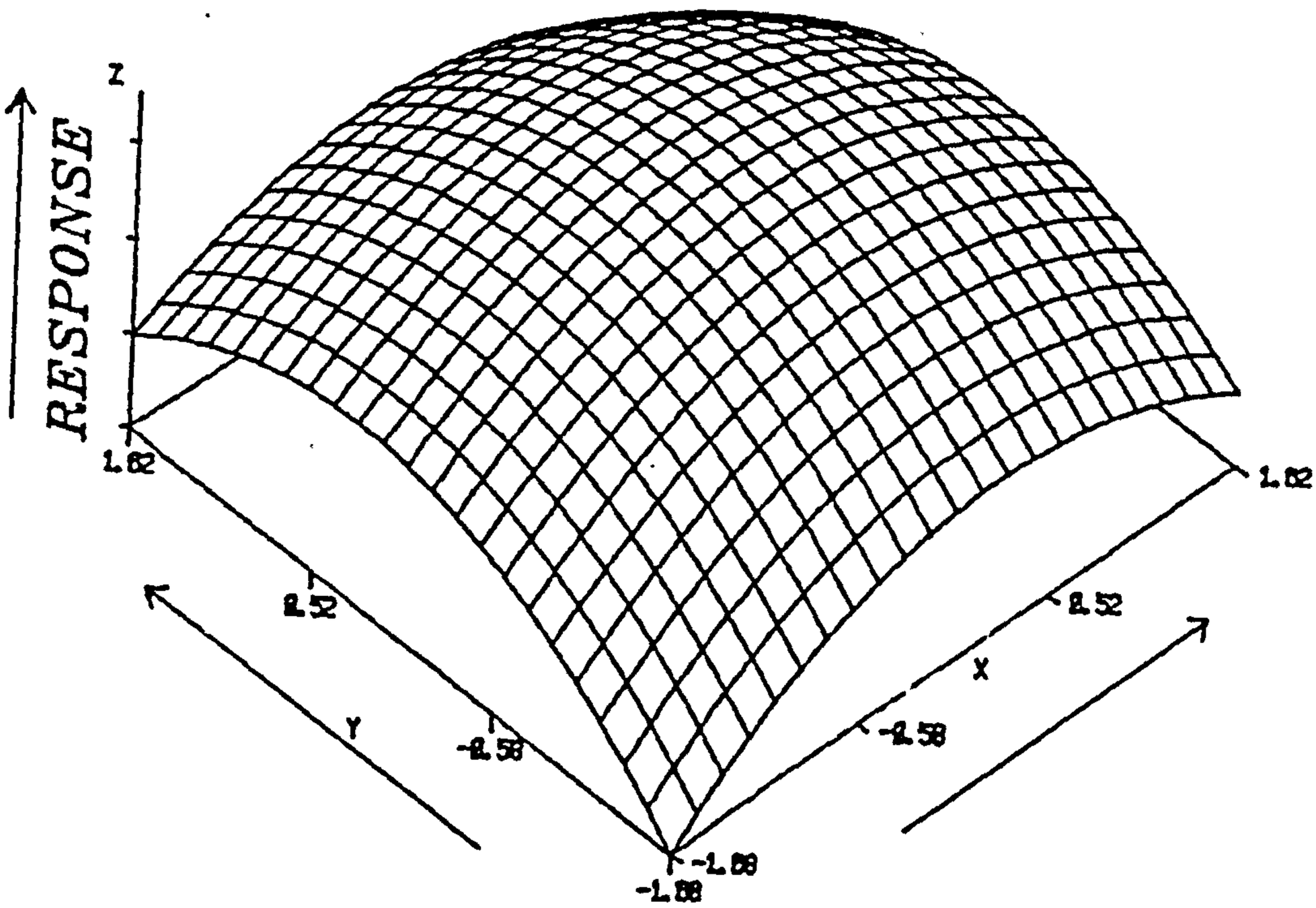


Table 6:1 Number of Coefficients with Different Degrees of Polynomial

Number of Factors	Degree of Polynomial			
	First	Second	Third	Fourth
2	3	6	10	15
3	4	10	20	35
4	5	15	35	70
5	6	21	56	126

The precise nature of the response surface is not known but it is approximated to by fitting the polynomial of the Kth degree (14) to the data.

For K equal to 2 and 3 respectively the first order polynomial and second order polynomials are illustrated in the equations below:

$$R = B_o + B_1x_1 + B_2x_2 \tag{6.8}$$

$$R = B_o + B_1x_1 + B_2x_2 + B_{12}x_1x_2 + B_{11}x_1^2 + B_{22}x_2^2 \tag{6.9}$$

For K = 3

$$R = B_o + B_1x_1 + B_2x_2 + B_3x_3 \tag{6.10}$$

$$R = B_o + B_1x_1 + B_2x_2 + B_3x_3 + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{23}x_2x_3 + B_{11}x_1^2 + B_{22}x_2^2 + B_{33}x_3^2 \tag{6.11}$$

where the parameters :

B_o = intercept

B_1, B_2, B_3 = linear

B_{11}, B_{22}, B_{33} = quadratic

B_{12}, B_{13}, B_{23} = interaction

The first degree equation represents a plane, if the degree of fit is inadequate, then a second degree equation should be used. Analysis of the fitted second degree equation might indicate certain features of the surface requiring further study. If the second degree equation is a poor fit, then steps should be taken to try to fit a third degree equation.

The goodness of the fitted polynomial can be examined by estimation. The term "lack of fit" is a component which measures the deviations of the responses from the fitted surfaces and experimental errors which are calculated from the replicated points at the centre of the polynomial (15).

In the present work, all the coefficient parameters of the fitted polynomial and "lack of fit" were determined by computational methods (SAS and GINO) (16,17). There are basically four types of surfaces which the second order polynomial can represent (3,5,7):

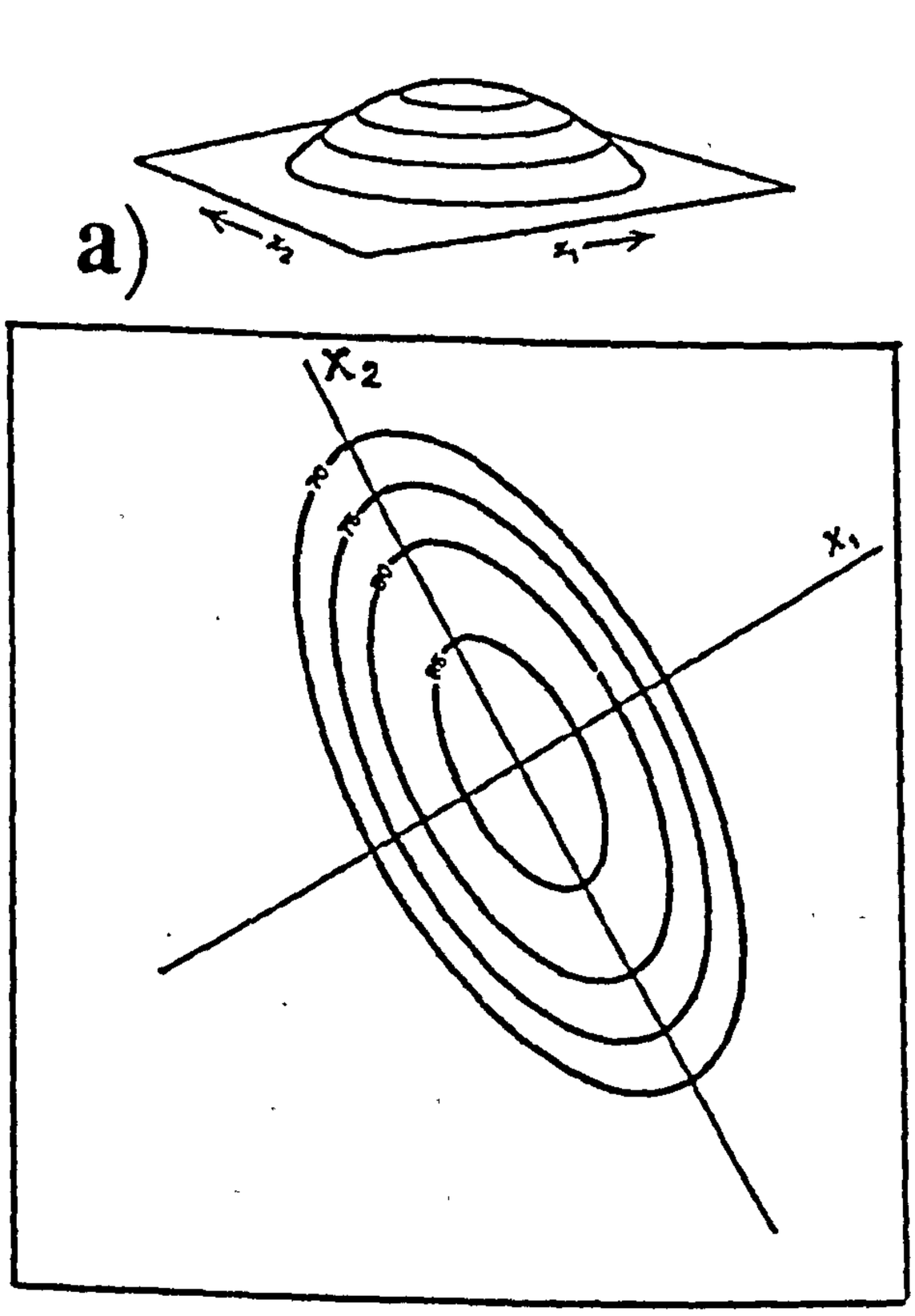
a) if B_{11} and B_{22} are of the same sign, the shape of surface is similar to Figure 6:2a. In this particular case B_{11} and B_{22} are both negative and the centre of the system is a maximum. But if they are positive, the surface is a minimum.

b) when B_{11} and B_{22} are of opposite sign, the shape of the surface is shown in Figure 6:2b. The surface is called a saddle point or minimax.

c) when B_{22} is zero, the shape of the surface is described by Figure 6:2c. The surface is called a stationary ridge surface.

d) if B_{22} is zero and the centre of the system were at infinity, Figure 6:2d which describes the shape of the surface is a rising ridge system.

Figure 6:2: FUNDAMENTAL AND LIMITING SURFACE GENERATED BY SECOND ORDER POLYNOMIAL IN TWO AND THREE DIMENSIONS



6:1:3 Applications of RSM

The objective of this chapter was to investigate the effects of some toxic metals and metalloids on Lolium perenne, and interactions (if any) between the metals or metalloid uptake by plants using RSM techniques.

RSM has been used in the solution of certain types of problems which are pertinent to scientific processes. The major applications have been in industrial research, particularly in situations where a large number of variables influence certain features of the system.

The feature (e.g. reaction yield, concentration of reactant, pH, cost of production, etc.) is termed the response. RSM usually helps the experimenter to make an efficient empirical explanation of the system in which he is interested.

Initially RSM was used in the chemical industry, where nearly all process-oriented problems involve an optimization phase; that is, finding through experimentation conditions of the variables that give rise to a desirable process yield (6).

A typical RSM study begins with a definition of the problem; those questions which become particularly critical in these situations are:-

i) are a large number of factors important?

ii) which responses are to be measured?

iii) how are they to be measured?

iv) over what ranges are they to be explored?

v) which variables are to be important?

One of the important applications of RSM, in a simplified form, has been to improve the performance of existing industrial processes by systematically varying process variables and gathering data while

the processes remain in operation, without upsetting normal production. This use of RSM for process improvement has been known as evolutionary operation (1).

The applications of RSM in the field of chemistry has been reported and discussed:-

1) An important application of RSM was by Hader and Moore (1957) to investigate the relationships between copper, iron and molybdenum on the growth and nutrition of lettuce (18,19).

2) Tidwell (1960) used RSM to improve the chemical process in the yield of product D in the reaction: $A + B + C \xrightarrow{\text{catalyst}} D + B + C +$ other products (20).

3) RSM has been discussed and used by Aia et al. (1961) to study the precipitation of the dihydrate of calcium hydrogen orthophosphate, $CaHPO_4.2H_2O$ at constant temperature and varying concentrations of calcium chloride and ammonium phosphate (21).

4) In 1971 Rubin et al. examined the effect of complexing agents on the yield of certain antibiotics, by fitting a second order model (central composite design for three variables) for both cost and yield responses (22).

5) RSM has been employed in gas chromatographic studies to find the relationships between the controlled factors in GLC (e.g. flow rate, temperature and stationary phase) and the measured response (23).

6) Deming et al. (1979) have used RSM (especially second order polynomials) in clinical chemistry, one example being the measurement of the absorbance response as a function of calcium and magnesium concentrations in a cresolphthalein complexone colorimetric study (13).

7) Very recently Kharnoob (1986) has studied the effect of pH and time on heavy metals released from contaminated sediments using RSM and investigated the interactions between metals of their uptake by plants using RSM (15).

6:1:4 Advantages of RSM

I. RSM has the advantages outlined below:

a) reducing the time spent in evaluating results;

b) if more than one factor is used then several responses may be evaluated simultaneously;

c) when a large number of factors are needed for a system study, the experiment can be designed to estimate the model parameters efficiently with the minimum number of experiments (trials). The response approach can help the researcher to determine the optimal level of factors and to predict responses within the limits of the experimental region;

d) from a visual inspection, one can elucidate factor interactions and effects not clearly apparent in the original data (3).

II. RSM can be used to answer a number of different questions (15):

i) how is a particular response affected by a set of variables over some specified region?

ii) what settings of the variables will yield a maximum or minimum response, and what is the local geography of the response surface near this maximal (or minimal) value?

iii) what settings of the variables will give a product or process satisfying desirable specifications?

6:2 EXPERIMENTAL AND RESULTS

6:2:1 Experimental Design

Experimental design is an important factor in the study of possible interactive effect of toxic metals or metalloids upon plant growth. A design is needed which will characterise the response and allow realistic evaluations of any response relationships.

The experimental design may be defined as the combination of each factor level in any set of trials corresponding to points in the space (18).

The designs for fitting a second order response surface must involve at least three levels of each variable, so that the coefficients in the model can be estimated.

Obviously the design that is suggested by the model requirement is the 3^K factorial, a factorial experiment with each factor at three levels. Generally for small values of K e.g. 2 or 3, this design is well suited. Therefore, for K equals 2, 3 and 4, the number of trials needed are 9, 27 and 81 respectively. However, when K is equal to 4 factors, from a practical point of view eighty-one design points may be unsatisfactory. Consequently a composite design system is more suitable (6).

Box and Wilson (2) have devised a workable alternative to the 3^K factorial ssytem through the development of a class of composite designs, a special type of which is called the central composite design. This design has been greatly used by workers applying second order response surface techniques (6).

Central composite design:

Composite designs to determine effects up to second order are built up from complete two-level factorials (coded to the usual -1, +1) or fractional factorials. The procedure is first to choose a two-level design so that all effects of the first order and all interaction effects of second order can be estimated. The design is then supplemented with further points which allows for the estimation of the quadratic effects (7).

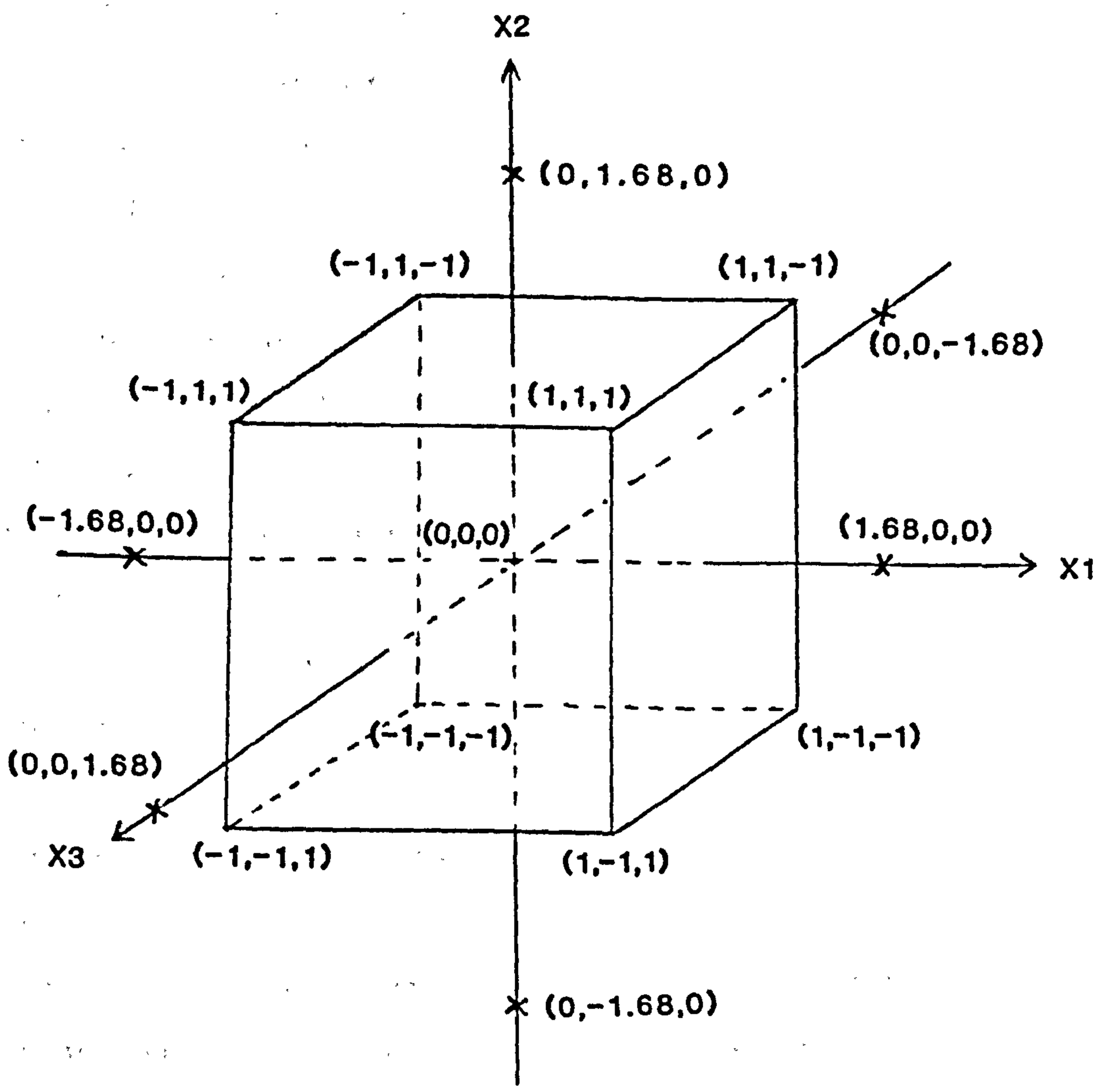
A composite design demonstrated for three factors may be applied for K factors. To the appropriate complete two-level factorial or fractional several (2K+1) supplementary points are added, to a total of 2^K + (2K+1) treatments. In this design for 2, 3 and 4 X-variables, the experiments required are 9, 15 and 25 treatments respectively as compared with 9, 27 and 81 in the 3^K series.

Composite designs can be fitted to second order response surfaces; this starts with a 2^K factorial to which a linear response surface is fitted. Treatment combinations (2K+1) are added to find a central composite design. The design uses the results of the initial 2^K experiment and the 2K+1 treatment combination to which a quadratic surface is fitted (7).

The estimated response in the composite rotatable design at a given point has a value which is dependent only on the distance of the point from the centre of the design (see Figure 6:3) and Table 6:3 shows the data arrangement and proposed coordinates for such a design. This type of design depicts a response surface with a smallish number of experiments. Consequently, in the present study a second order rotatable design was used (after Hader et al. (18)).

The resultant metal concentrations were processed using the SAS or GINO software packages, and are displayed as a series of 3-D surfaces, plus the accompanying statistical information for the fitting of the second degree polynomial equation. The nature of surfaces generated may also be represented by contour diagrams. Three dimensional surfaces have an enormous advantage over contour diagrams in that they are much clearer and easier to understand, enabling the experimenter to assess the results with greater clarity and ease.

Figure 6:3: CENTRAL COMPOSITE ROTATABLE DESIGN IN THREE X VARIABLES WITH FIVE LEVELS



6:2:2 The Use of RSM to Study the Uptake of Elements by Plants

Lolium perenne (a ryegrass) was chosen for the study of the uptake of metallic elements by plants. The grass is very quick growing and a typical grass, and hence ideal for this type of study. Lolium seeds were germinated in acid washed silver sand placed in a polyethylene potting tray which had been rinsed with DDW to eliminate any chance of element contamination of the seedlings. After two weeks of development in the sand, the seedlings were transferred to nutrient solutions (Hoagland's solution) which contained the essential elements for the growth of the plants, and were left to grow for a further two weeks. The containers, or pots, used were the bottom halves of a series of sawn-off Winchester acid bottles and each held one litre of solution. The germinated seedlings were removed from the sand and their roots washed with DDW.

Then the roots of the plants were cut to 7 cm and the shoots to 10 cm; and the fresh weight of each bunch (about 1.0 g) were recorded. Five seedlings were taken for Experiment 1 and six seedlings for Experiment 2 (see Figures 6.3(A) and 6.3(B)). After the two week acclimatisation and growth period, the 'doping' experiments were initiated. In both experiments Se(VI) at various levels was added, additionally in Experiment 1 cadmium (II) and mercury (II) were added. However, in Experiment 2, tellurium (VI) and thallium (I) were used instead. The plants were allowed to grow for the next three weeks.

Each week, a fresh nutrient solution containing the three spiking elements was prepared and the old solution discarded. After the end of the third week, the seedlings were removed from the nutrient solution and each bunch dried between filter papers. The fresh weight, and root and shoot lengths were recorded. The roots and


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Figure 6.3(A) Interactive effects of Selenium (VI) , Cadmium (II) and Mercury (II) upon growth of Lolium perenne.

Note : Concentration (ug/ml).

Interactive effects of Selenium [VI] ,Tellurium [VI] and Thallium [I] upon growth of Lolium perenne

Note : Conc. [ug/ml]

November 1983 1985

Figure 6.3(B) Interactive effects of Selenium (VI) , Tellurium (VI) and Thallium (I) upon growth of Lolium perenne.

Note : Concentration (ug/ml).

shoots of individual bunches were placed in beakers and left overnight at 35°C for Experiment 1 and 50°C for Experiment 2. Next day the dry weight of each sample was recorded.

The two experiments were run, as follows:-

Experiment Number	Elements Ions Added to Nutrient Solution
1	Se(VI), Cd(II), Hg(II)
2	Se(VI), Te(VI), Tl(I)

The elements were added as :-

Se(VI) - Sodium selenate, $Na_2SeO_4.10H_2O$

Te(VI) - Telluric acid, $H_2TeO_4.2H_2O$

Cd(II) - Cadmium acetate, $Cd(CH_3COO)_2.2H_2O$

Hg(II) - Mercuric acetate, $Hg(CH_3COO)_2$

Tl(I) - Thallium (1) nitrate, $TlNO_3$

Table 6:2 : The Coded Values with Corresponding Levels of Se, Te, Cd, Hg and Tl

Coded Value	Experiment 1			Experiment 2		
	Concentration (ug/ml)			Concentration (ug/ml)		
	Se	Cd	Hg	Se	Te	Tl
-1.68	$5x10^{-4}$	$1x10^{-4}$	$1x10^{-4}$	$1x10^{-3}$	$1x10^{-3}$	$5x10^{-4}$
-1.00	$3.25x10^{-2}$	$6.5x10^{-3}$	$6.5x10^{-3}$	0.065	0.065	$3.25x10^{-2}$
0.0	0.050	0.010	0.010	0.100	0.100	0.050
+1.00	0.775	0.155	0.155	1.55	1.55	0.775
+1.68	5.000	1.00	1.00	10.00	10.00	5.000

A composite rotatable design with three variables was used (see Table 6:3). The roots and shoots of plants were digested with concentrated HNO_3 using a Bomb/Teflon system or in a Teflon stoppered test tube at 140°C for 75 minutes in an oven. The digestion procedure was used for both experiments.

Mercury was determined in plant tissue using Cold Vapour AAS or Graphite Furnace AAS.

Selenium was determined in Experiment 1 using GLC + ECD with the 3-bromo-5-trifluoromethyl-1,2-diaminobenzene ligand and GFAAS, while in Experiment 2, selenium along with tellurium, thallium and cadmium were determined by GFAAS.

The other metals such as Mn, Fe, Cu and Zn (in the plant tissues) were determined by FAAS.

The results obtained are given in Tables 6:4 to 6:7. The data were fitted to second order polynomials (see equation 6.11) and analysed by SAS and the Minitab packages. The results are presented in Tables 6:8 to 6:11. The 3-D plots of measured responses with two elements were drawn by the SAS and GINO packages while the other element was kept constant. Typical examples of the diagrams obtained are shown in Figures 6:4 to 6:63.

Table 6:3 : Composite Rotatable Design

Trials	x_1	x_2	x_3	
1	-1	-1	-1	Factorial
2	+1	-1	-1	
3	-1	+1	-1	
4	-1	-1	+1	
5	+1	+1	-1	
6	+1	-1	+1	
7	-1	+1	+1	
8	+1	+1	+1	
9	+1.68	0.0	0.0	Additional points to form a central composite design
10	-1.68	0.0	0.0	
11	0.0	+1.68	0.0	
12	0.0	-1.68	0.0	
13	0.0	0.0	+1.68	
14	0.0	0.0	-1.68	
15	0.0	0.0	0.0	Central point
16	0.0	0.0	0.0	
17	0.0	0.0	0.0	
18	0.0	0.0	0.0	
19	0.0	0.0	0.0	
20	0.0	0.0	0.0	

Table 6:4: Tissue Concentrations of Se, Cd, Hg, Mn, Fe, Cu and Zn in Shoots of Lolium perenne Seedlings

Trials	Independent Vari-			Dependent Variables								
	(Se)	(Cd)	(Hg)	Sh.L.	Se	Cd	Hg	Mn	Fe	Cu	Zn	D.Wt
	x1	x2	x3	y1	y2	y3	y4	y5	y6	y7	y8	y9
1	-1	-1	-1	30.00	1.30	1.73	1.04	85.15	156.77	12.52	73.33	1.1910
2	+1	-1	-1	26.00	26.33	1.40	1.25	88.16	98.00	13.34	77.50	0.8880
3	-1	+1	-1	22.50	1.40	6.50	0.97	86.74	153.22	12.05	68.66	1.092
4	-1	-1	+1	25.10	1.62	1.52	2.59	91.40	222.33	34.80	74.00	0.7975
5	+1	+1	-1	24.50	26.11	7.07	1.28	86.15	101.00	20.70	81.60	0.968
6	+1	-1	+1	25.40	27.55	1.86	1.95	84.60	78.50	12.20	52.15	0.9610
7	-1	+1	+1	22.00	1.51	6.73	2.21	82.16	68.60	17.80	78.40	0.7110
8	+1	+1	+1	23.80	31.64	16.47	3.61	85.28	114.90	13.00	62.77	0.519
9	+1.68	0.0	0.0	22.90	355.60	2.44	1.61	80.00	121.30	16.25	56.25	0.5150
10	-1.68	0.0	0.0	26.00	5.32	2.24	0.67	81.10	127.90	14.75	57.50	0.4000
11	0.0	+1.68	0.0	17.10	5.93	63.10	2.69	73.17	171.30	23.50	148.00	0.2500
12	0.0	-1.68	0.0	26.45	1.78	2.50	0.37	82.67	85.40	11.10	65.30	0.6110
13	0.0	0.0	+1.68	10.70	3.60	3.53	33.34	106.20	173.90	15.30	46.00	0.2465
14	0.0	0.0	-1.68	22.30	2.70	3.15	0.58	79.29	122.00	14.00	69.15	0.4335
15	0.0	0.0	0.0	23.00	2.44	1.28	1.31	89.32	103.60	15.10	82.35	0.6525
16	0.0	0.0	0.0	21.80	3.16	1.04	1.05	106.24	138.30	17.44	71.70	0.7275
17	0.0	0.0	0.0	24.20	3.46	1.97	1.20	98.13	118.20	14.22	57.60	0.7900
18	0.0	0.0	0.0	23.50	3.75	2.51	1.15	105.50	108.20	16.50	58.70	0.516
19	0.0	0.0	0.0	25.40	2.76	1.96	0.90	101.80	110.30	17.20	60.15	0.5270
20	0.0	0.0	0.0	23.70	3.62	2.21	1.11	103.95	108.60	17.00	60.75	0.6295

Sh.L. = Shoot length (cm)

D.Wt. = Dry weight of plant per treatment (g)

* = Significant from control

Concentration of Element = ug/g per dry weight of plant

Table 6:5: Tissue Concentrations of Se,Cd,Hg,Mn,Fe,Cu and Zn in Roots of Lolium perenne Seedlings

Trials	Independent Vari-			Dependent Variables								
	(Se)	(Cd)	(Hg)	R. L.	Se	Cd	Hg	Mn	Fe	Cu	Zn	D. Wt
	x1	x2	x3	y1	y2	y3	y4	y5	y6	y7	y8	y9
1	−1	−1	−1	20.00	3.86	6.87	8.51	334.40	1720.00	43.00	104.90	0.259
2	+1	−1	−1	18.00	15.50	10.84	7.10	362.33	1343.15	64.66	60.23	0.260
3	−1	+1	−1	16.60	4.10	115.38	7.77	530.00	1901.00	61.74	115.25	0.260
4	−1	−1	+1	15.50	4.75	9.25	136.73	504.00	1700.00	57.36	75.30	0.200
5	+1	+1	−1	19.40	15.92	103.25	5.69	417.80	1560.50	56.60	56.10	0.284
6	+1	−1	+1	16.80	19.36	7.13	194.94	362.35	1956.60	61.73	52.40	0.234
7	−1	+1	+1	12.50	3.88	120.70	197.24	488.72	2080.66	60.00	48.45	0.174
8	+1	+1	+1	16.20	16.90	96.33	249.05	355.00	2582.50	69.66	94.80	0.164
9	+1.68	0.0	0.0	12.00	72.30	13.86	15.12	178.80	2573.00	67.56	52.10	0.119
10	−1.68	0.0	0.0	13.60	2.75	11.05	28.66	227.93	2377.70	103.50	60.20	0.095
11	0.0	+1.68	0.0	9.30	5.50	212.77	26.34	42.82	4494.50	105.10	82.57	0.071
12	0.0	−1.68	0.0	16.50	1.66	5.01	32.27	181.30	3233.50	117.66	93.90	0.105
13	0.0	0.0	+1.68	7.70	2.86	6.79	1532.77	38.36	4815.50	103.00	31.40	0.070
14	0.0	0.0	−1.68	19.10	2.77	7.14	4.34	167.33	4041.80	138.42	75.55	0.081
15	0.0	0.0	0.0	16.70	5.47	11.46	11.83	392.43	2409.50	62.50	64.60	0.157
16	0.0	0.0	0.0	16.80	4.83	18.60	9.36	418.93	2189.30	64.50	50.00	0.215
17	0.0	0.0	0.0	17.40	5.65	11.00	8.87	395.65	2172.00	56.50	68.22	0.150
18	0.0	0.0	0.0	17.10	4.48	11.03	8.75	367.40	2344.00	64.40	44.70	0.165
19	0.0	0.0	0.0	17.80	5.56	12.33	9.28	382.00	2384.20	64.60	53.70	0.175
20	0.0	0.0	0.0	16.50	4.60	9.45	11.87	357.45	2788.00	65.10	55.40	0.157

Table 6:6: Tissue Concentrations of Se,Te,Tl,Mn,Fe,Cu and Zn in Shoots of Lolium perenne Seedlings

Trials	Independent Vari-			Dependent Variables								
	(Se)	(Te)	(Tl)	Sh.L.	Se	Te	Tl	Mn	Fe	Cu	Zn	Dry Wt.
	x1	x2	x3	y1	y2	y3	y4	y5	y6	y7	y8	y9
1	-1	-1	-1	18.18	4.21	2.73	1.31	123.54	168.63	32.78	168.14	0.3295
2	+1	-1	-1	22.08	166.66	3.03	2.74	120.55	142.53	17.66	117.80	0.5625
3	-1	+1	-1	16.25	4.00	126.14	1.55	121.30	144.89	24.77	123.23	0.3835
4	-1	-1	+1	11.66	2.73	3.10	39.84	124.00	235.96	15.49	80.20	0.3050
5	+1	+1	-1	12.25	220.29	172.31	1.09	175.57	392.73	25.64	133.95	0.3366
6	+1	-1	+1	13.00	169.22	2.00	42.97	128.05	179.17	16.79	138.66	0.3330
7	-1	+1	+1	12.50	6.20	81.25	26.70	114.26	52.00	18.42	89.30	0.2800
8	+1	+1	+1	13.00	130.00	47.46	28.18	94.02	131.10	16.95	102.10	0.3300
9	+1.68	0	0	16.42	919.12	1.80	2.20	103.64	270.00	17.30	77.50	0.4488
10	-1.68	0	0	20.42	6.21	2.50	2.00	108.17	385.90	24.86	99.00	0.6090
11	0	+1.68	0	6.75	12.81	990.05	2.73	202.77	175.53	19.54	71.18	0.1530
12	0	-1.68	0	14.92	10.76	3.30	3.64	212.31	450.13	25.74	162.33	0.2510
13	0	0	+1.68	5.75	13.98	10.06	204.02	222.82	366.48	22.17	290.33	0.1740
14	0	0	-1.68	19.92	6.60	2.10	2.00	130.10	291.10	20.38	90.50	0.4022
15	0	0	0	21.00	5.21	1.95	2.68	111.17	195.23	13.14	77.93	0.5577
16	0	0	0	20.05	6.13	2.38	2.05	111.58	216.40	14.30	76.90	0.5730
17	0	0	0	22.92	6.90	2.45	1.31	96.36	177.86	15.10	74.04	0.5430
18	0	0	0	19.50	7.71	1.96	1.44	110.77	314.65	13.20	67.81	0.5265
19	0	0	0	19.86	7.45	2.53	1.54	87.73	365.00	18.07	65.60	0.3095
20	0	0	0	21.00	6.95	2.50	2.89	87.91	286.74	19.15	62.10	0.4300

Table 6:7: Tissue Concentrations of Se,Te,Tl,Mn,Fe,Cu and Zn in Roots of Lolium perenne Seedlings

Trials	Independent Vari-			Dependent Variables								
	(Se)	(Te)	(Tl)	R.L.	Se	Te	Tl	Mn	Fe	Cu	Zn	D Wt.
	x1	x2	x3	y1	y2	y3	y4	y5	y6	y7	y8	y9
1	−1	−1	−1	9.50	6.58	3.15	5.81	78.12	4047.62	152.03	352.41	0.111
2	+1	−1	−1	10.66	46.45	2.60	10.56	98.98	3148.52	111.16	366.00	0.144
3	−1	+1	−1	11.06	4.23	25.38	6.50	39.58	3648.81	115.57	103.85	0.131
4	−1	−1	+1	0.50	4.62	6.10	76.20	12.20	5929.23	137.00	807.93	0.082
5	+1	+1	−1	2.42	44.10	26.12	10.21	54.62	4324.00	152.66	716.02	0.1077
6	+1	−1	+1	1.08	67.50	5.31	93.75	38.65	5718.60	102.45	651.82	0.080
7	−1	+1	+1	0.50	6.00	32.14	92.86	14.28	5949.65	117.10	737.18	0.070
8	+1	+1	+1	0.83	43.13	27.47	82.42	11.00	5252.70	109.15	55.00	0.091
9	+1.68	0	0	3.50	108.00	6.00	3.50	46.08	4577.00	119.15	106.45	0.1125
10	−1.68	0	0	11.00	8.01	6.03	20.93	93.12	3444.00	184.11	215.34	0.1388
11	0	+1.68	0	0.50	8.00	164.84	6.35	49.31	5658.30	156.84	55.55	0.091
12	0	−1.68	0	3.16	2.76	5.12	7.07	67.70	4637.22	173.13	126.55	0.1075
13	0	0	+1.68	1.00	3.37	6.40	401.36	61.43	5420.50	179.38	77.60	0.0844
14	0	0	−1.68	8.00	3.96	2.46	6.71	45.85	3531.00	111.55	219.64	0.1495
15	0	0	0	10.83	3.03	3.30	21.61	69.71	3509.00	122.88	110.40	0.1444
16	0	0	0	10.83	2.72	3.60	19.08	69.10	3972.12	113.72	106.77	0.1255
17	0	0	0	12.92	4.53	4.72	24.43	93.95	4119.70	138.33	95.82	0.1220
18	0	0	0	12.83	3.33	4.10	24.30	104.00	4153.30	134.77	111.52	0.1220
19	0	0	0	9.95	4.85	5.16	25.27	94.50	4584.75	122.11	101.52	0.092
20	0	0	0	9.50	5.25	3.56	22.87	81.80	4625.38	142.60	123.35	0.108

R.L. = Root length (cm)

* = *Significant from control*

D.Wt. = Dry weight of plant per treatment (g)

Concentration of Element = *ug/g per dry weight of plant*

Table 6:8. Response Surface for Se, Cd, Hg, Mn, Fe, Cu and Zn taken up by Shoots of Lolium perenne seedlings. Analysis of Data by SAS.

	Shoot length	Se	Cd	Hg	Mn	Fe	Cu	Zn	Dry wt.
1. Coefficient									
B_0	23.461	4.9198	2.099	1.2877	100.705	114.89	16.201	65.31	0.6238
prob	0.0001	0.8444	0.5996	0.6058	0.0001	0.0001	0.0001	0.0001	0.0005
B_1	-0.3744	50.882	0.7809	0.2095	-0.228	-16.095	-1.1294	-1.6468	-0.0192
prob	0.6625	0.0106	0.7675	0.8987	0.9081	0.1406	0.2928	0.6952	0.8212
B_2	-2.1553	0.7938	9.679	0.3765	-1.828	1.937	0.8444	11.241	-0.0846
prob	0.0270	0.9619	0.0037	0.8192	0.3645	0.8511	0.4258	0.0204	0.3314
B_3	-1.9193	0.6370	0.7708	4.460	3.111	4.5828	1.5665	-5.325	-0.1073
prob	0.0438	0.9694	0.7704	0.0195	0.1369	0.6583	0.1546	0.2214	0.2242
B_{11}	1.1849	51.892	-1.5358	-1.0564	-6.433	1.3072	0.0037	-3.627	0.0403
prob	0.1748	0.0082	0.5535	0.5147	0.0064	0.8965	0.9971	0.3836	0.6289
B_{22}	0.2372	-10.680	9.2564	-0.9182	-7.365	2.6358	0.6415	14.009	0.0307
prob	0.7760	0.5145	0.0041	0.5701	0.0028	0.7934	0.5321	0.0055	0.7119
B_{33}	-1.6318	-10.930	-1.1815	4.5488	-2.112	9.5803	-0.2974	-3.3786	-0.0014
prob	0.0720	0.5050	0.6473	0.0156	0.2862	0.3514	0.7703	0.4158	0.9867
B_{12}	0.9375	0.485	1.2875	0.2675	0.79	24.585	3.2037	1.8737	-0.0221
prob	0.4087	0.9822	0.7094	0.9010	0.7597	0.0907	0.0366	0.7326	0.8425
B_{13}	0.5125	0.79	1.23	0.03	-0.762	1.6825	-4.6087	-6.824	0.0498
prob	0.6474	0.9710	0.7217	0.9889	0.7677	0.9006	0.0060	0.2296	0.6551
B_{23}	0.5375	0.5125	1.1725	0.165	-1.0175	-14.597	-2.8862	1.9487	-0.0637
prob	0.6317	0.9812	0.7341	0.9388	0.6940	0.2924	0.0549	0.7224	0.5692
2. Regression									
linear (SS)	115.555	35339.78	1294.716	273.959	178.351	3872.29	60.614	2148.21	0.2598
prob	0.0394	0.0668	0.0256	0.1102	0.3667	0.4593	0.2911	0.0735	0.4639
quadratic (SS)	65.6318	45421.74	1368.915	352.19	1263.211	1360.79	7.8075	3453.89	0.0345
prob	0.1378	0.0360	0.0218	0.0641	0.0045	0.8048	0.9046	0.0219	0.9445
cross product (SS)	11.444	8.9758	36.362	0.7974	17.926	6562.72	318.68	430.977	0.0562
prob	0.7538	1.0000	0.9373	0.9990	0.9473	0.2539	0.0064	0.6113	0.8940
total regress (SS)	192.63	80770.5	2699.99	626.945	1459.488	11795.8	387.10	6033.08	0.3506
prob	0.1096	0.0848	0.0374	0.1505	0.0417	0.5262	0.487	0.0538	0.8988
3. Residual									
lack of fit (SS)	87.325	35887.06	899.986	351.213	303.486	13009.09	132.78	1793.78	0.8780
prob	0.0080	0.0001	0.0001	0.0001	0.3321	0.0040	0.0044	0.0877	0.0050
pure error (SS)	7.22	1.3193	1.5699	0.0968	201.52	791.07	8.387	481.687	0.0586
total error (SS)	94.545	35888.38	901.556	351.314	505.01	13500.16	141.167	2275.46	0.9366
4. Solution	Saddle point	Saddle point	Saddle point	Saddle point	Maximum	Saddle point	Saddle point	Saddle point	Saddle point
5. R-squared	0.67077	0.69236	0.7497	0.64085	0.7429	0.4608	0.7328	0.7261	0.2723

SS = sum of squares

Table 6:9. Response Surface for Se, Cd, Hg, Mn, Fe, Cu and Zn taken up by Roots of Lolium perenne seedlings.
Analysis of Data by SAS.

	Root length	Se	Cd	Hg	Mn	Fe	Cu	Zn	Dry wt.
1. Coefficient									
B_0	16.913	5.256	11.843	16.304	375.77	2441.62	64.578	55.791	0.16502
prob	0.0001	0.1699	0.0252	0.8677	0.0004	0.0002	0.0004	0.0001	0.0008
B_1	0.2281	12.307	-2.193	6.140	-32.332	27.057	-2.186	-6.887	0.00655
prob	0.7681	0.0004	0.4802	0.9247	0.5127	0.9254	.7968	0.0981	0.7833
B_2	-1.2969	0.277	55.010	7.5126	-0.3083	258.22	0.0109	0.2005	-0.00939
prob	0.1156	0.9088	0.0001	0.9079	0.9950	0.3809	0.9990	0.9587	0.6938
B_3	-2.356	0.4149	-0.258	243.071	-11.076	226.82	-2.694	-10.238	-0.02268
prob	0.0107	0.8639	0.9330	0.0033	0.8208	0.4394	0.7513	0.0219	0.3507
B_{11}	-0.6388	10.492	3.022	-35.793	-2.115	-349.87	-2.419	1.9976	0.008277
prob	0.4043	0.0010	0.3242	0.5749	0.9646	0.2313	0.7702	0.5991	0.7216
B_{22}	-0.6034	-1.535	37.190	-33.166	-34.359	142.144	6.740	13.366	0.001545
prob	0.4300	0.5195	0.0001	0.6029	0.4763	0.6159	0.4225	0.0046	0.9468
B_{33}	-0.4262	-1.8056	1.077	228.76	-37.624	342.20	10.046	1.050	-0.00288
prob	0.5741	0.4503	0.7195	0.0041	0.4366	0.2410	0.2409	0.7812	0.9009
B_{12}	0.900	-0.176	-4.794	-0.884	-16.525	35.198	-2.688	6.846	-0.0026
prob	0.3814	0.9555	0.2478	0.9917	0.7959	0.9257	0.8084	0.1951	0.9326
B_{13}	0.525	0.521	-2.291	14.189	-23.887	184.47	-0.311	15.909	-0.000125
prob	0.6049	0.8690	0.5705	0.8672	0.7090	0.6269	0.9776	0.0091	0.9968
B_{23}	-0.200	-0.499	-0.034	14.596	-34.212	76.026	-0.0137	1.166	-0.015125
prob	0.8429	0.8746	0.9933	0.8635	0.5944	0.8404	0.9990	0.8178	0.6280
2. Regression									
linear (SS)	99.421	2070.252	41357.78	807465.9	15939.34	1621831	104.22	2078.15	0.0033
prob	0.0345	0.0033	0.0001	0.0237	0.9132	0.6911	0.9803	0.0551	0.7550
quadratic (SS)	11.552	1776.447	19967.58	838217.4	34244.6	4114077	2154.56	2574.56	0.00119
prob	0.6918	0.0056	0.0001	0.0213	0.7773	0.3379	0.5367	0.0319	0.9823
cross product (SS)	9.005	4.412	225.848	3321.22	16113.47	328396.1	58.612	2410.56	0.00188
prob	0.7640	0.9961	0.6197	0.9958	0.9119	0.9575	0.9956	0.0380	0.9662
total regress (SS)	119.978	3851.11	61551.22	1649004	66297.41	6064274	2377.39	7063.27	0.01189
prob	0.2040	0.0062	0.0001	0.0367	0.9792	0.7560	0.9645	0.0201	0.9917
3. Residual									
lack of fit (SS)	76.125	757.636	1168.67	547499.8	307237.7	10579307	9274.2	1552.00	0.0704
prob	0.0002	0.0001	0.0019	0.0001	0.0001	0.0004	0.0001	0.0788	0.0015
pure error (SS)	1.175	1.358	51.821	10.613	2395.15	248270.2	53.653	392.56	0.0028
total error (SS)	77.2995	758.994	1220.49	547510.4	309632.9	10827578	9327.85	1944.56	0.0733
4. Solution	Maximum	Saddle point	Minimum	Saddle point	Saddle point	Saddle point	Saddle point	Saddle point	Saddle point
5. R-squared	0.6082	0.8354	0.9805	0.7507	0.1763	0.3590	0.2031	0.7841	0.140

SS = sum of squares

Table 6:10. Response Surface fo Se, Te, Tl, Mn, Fe, Cu and Zn taken up by Shoots of Lolium perenne seedlings. Analysis of Data by SAS.

	Shoot length	Se	Te	Tl	Mn	Fe	Cu	Zn	Dry wt.
1. Coefficient									
B_0	20.6798	9.5112	6.4369	2.6597	102.3252	264.2014	15.5264	71.2804	0.4894
prob	0.0001	0.8482	0.9216	0.8187	0.0001	0.0003	0.0001	0.0047	0.0001
B_1	-0.3635	161.4328	0.7625	0.4336	2.0139	3.6158	-1.9876	-0.3283	0.0057
prob	0.5232	0.0005	0.9860	0.9550	0.8343	0.9134	0.1046	0.9805	0.8272
B_2	-1.80476	1.5474	152.0022	-2.2623	-0.5143	-34.2180	-0.5391	-15.3430	-0.0328
prob	0.0082	0.9626	0.0049	0.7691	0.9574	0.3161	0.6387	0.2680	0.2296
B_3	-3.10636	-5.4681	-11.5082	34.4742	5.5068	-9.0812	-2.2128	14.8668	-0.0486
prob	0.0002	0.8683	0.7914	0.0010	0.5702	0.7851	0.0750	0.2822	0.0871
B_{11}	-0.5500	143.8807	-26.3080	-4.2362	-7.1414	-6.6683	1.7696	2.7183	0.01737
prob	0.3285	0.0010	0.5383	0.5751	0.4528	0.8371	0.1340	0.8354	0.5031
B_{22}	-3.2375	-15.8698	148.9063	-3.8517	28.8687	-12.0254	2.3223	12.8179	-0.0984
prob	0.0001	0.6234	0.0048	0.6097	0.0102	0.7115	0.0580	0.3384	0.0028
B_{33}	-2.5288	-16.3995	-24.9156	31.5171	17.8568	-6.3707	1.8387	38.9162	-0.0679
prob	0.0008	0.6120	0.5596	0.0015	0.0793	0.8443	0.1211	0.0122	0.0217
B_{12}	-1.095	1.3937	1.6475	-0.4425	4.1212	51.2287	1.6525	1.9250	-0.0218
prob	0.1580	0.9742	0.9768	0.9649	0.7435	0.2542	0.2823	0.9125	0.5300
B_{13}	0.24	-11.0563	-10.1700	0.4550	-8.4337	-24.9288	1.7600	13.8600	-0.0239
prob	0.7449	0.7976	0.8578	0.9639	0.5068	0.5691	0.2540	0.4361	0.4912
B_{23}	1.5725	-11.1463	-21.135	-3.3150	-12.0688	-57.3113	0.3900	0.1625	0.0284
prob	0.0532	0.7960	0.7104	0.7420	0.3478	0.2057	0.7940	0.9926	0.4162
2. Regression									
linear (SS)	177.91	356031.8	317073.7	16288.84	472.73	17279.99	124.68	6229.35	0.04747
prob	0.0006	0.0043	0.0339	0.0078	0.9393	0.7550	0.1231	0.4796	0.2179
quadratic (SS)	221.47	321807.1	361685.5	15629.28	17119.2	2836.59	143.63	22982.33	0.2037
prob	0.0002	0.0061	0.0236	0.0089	0.0261	0.9767	0.0926	0.0669	0.0063
cross product (SS)	29.83	1987.38	4422.65	91.136	1870.14	52243.25	47.84	1566.65	0.01485
prob	0.1273	0.9857	0.9796	0.9888	0.6785	0.3545	0.4562	0.8778	0.6588
total regress (SS)	429.21	679826.3	683181.9	32009.26	19462.07	72359.84	316.15	30778.34	0.26604
prob	0.0003	0.0075	0.0463	0.0126	0.1862	0.8013	0.1352	0.2796	0.0387
3. Residual									
lack of fit (SS)	33.52	140959.9	244847.2	7673.576	11325.68	115862	136.78	23137.06	0.03805
prob	0.0657	0.0001	0.0001	0.0001	0.0039	0.0706	0.0699	0.0001	0.6283
pure error (SS)	7.674	4.226	0.3597	2.2569	679.75	27566.91	32.386	210.185	0.05179
total error (SS)	41.19	140964.1	244847.6	7675.83	12005.43	143428.9	169.166	23347.25	0.0898
4. Solution	Maximum	Saddle point	Saddle point	Saddle point	Saddle point	Saddle point	Minimum	Minimum	Saddle point
5. R-squared	0.9124	0.8283	0.7362	0.8066	0.6185	0.3353	0.6514	0.5686	0.7475

SS = sum of squares

Table 6:11. Response Surface for Se, Te, Tl, Mn, Fe, Cu and Zn taken up by Roots of Lolium perenne seedlings. Analysis of Data by SAS.

	Root length	Se	Te	Tl	Mn	Fe	Cu	Zn	Dry wt.
1. Coefficient									
B_0	11.128	3.807	4.6585	23.937	86.03	4154.58	130.10	96.917	0.1190
prob	0.0001	0.1853	0.6281	0.2544	0.0001	0.0001	0.0001	0.3507	0.0001
B_1	-1.4049	25.485	-0.39	-1.0049	-1.463	56.575	-11.39	-28.98	-0.0011
prob	0.0256	0.0001	0.9510	0.9406	0.8351	0.7110	0.1513	0.6686	0.8206
B_2	-0.8354	-1.384	26.551	0.3269	-10.214	149.99	-2.604	-50.23	-0.0033
prob	0.1503	0.4539	0.0016	0.9806	0.1665	0.3360	0.7298	0.4623	0.5139
B_3	-3.1140	1.385	1.494	71.4677	-12.385	795.584	3.535	34.81	-0.0198
prob	0.0002	0.4537	0.8141	0.0003	0.1005	0.0003	0.6400	0.6079	0.0023
B_{11}	-1.2824	20.066	-3.022	-10.204	-8.937	-14.35	1.438	90.37	0.00025
prob	0.0340	0.0001	0.6272	0.4441	0.2099	0.9229	0.8445	0.1886	0.9581
B_{22}	-3.2027	1.4205	24.956	-12.1545	-12.868	388.59	6.170	65.627	-0.0091
prob	0.0001	0.4311	0.0020	0.3650	0.0825	0.0228	0.4080	0.3297	0.0845
B_{33}	-2.2567	0.8129	-3.584	57.7594	-14.592	150.49	-0.746	86.024	-0.0039
prob	0.0015	0.6488	0.5656	0.0011	0.0535	0.3227	0.9189	0.2089	0.4321
B_{12}	-1.2562	-3.2187	-0.324	-3.6287	-4.444	136.00	13.07	9.06	-0.0042
prob	0.1030	0.1955	0.9688	0.8368	0.6298	0.4989	0.2021	0.9180	0.5280
B_{13}	1.0487	2.5337	-0.706	-0.1687	-1.591	-85.46	-4.84	-183.01	0.0012
prob	0.1651	0.3004	0.9321	0.9923	0.8623	0.6687	0.6241	0.0588	0.8588
B_{23}	0.8037	-2.286	0.306	0.6237	7.166	-152.77	-2.28	-96.129	0.0019
prob	0.2778	0.3477	0.9705	0.9717	0.4413	0.4489	0.8166	0.2889	0.7700
2. Regression									
linear (SS)	168.77	8914.18	9651.335	69707.79	3545.71	8987184	2033.15	62426.63	0.0055
prob	0.0006	0.0001	0.0122	0.0025	0.2021	0.0024	0.4641	0.7880	0.0157
quadratic (SS)	211.26	5806.64	9831.69	55523.13	5568.78	2409426	586.73	239958.9	0.00135
prob	0.0002	0.0001	0.0115	0.0055	0.0877	0.1043	0.8479	0.3112	0.3031
cross product (SS)	26.59	176.057	5.579	108.683	589.07	393091.8	1595.59	342513.4	0.0002
prob	0.1439	0.3096	0.9997	0.9972	0.8197	0.7321	0.5596	0.1876	0.9051
total regress (SS)	406.62	14896.88	19488.61	125339.6	9703.56	11789702	4215.47	644899	0.0070
prob	0.0003	0.0001	0.0184	0.0052	0.2134	0.0155	0.7437	0.3803	0.0934
3. Residual									
lack of fit (SS)	28.90	425.055	5223.15	23540.99	5364.46	2148058	6707.99	588891.1	0.0016
prob	0.1415	0.0001	0.0001	0.0001	0.0468	0.1682	0.0104	0.0001	0.4902
pure error (SS)	10.32	5.580	2.685	26.171	1026.63	857757	623.68	445.31	0.0016
total error (SS)	39.23	430.63	5225.84	23567.16	6391.08	3005815	7331.67	589336.4	0.00324
4. Solution	Maximum	Saddle point	Saddle point	Saddle point	Maximum	Saddle point	Saddle point	Saddle point	Saddle point
5. R-squared	0.9120	0.9719	0.7885	0.8417	0.6029	0.7968	0.3651	0.5225	0.6845

SS = sum of squares

6.3 DISCUSSION

Two major experiments were carried out, Experiment 1 and Experiment 2. In both cases, increasing concentrations of two metals and selenium were fed in hydroponic solution to Lolium perenne seedlings. Experiment 1 was carried out first chronologically. However, because Experiment 2 generated greater scientific interest and effects, it will be discussed in detail while Experiment 1 will only be discussed in outline (after Experiment 2) except where points of scientific merit occur.

i) Interactive Effects of Se(VI), Te(VI) and Tl(I) on Plants

In Experiment 2, the elements fed to the seedlings were tellurium (as tellurate) and thallium (I) in conjunction with selenium (VI). The plants were grown for a period of three weeks and then two main types of measurement were undertaken:-

(a) gross parameters such as length of root, length of shoot, biomass; and

(b) elemental analyses for various metals and selenium content of the plant material.

An experimental approach such as the rotatable design used here generates a great amount of data and diagrams. In order to explain fully the type of data produced it is thought appropriate to discuss one printout from the least-squares RSREG (SAS package) and the resultant series of response surfaces which can be generated. The response surfaces were generated using RSREG and G3D programs in the SAS package and also by using MINITAB and GINO programs available on the University of Bristol MULTICS system. In all cases the surfaces produced were very similar, so examples of both surfaces will only be given for the gross parameter responses. The SAS system is much more convenient to plot and is the preferred method, and therefore will be used predominantly through the discussion section.

A typical example of the printed output is shown in Table 6.12(a) which describes the response variable shoot length (Y1) in terms of the three experimental variables, X_1, X_2, and X_3 which represent the concentrations of

TABLE 6.12(a)

RESPONSE SURFACE FOR VARIABLE Y1 *Shoot Length (Se, Te, Tl)*

(1)	RESPONSE MEAN	16.3705
(2)	ROOT MSE	2.029607
(3)	R-SQUARE	0.9124309
(4)	COEF OF VARIATION	0.1239795

	REGRESSION	(6) DF	(7) TYPE I SS	(8) R-SQUARE	(9) F-RATIO	(10) PROB
	LINEAR	3	177.9111	0.3782	14.40	0.0006
(5)	QUADRATIC	3	221.4671	0.4708	17.92	0.0002
	CROSSPRODUCT	3	29.83505	0.0634	2.41	0.1273
	TOTAL REGRESS	9	429.2132	0.9124	11.58	0.0003

	RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
	LACK OF FIT	5	33.51936	6.703873	4.368	0.0657
(11)	PURE ERROR	5	7.673683	1.534737 (12)		
	TOTAL ERROR	10	41.19305	4.119305		

	PARAMETER	DF	(14) ESTIMATE	(15) STD DEV	(16) T-RATIO	(17) PROB
	INTERCEPT	1	20.67979	0.8277123	24.98	0.0001
	X1	1	-0.363508	0.5494502	-0.66	0.5232
	X2	1	-1.80476	0.5494502	-3.28	0.0082
	X3	1	-3.10636	0.5494502	-5.65	0.0002
	X1*X1	1	-0.550049	0.5354913	-1.03	0.3285
	X2*X1	1	-1.095	0.7175744	-1.53	0.1580
	X2*X2	1	-3.23748	0.5354913	-6.05	0.0001
	X3*X1	1	0.24	0.7175744	0.33	0.7449
	X3*X2	1	1.5725	0.7175744	2.19	0.0532
	X3*X3	1	-2.52866	0.5354913	-4.72	0.0008

	FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
	X1	4	16.20232	4.05058	0.98	0.4592
(18)	X2	4	224.3856	56.0964	13.62	0.0005
	X3	4	243.7766	60.94415	14.79	0.0003

SOLUTION FOR OPTIMUM RESPONSE

	FACTOR	CRITICAL VALUE	
	X1	-0.0422425	≡ 0.0985 µg Se/ml
(19)	X2	-0.455638	≡ 0.085 µg Te/ml
	X3	-0.757847	≡ 0.0367 ug Tl/ml

PREDICTED VALUE AT OPTIMUM 22.2757

(20)

EIGENVALUES	EIGENVECTORS X1	X2	X3
-0.442193	0.9802368	-0.19702	-0.017865
-2.04406	0.1176787	0.5081246	0.8532064
-3.83013	0.1590207	0.8384466	-0.521267

SOLUTION WAS A MAXIMUM

TABLE 6.12(b): RESPONSE SURFACE FOR SHOOT LENGTH (Se, Te, Tl)

```
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 -- read 'AdlyShoot' c1-c8
column        c1          c2          c3          c4          c5
c6
count         20          20          20          20          20
20
row
   1    -1.00000    -1.00000    -1.00000     18.1600      4.210
2.730
   2     1.00000    -1.00000    -1.00000     22.0800    166.660
3.030
   3    -1.00000     1.00000    -1.00000     16.2500      4.000
126.140
   4    -1.00000    -1.00000     1.00000     11.6600      2.730
3.100
   .  .  .

column        c7          c8
count         20          20
row
   1       1.310    0.329500
   2       2.740    0.562500
   3       1.550    0.383500
   4      39.840    0.305000
   .  .  .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c4 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    20.7 - 0.364 x1 -  1.80 x2
    -  3.11 x3 - 0.550 x4 -  1.09 x5
    + 0.240 x6 -  3.24 x7 +  1.57 x8
    -  2.53 x 9

                                        st. dev.    t-ratio =
      column      coefficient           of coef.    coef/s.d.
      --            20.6798             0.8277       24.98
x1    c1            -0.3635             0.5495       -0.66
x2    c2            -1.8048             0.5495       -3.28
x3    c3            -3.1064             0.5495       -5.65
x4    c11           -0.5500             0.5355       -1.03
x5    c12           -1.0950             0.7176       -1.53
x6    c13            0.2400             0.7176        0.33
x7    c22           -3.2375             0.5355       -6.05
x8    c23            1.5725             0.7176        2.19
x9    c33           -2.5289             0.5355       -4.72

the st. dev. of y about regression line is
s = 2.030

with (  20-10) =  10 degrees of freedom

r-squared = 91.2 percent
r-squared = 83.4 percent, adjusted for d.f.

analysis of variance

 due to      df          ss        ms=ss/df
regression    9      429.213         47.690
residual     10       41.193          4.119
total        19      470.406

further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9      429.213
c1            1        1.803
c2            1       44.443
c3            1      131.665
c11           1        0.017
c12           1        9.592
c13           1        0.461
c22           1      129.581
c23           1       19.782
c33           1       91.869

              x1          y     pred. y     st.dev.
row           c1         c4       value   pred. y    residual     st.res.
  8         1.00     13.000       9.806     1.661       3.194       2.74r
 13         0.00      5.750       8.324     1.581      -2.574      -2.02r

r denotes an obs. with a large st. res.

durbin-watson statistic = 2.71
 -- end
 -- stop

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selenium (VI), tellurium (VI) and thallium (I) respectively.

All estimates and hypothesis tests depend on the correctness of the model and the error distributed according to classical statistical assumptions.

The individual items in the output of RSREG are:-

1. RESPONSE MEAN is the mean of the response variable in the sample, in this case shoot length.

2. ROOT MSE estimates the standard deviation of the response variable by means of the square root of the TOTAL ERROR mean square.

3. R-SQUARE is R^2 or the coefficient of determination. R^2 measures the portion of the variation in the response that is attributed to the model rather than to random error. In this case, a value of 0.9124 is a very high value, in that 91% of the variation is due to the model rather than random error. Thus the model used involving linear, quadratic and cross-terms appears to be a good descriptor for the length of shoot versus metal concentration.

4. COEF OF VARIATION is the coefficient of variation, which is equal to 100*rootmse/mean for the response variable.

5. The terms are brought into the regression equation in four steps.

   (1) INTERCEPT;

   (2) LINEAR terms like X_1, X_2 and X_3;

   (3) QUADRATIC terms X1*X1; and

   (4) CROSS PRODUCT terms like X1*X2.

6. DF indicates degrees of freedom and should be the same as the number of parameters unless one or more of the parameters is not estimable.

7. TYPE 1 SS, sequential sum of squares, measures reduction in error sum of squares as terms are added to the model individually (in the order given in Model Equation).

8. These R-SQUAREs measure the portion of the R^2 term, contributed as each set of terms (Linear, Quadratic, Cross-Product) is added to the model. In the present case, the R^2 term of 0.9124 is made up primarily of contributions from the Linear and Quadratic terms (0.3782 and 0.4708 respectively) with only a minor contribution from the Cross-Product terms. In other words, there appears to be little interaction between the three metals at the particular levels used in this experiment.

9. Each F-RATIO tests the hypothesis that all parameters in the term are zero using the TOTAL ERROR mean square as the denominator. This is a test of a TYPE 1 hypothesis, which is the usual F test numerator conditional on the effects below it not being in the model. Here, both the Linear and Quadratic terms are significant while the Cross-Product term is not significant at, say, the 95% level. For 3 degrees of freedom the critical value is 9.28; values less than that being insignificant. Thus the Linear and Quadratic terms are present in the model.

10. PROB is the significance value or probability of obtaining at least as great an F ratio given that the hypothesis is true. When PROB < 0.05, the effect is usually termed "significant". In this case it is obvious that the Linear and Quadratic terms are significant but not the Cross-Product term, giving further evidence to support the small R^2 term in [8].

11. The TOTAL ERROR sum of squares can be partitioned into LACK OF FIT and PURE ERROR. When LACK OF FIT is significantly different from PURE ERROR, then there is variation in the model not accounted for by random error. In this case there is not a significant difference between the LACK OF FIT and PURE ERROR; however if the probability value is less than 0.05 then there is a significant difference.

12. The TOTAL ERROR MEAN SQUARE estimates σ^2, the variance.

13. If an effect is a linear combination of previous effects, the parameter for it is not estimable. When this happens, the DF is zero, the parameter estimate is set to zero, and the estimates and tests on other parameters are conditional on this parameter being zero (not shown). In our case the DF > 0, and therefore the response is not a linear response.

14. The ESTIMATE column contains the value of the estimates, be they intercept (β_o), magnitude of 'the slope' of X_1,X_2, etc.

15. The STD DEV column contains the estimated standard deviations of the parameter estimates and reflect the error in the system.

16. The T-RATIO column contains $\underline{t}$ values of a test of the hypothesis that each particular parameter value is zero, in other words that there is no effect on the model due to that particular variable.

17. PROB, similar to [10], gives a significance value or probability of a greater absolute $\underline{t}$ value given that the hypothesis is true. When PROB is $<$ 0.05 the effect may be termed 'significant'. In our case, for the short length situation, clearly the tellurium (VI) and thallium (I) have a significant effect whilst Se(VI) does not. Notice that this is also carried on into the X2*X2 and X3*X3 terms which are significant, while the X1*X1 term is not. Similar reasoning can be applied to the Cross-Product terms where none are significant. In a way this particular solution was expected because

(a) it has yet to be demonstrated that there is an interaction between Se(VI) and either Te(VI) or Tl(I) on plant growth;

(b) thallium (I) is supposed to interfere with the movement of potassium ions in the functioning of the cell and it is unlikely that Te(VI) or Se(VI) (because of their large size

compared to K^+ ion) would cause interference, by competition for the same enzyme system as Tl(I).

The effect (or lack of fit) can be seen in the response surfaces in Figures 6.4, 6.6 and 6.8, where in each case increasing selenium concentration has only a small effect on growth. However, both Tl(I) and Te(VI) have a marked effect on growth, firstly appearing to stimulate growth and elongation but then rapidly becoming detrimental and eventually quite toxic to the Lolium perenne seedlings. All the surfaces are plain showing curvature in the expected direction, for

i) Te v Se in the direction of increasing Te,

ii) Tl versus Se in the direction of increasing Tl,

iii) Tl versus Te curvature in both directions of increasing concentration, i.e. dome-shaped.

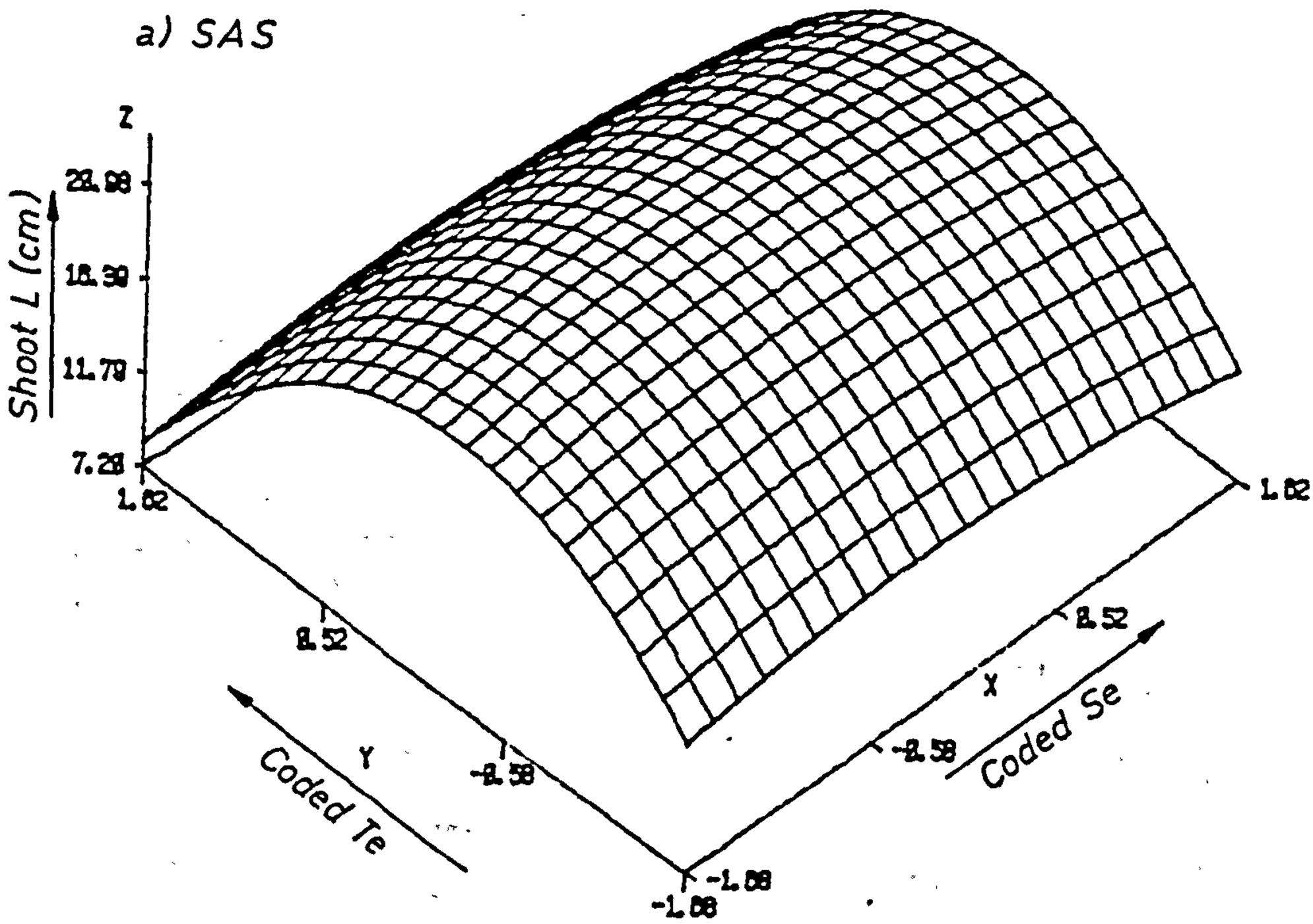
18. The test on a factor, say X1, is a joint test on all parameters involving that factor. Thus, the test for X1 tests the hypothesis that X1, X1*X1, X1*X2, etc., are all zero. In our situation, the X1 factor is not significant, in other words, the level of Se(VI) is not a critical factor in the Lolium perenne seedlings. However, both the X2 and X3 factors are.

19. The CRITICAL VALUES for the factor variables are solved to find the factor combinations that yield optimum response, in this case optimum shoot elongation. The critical values can either be a maximum, minimum or saddle point. Here we have a maximum response because of the sign of the various estimates and the actual coded values are given. For elongation, the optimum response is clearly within the boundaries of the experimental design, representing 0.0985 µg/ml Se(VI), 0.084 µg/ml Te(VI) and 0.0367 µg/ml Tl(I). These values reflect the relative needs or toxicity

towards the Lolium perenne seedlings of these variables. Thus the seedlings require some Se(VI), which acts as a source of an essential trace element Se, while Te(VI) and Tl(I) are both toxic to the plant. The lower value for the Tl(I) reflects the extremely toxic nature of this element to plant growth. Obviously such values may only be used for comparison purposes but demonstrate the large quantity of information that can be gleaned from one experiment.

20. Finally, the EIGENVALUES and EIGENVECTORS are derived from the matrix of quadratic parameter estimates which determine the curvature of the response surface. When all three eigenvalues are negative, the solution is a maximum. In general, if the first eigenvalue is large and positive the solution is a saddle point; whilst when all three values are positive the answer is a minimum.

FIG.6.4 RESPONSE SURFACE FOR SHOOT LENGTH AT CODED TI = 0



Se(VI) , Te(VI), Shoot length

FIG. 6.5 CONTOUR PLOT OF RESPONSE SURFACE FOR SHOOT LENGTH SEEDLING OF LOLIUM PERENNE AT CODED T1 =0

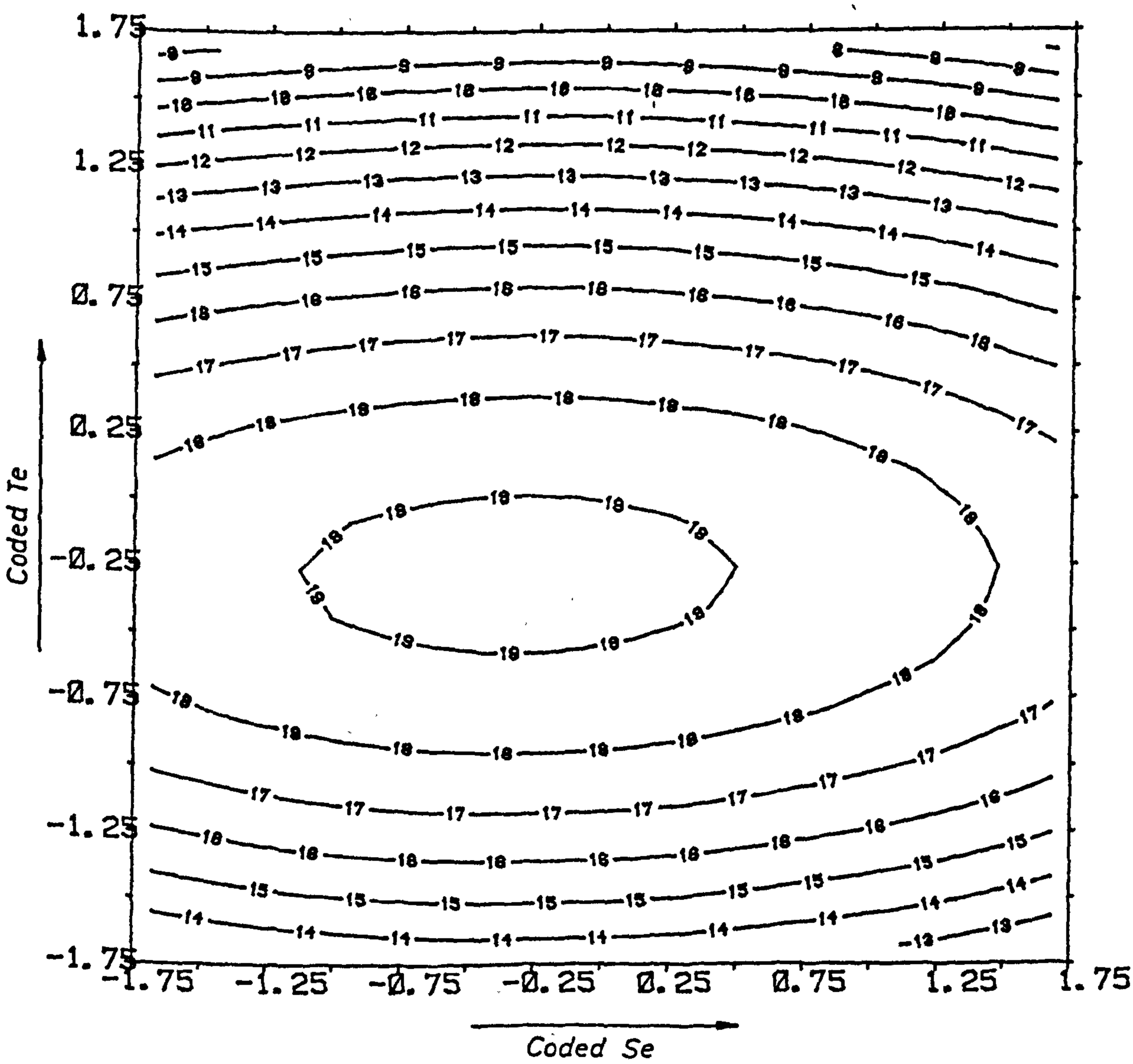
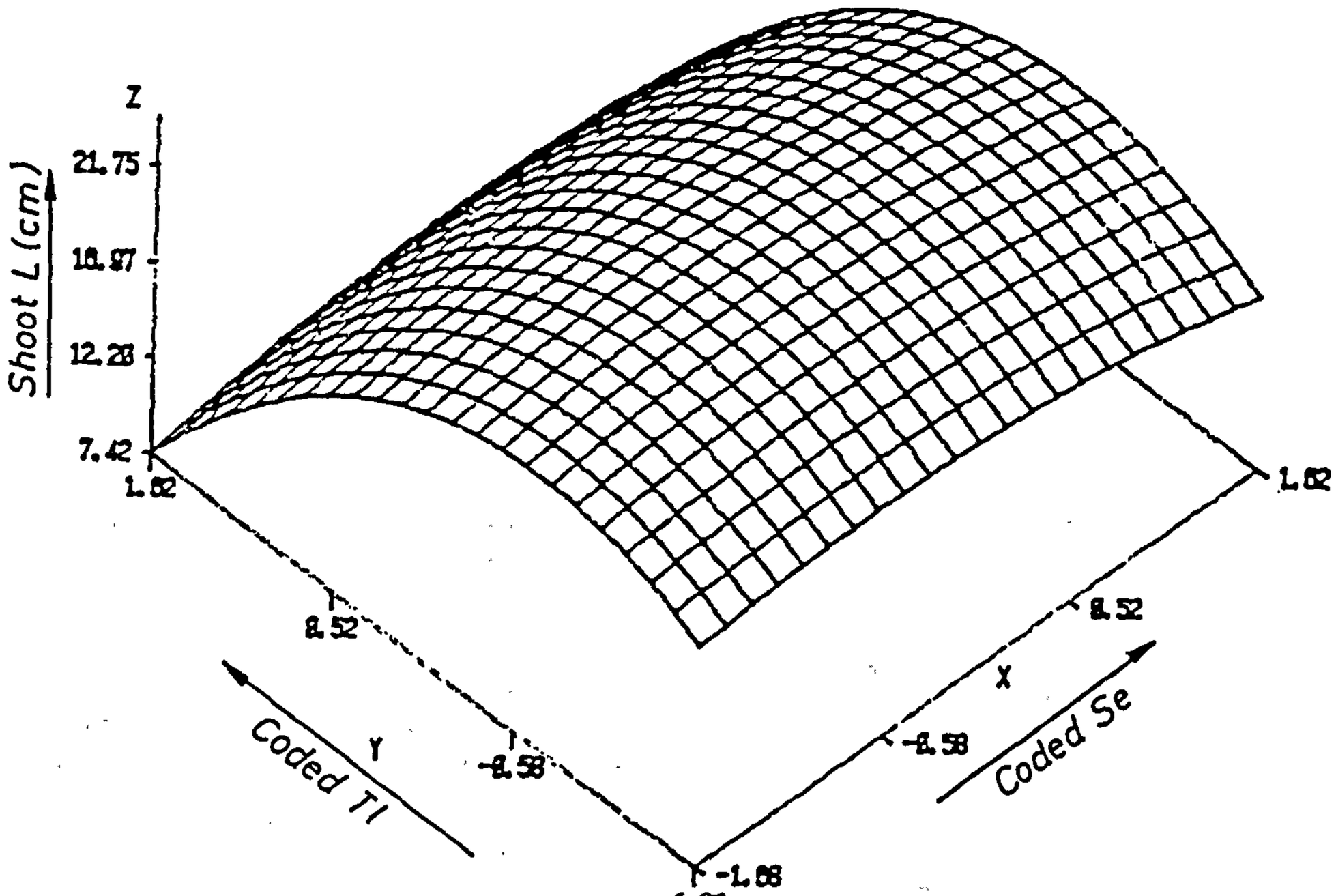
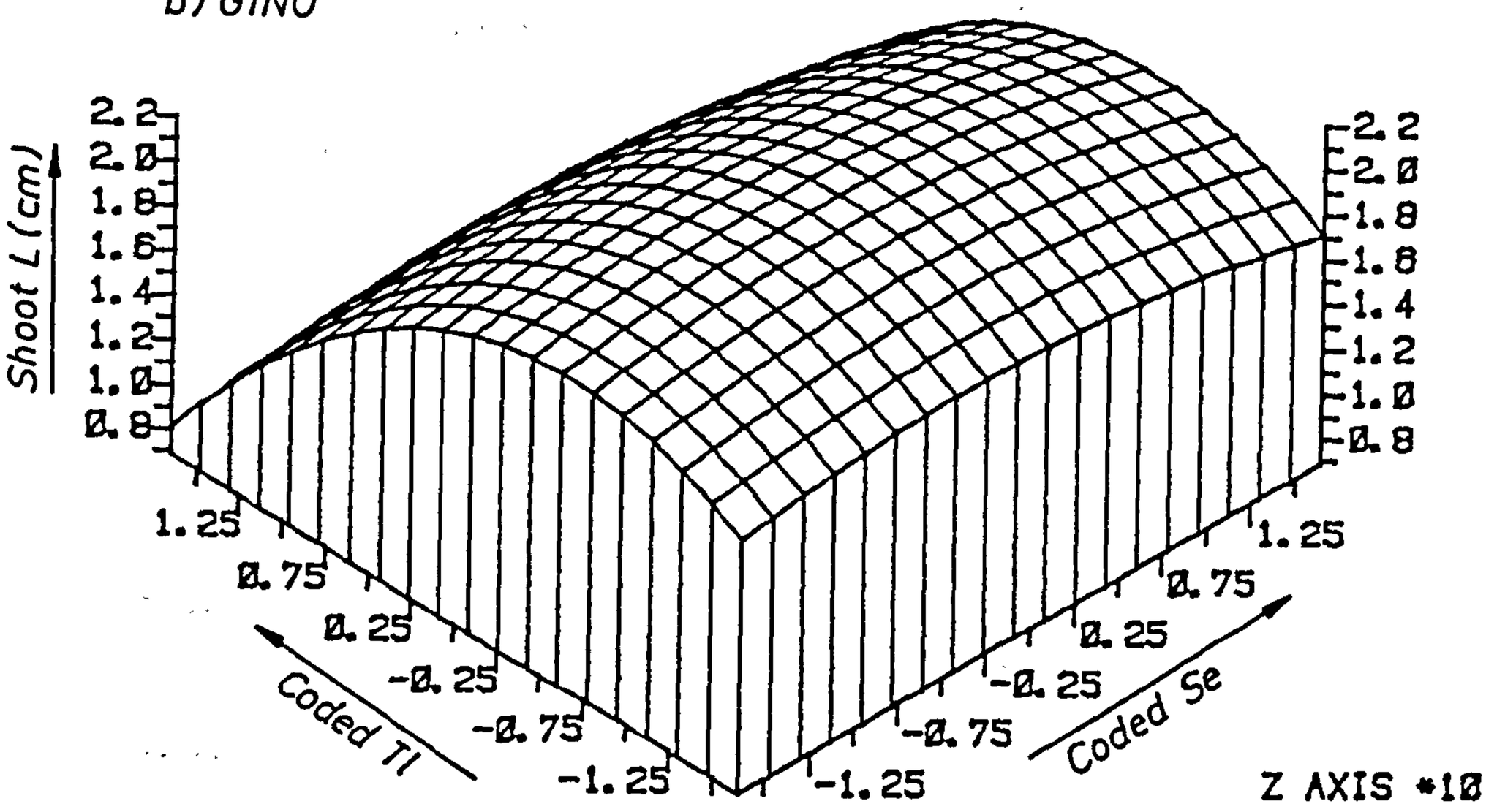


FIG. 6.6 RESPONSE SURFACE FOR SHOOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Te =0

a) SAS



b)'GINO



Se(VI), Tl(I), Shoot length

FIG. 6.7 CONTOUR PLOT OF RESPONSE SURFACE FOR SHOOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Te =0

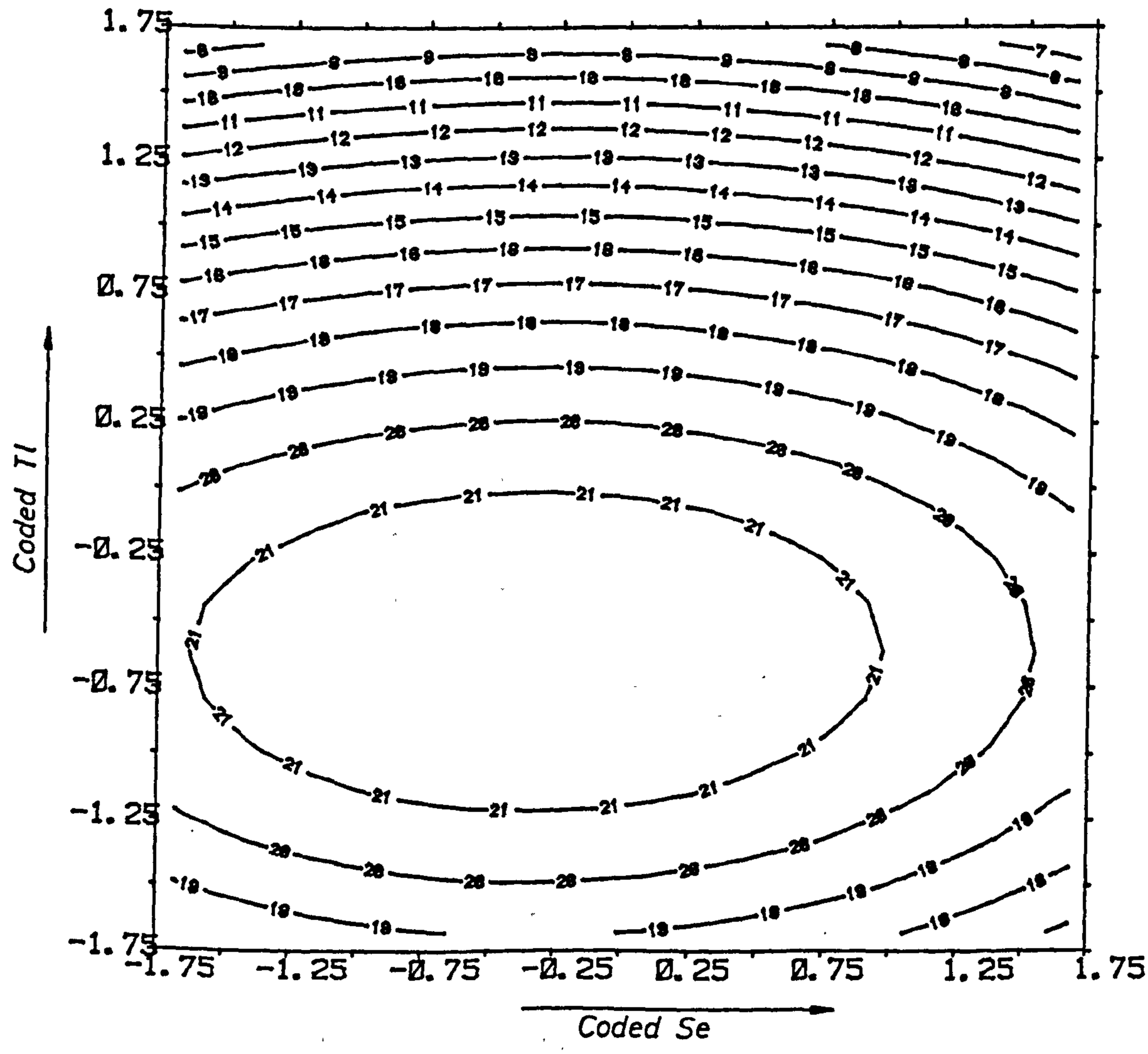
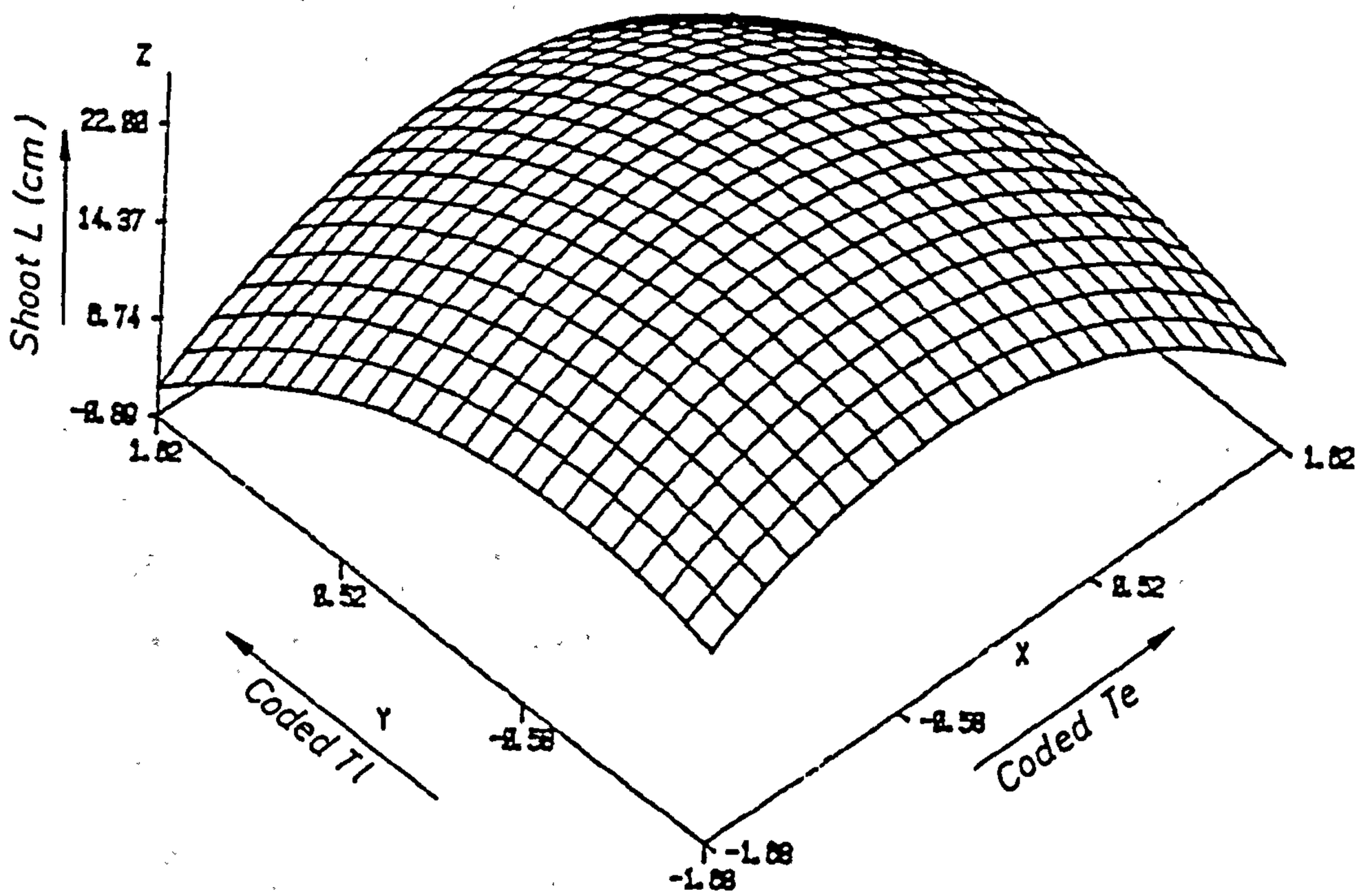
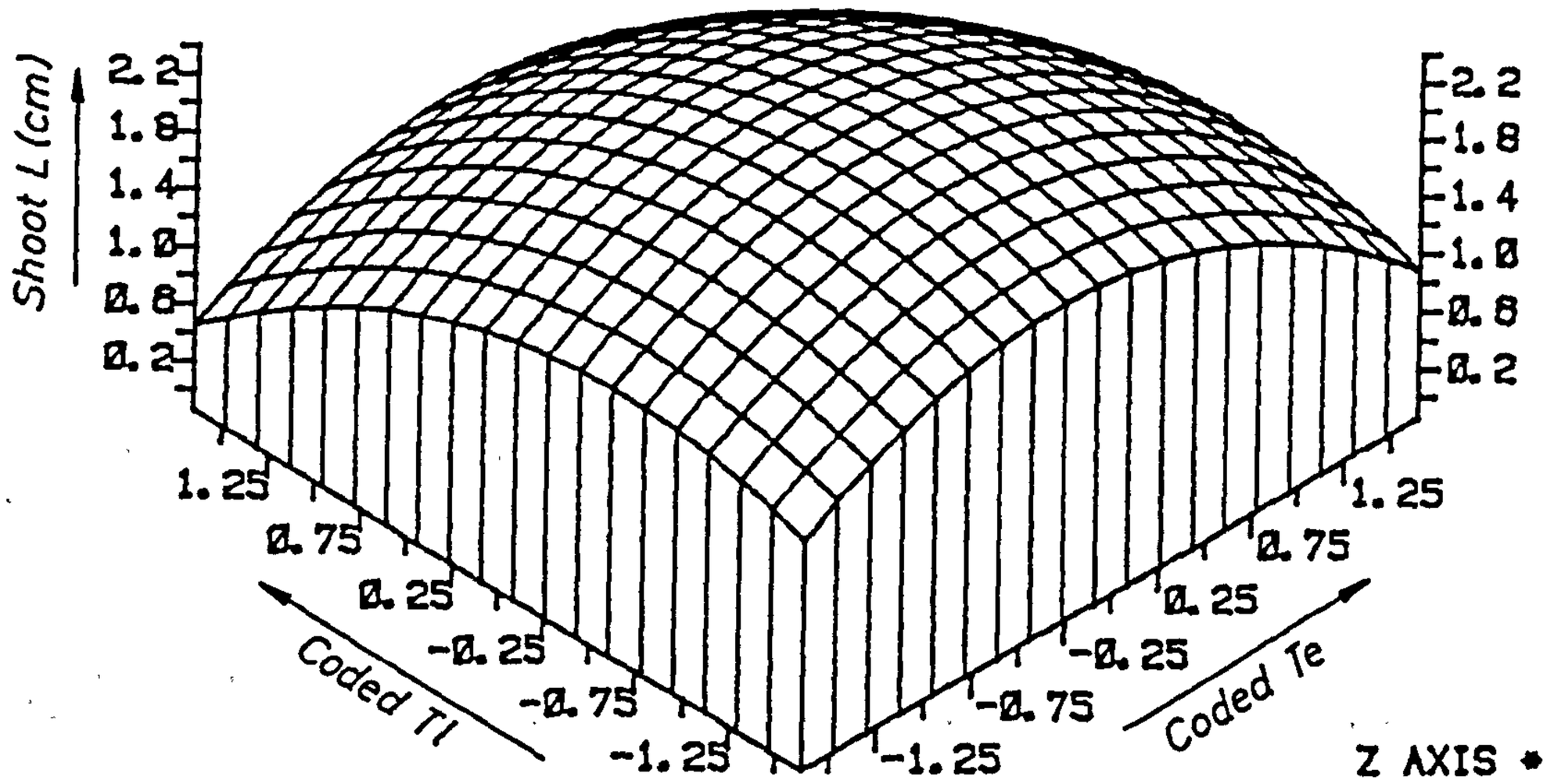


FIG. 6.8 RESPONSE SURFACE FOR SHOOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

a) SAS

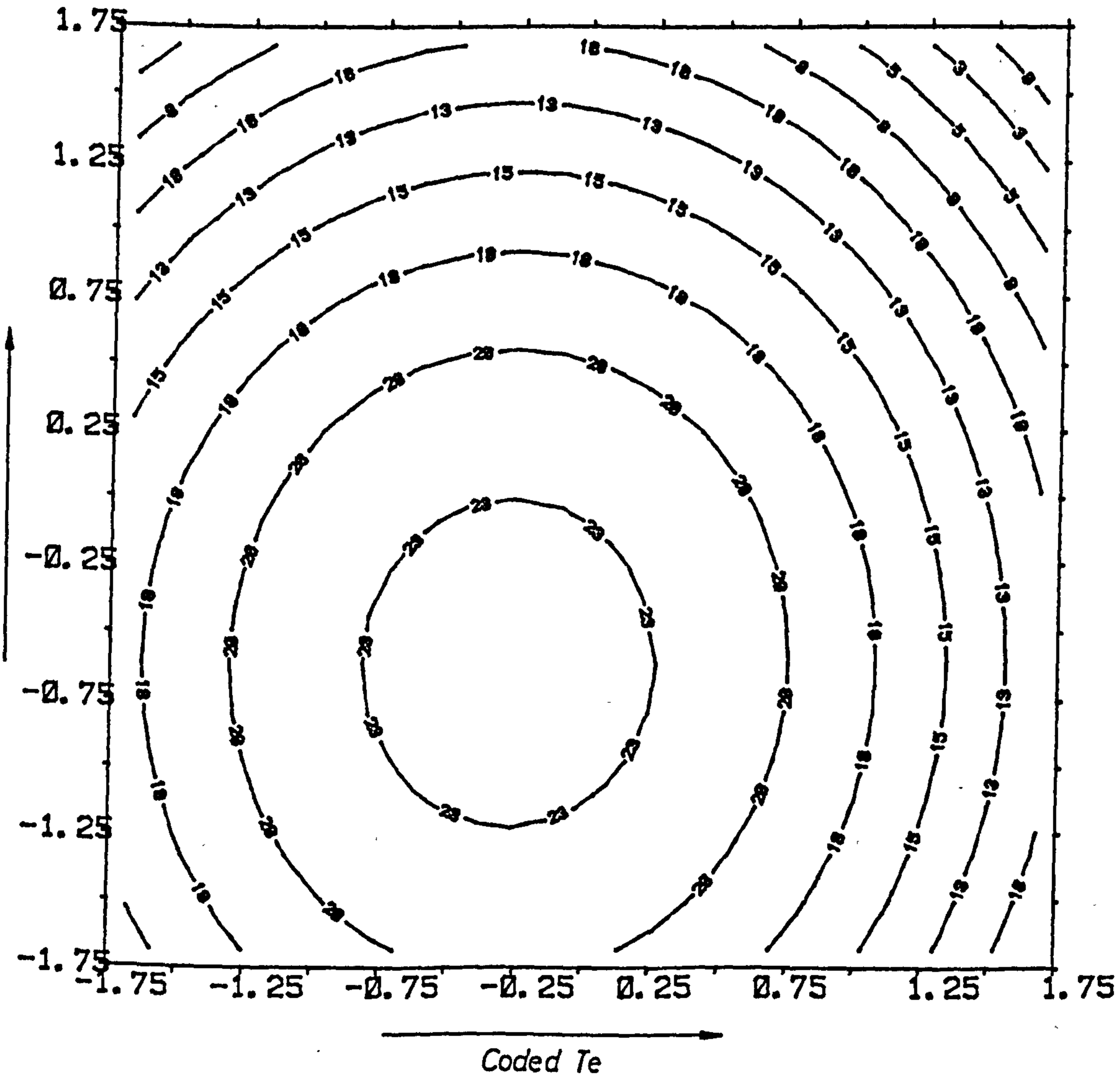


b) GINO



Te(VI), Tl(I), Shoot length

FIG. 6.9 CONTOUR PLOT OF RESPONSE SURFACE FOR SHOOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Se =0



Because the discussion has been initiated using the shoot length parameter, it is thought best now to discuss the dry weight of the shoots as a response variable (response Y9, Table 6.6), and then to proceed to the elemental responses.

In the dry weight case, the fit of R^2 is only 0.747, being almost a 20% lesser fit than for the shoot length response, but allowing an R value of about 0.86, showing the extreme sensitivity of the R^2 value to slight changes in the fit of the model. The coefficient of variation is quite high and this is reflected in the Type I SS term [11] where the pure error term derived from the six-coded (0,0,0) experiments (Trials 15-20) is greater than the lack of fit to the model term. Thus the effect in the model of changing the values of the metal concentrations and selenium is not significant when examined with respect to the pure error term. The value of 0.6283 for the PROB [12] is not significant. The variation in the biomass at the coded (0,0,0) values is greater than the variation in the model. Obviously other parameters which have not been included in the model, for example temperature or light intensity, have probably played a major part in the biomass response. Because of this large variation, it would be unexpected to find significant probability levels for the variables X_1, X_2 and X_3 at any stage, either linear, quadratic or cross-product. Examination of the PROB values [17] seems to confirm this proposal, certainly Se(VI), Te(VI) and Tl(I) in linear combination are not significant, while Te(VI) and Tl(I) appear to be significant at the quadratic combination level. But none of the cross-product terms are significant. When considering the factors as a whole only the Te(VI) variable appears to be significant, the Tl(I) total value being just outside the 95% confidence level. Examination of the response surfaces

TABLE 6.13(a)

RESPONSE SURFACE FOR VARIABLE Y9 SHOOT DRY WEIGHT (Se, Te, Tl)

RESPONSE MEAN	0.387715
ROOT MSE	0.09478676
R-SQUARE	0.7475438
COEF OF VARIATION	0.2444754

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	0.04747307	0.1334	1.76	0.2179
QUADRATIC	3	0.2037117	0.5724	7.56	0.0063
CROSSPRODUCT	3	0.01485459	0.0417	0.55	0.6588
TOTAL REGRESS	9	0.2660394	0.7475	3.29	0.0387

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	0.03805152	0.007610305	0.735	0.6283
PURE ERROR	5	0.05179377	0.01035876		
TOTAL ERROR	10	0.0898453	0.00898453		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	0.4893866	0.03865584	12.66	0.0001
X1	1	0.005750469	0.02566044	0.22	0.8272
X2	1	-0.032836	0.02566044	-1.28	0.2296
X3	1	-0.0486615	0.02566044	-1.90	0.0871
X1*X1	1	0.01737155	0.02500853	0.69	0.5031
X2*X1	1	-0.0218	0.03351218	-0.65	0.5300
X2*X2	1	-0.0984519	0.02500853	-3.94	0.0028
X3*X1	1	-0.02395	0.03351218	-0.71	0.4912
X3*X2	1	0.028425	0.03351218	0.85	0.4162
X3*X3	1	-0.0679459	0.02500853	-2.72	0.0217

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	0.01317702	0.003294254	0.37	0.8271
X2	4	0.1642185	0.04105463	4.57	0.0234
X3	4	0.1096829	0.02742072	3.05	0.0694

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE
X1	-0.476637 = 0.083 µg Se/ml
X2	-0.158339 = 0.094 µg Te/ml
X3	-0.307206 = 0.045 µg Tl/ml

PREDICTED VALUE AT OPTIMUM 0.4980904

EIGENVALUES	EIGENVECTORS X1	X2	X3
0.02040589	0.9826672	-0.108123	-0.150581
-0.0651	0.1786632	0.335728	0.9248601
-0.104332	0.0494439	0.935733	-0.349226

SOLUTION WAS A SADDLE POINT

TABLE 6.13(b) Y9 SHOOT DRY WEIGHT (Se,Te,Tl)

```
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 -- read 'AdlyDryWt' c1-c8
column        c1          c2          c3          c4          c5
c6
count         20          20          20          20          20
20
row
   1    -1.00000    -1.00000    -1.00000     18.1600      4.210
2.730
   2     1.00000    -1.00000    -1.00000     22.0800    166.660
3.030
   3    -1.00000     1.00000    -1.00000     16.2500      4.000
126.140
   4    -1.00000    -1.00000     1.00000     11.6600      2.730
3.100
   . . .

column        c7          c8
count         20          20
row
   1      1.310    0.329500
   2      2.740    0.562500
   3      1.550    0.383500
   4     39.840    0.305000
   . . .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c8 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =   0.489 -0.0004 x1 -0.0267 x2
    -0.0548 x3 +0.0196 x4 -0.0322 x5
    -0.0135 x6 -0.0962 x7 +0.0180 x8
    -0.0657 x 9

                                        st. dev.    t-ratio =
       column      coefficient        of coef.    coef/s.d.
       --            0.48904          0.03844        12.72
x1     c1           -0.00037          0.02551        -0.01
x2     c2           -0.02672          0.02551        -1.05
x3     c3           -0.05478          0.02551        -2.15
x4     c11           0.01958          0.02487         0.79
x5     c12          -0.03224          0.03332        -0.97
x6     c13          -0.01351          0.03332        -0.41
x7     c22          -0.09624          0.02487        -3.87
x8     c23           0.01799          0.03332         0.54
x9     c33          -0.06574          0.02487        -2.64

the st. dev. of y about regression line is
s = 0.09425

with (  20-10) =  10 degrees of freedom

r-squared = 74.5 percent
r-squared = 51.5 percent, adjusted for d.f.

analysis of variance

 due to      df          ss          ms=ss/df
regression    9     0.259031        0.028781
residual     10     0.088829        0.008883
total        19     0.347860

further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9     0.259031
c1            1     0.000002
c2            1     0.009739
c3            1     0.040947
c11           1     0.016986
c12           1     0.008314
c13           1     0.001461
c22           1     0.116918
c23           1     0.002588
c33           1     0.062076

            x1          y     pred. y    st.dev.
row         c1         c8      value   pred. y   residual     st.res.
 19       0.00     0.3095     0.4890    0.0384    -0.1795      -2.09r

r denotes an obs. with a large st. res.

durbin-watson statistic = 2.10
 -- end
 -- stop

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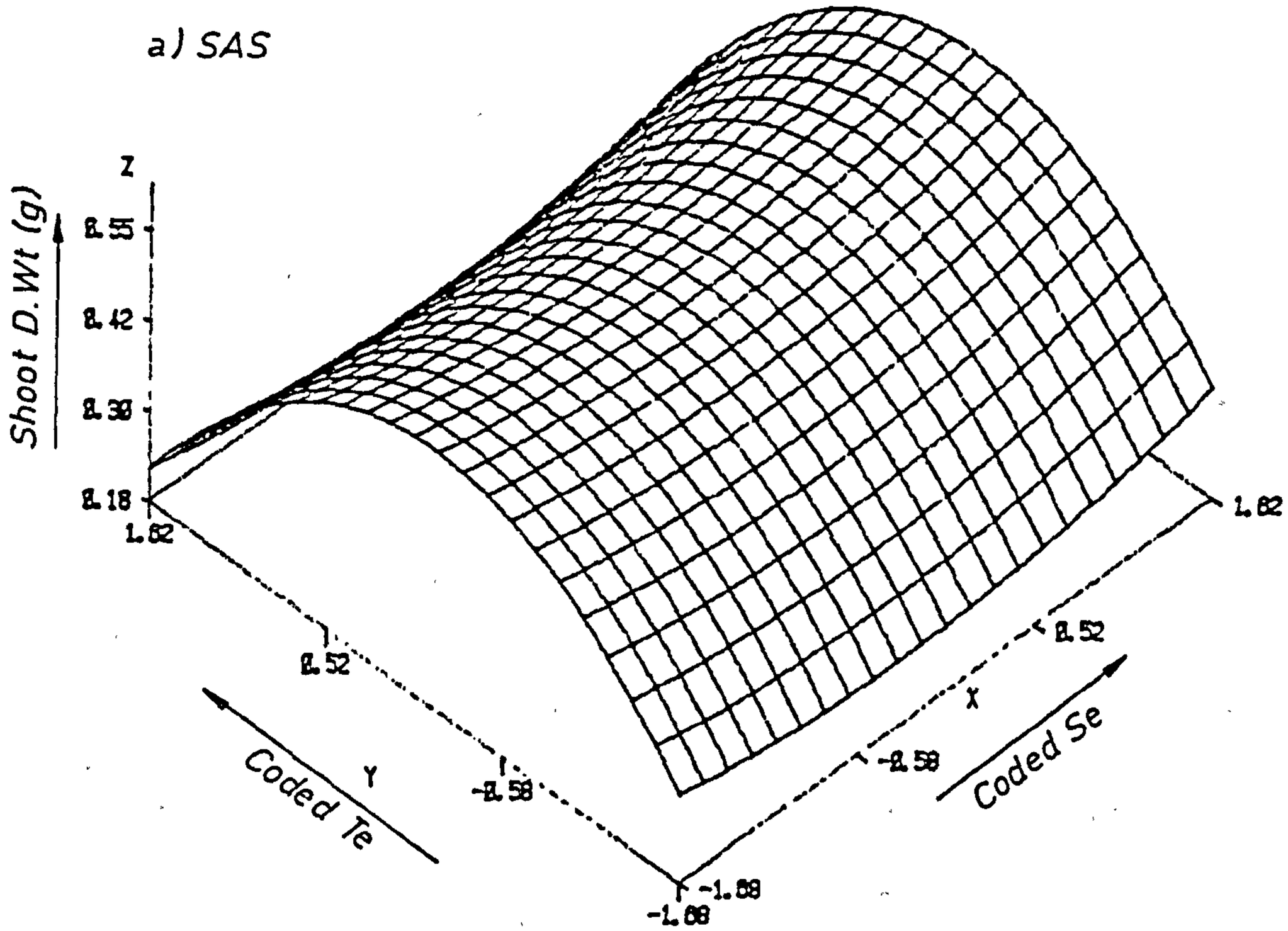
confirm these findings; in Figure 6.10 and 6.12 the effect of changing Se concentration is almost negligible, a very shallow saddle point surface being suggested. In both situations the Te(VI) and Tl(I) seem firstly to stimulate growth and then become rather toxic; with Te(VI) appearing to be more detrimental to biomass production than Tl(I). A reversal of roles when compared to the elongation phenomenon of shoot length response. In Figure 6.14, the response surface at coded Se = 0 values is the expected dome shape with the maximum value well within the experimental area. The critical factor values for Se(VI), Te(VI) and Tl(I) are 0.083 µg/ml, 0.094 µg/ml and 0.045 µg/ml respectively. These values are very similar to those recorded for the shoot length response. Again, notice the extreme toxicity of Tl(I) to plant growth, in this case biomass, while the toxicity of Te(VI) is slightly lower. Notice for Se(VI) the optimum respsonse is obtained at an almost equivalent level as for the Te(VI), but whereas increasing Te(VI) concentrations become increasingly toxic to the seedlings, similar increase in the Se(VI) concentration produces little change. See Figure 6.14 and the corresponding contour diagram, Figure 6.11, at Tl = 0 (coded value), where the steepness of the "contour lines" at increasing Te(VI) concentration is clearly visible.

FIG. 6.10 RESPONSE SURFACE FOR DRY WT. SHOOT AT CODED TI =0



Se(VI), Te(VI), DryWt. Shoot

FIG. 6.11 CONTOUR PLOT OF RESPONSE SURFACE FOR DRY WT. SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED TI = 0

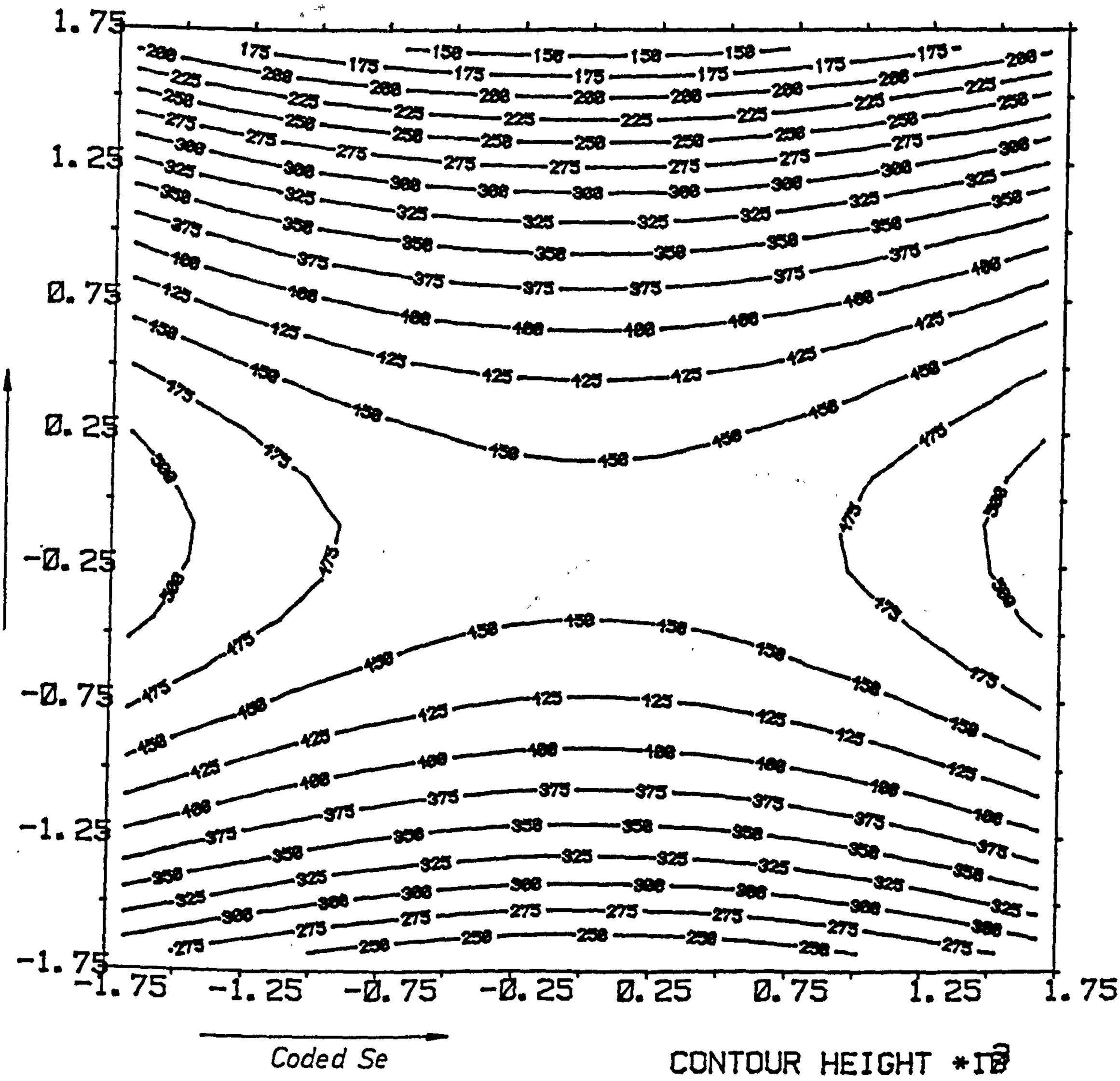
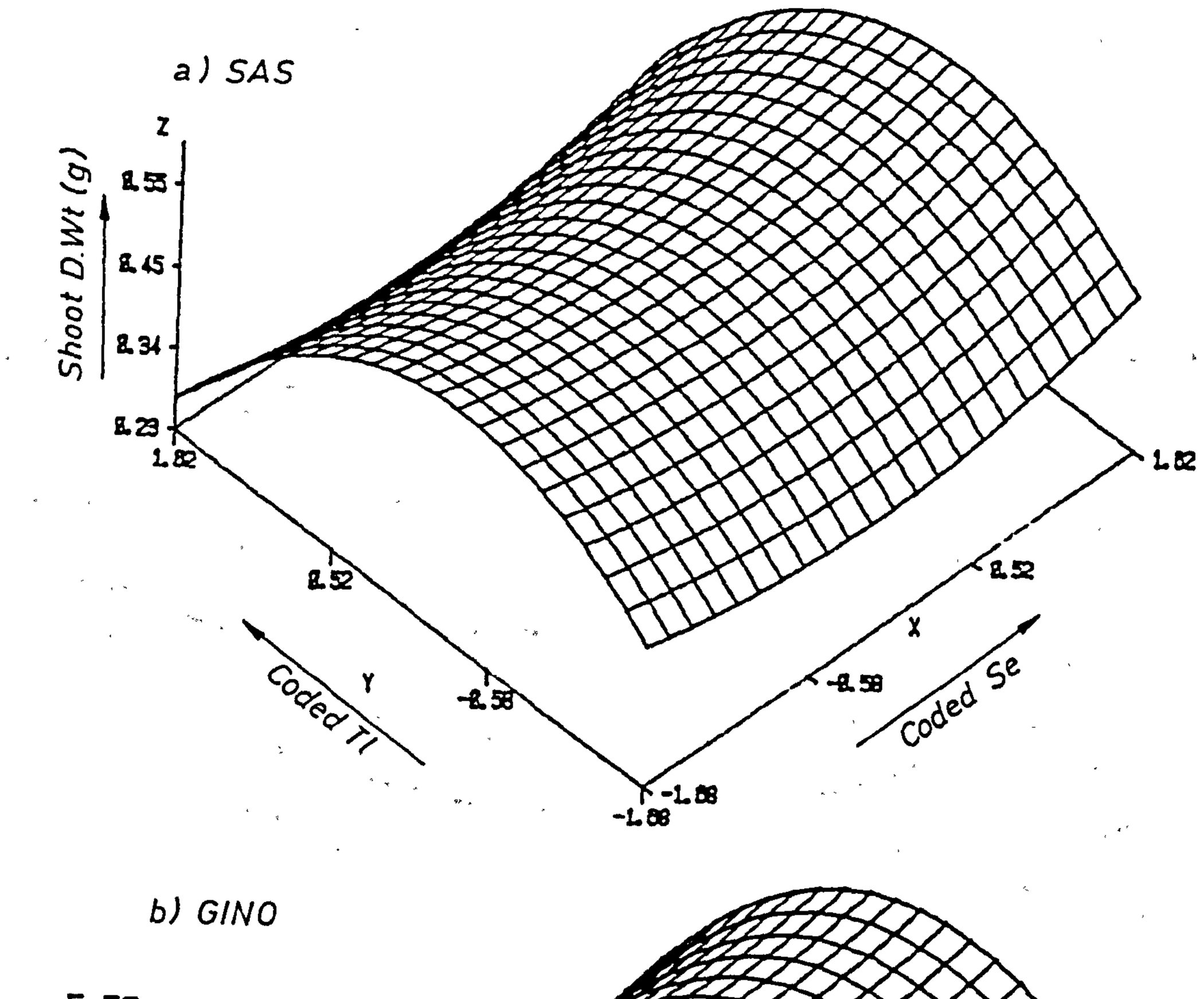


FIG. 6.12 RESPONSE SURFACE FOR DRY WT. SHOOT AT CODED Te = 0



Se(VI), Tl(I), Dry Wt. Shoot

FIG. 6.13 CONTOUR PLOT OF RESPONSE SURFACE FOR DRY WT. SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED Te = 0

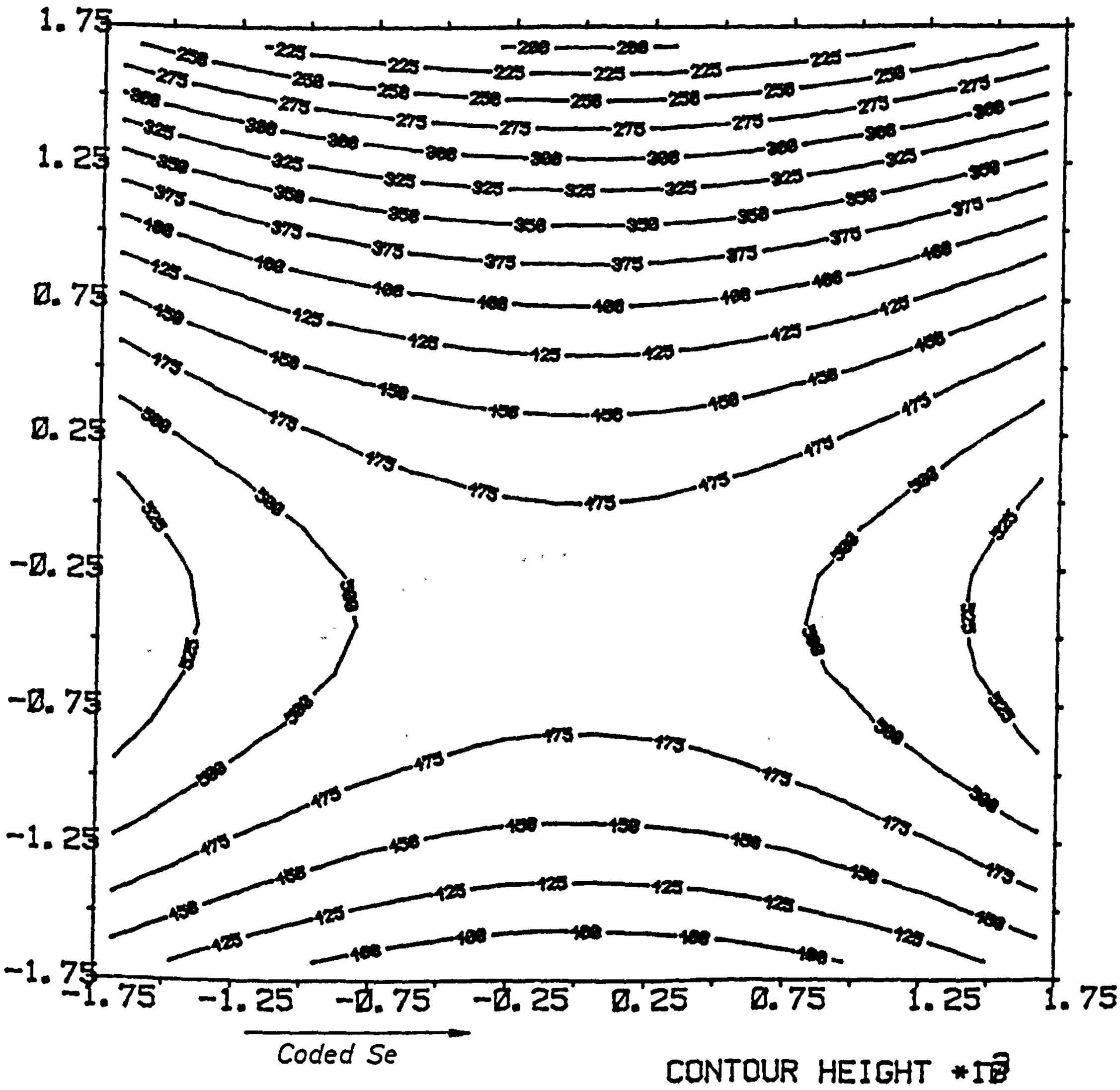
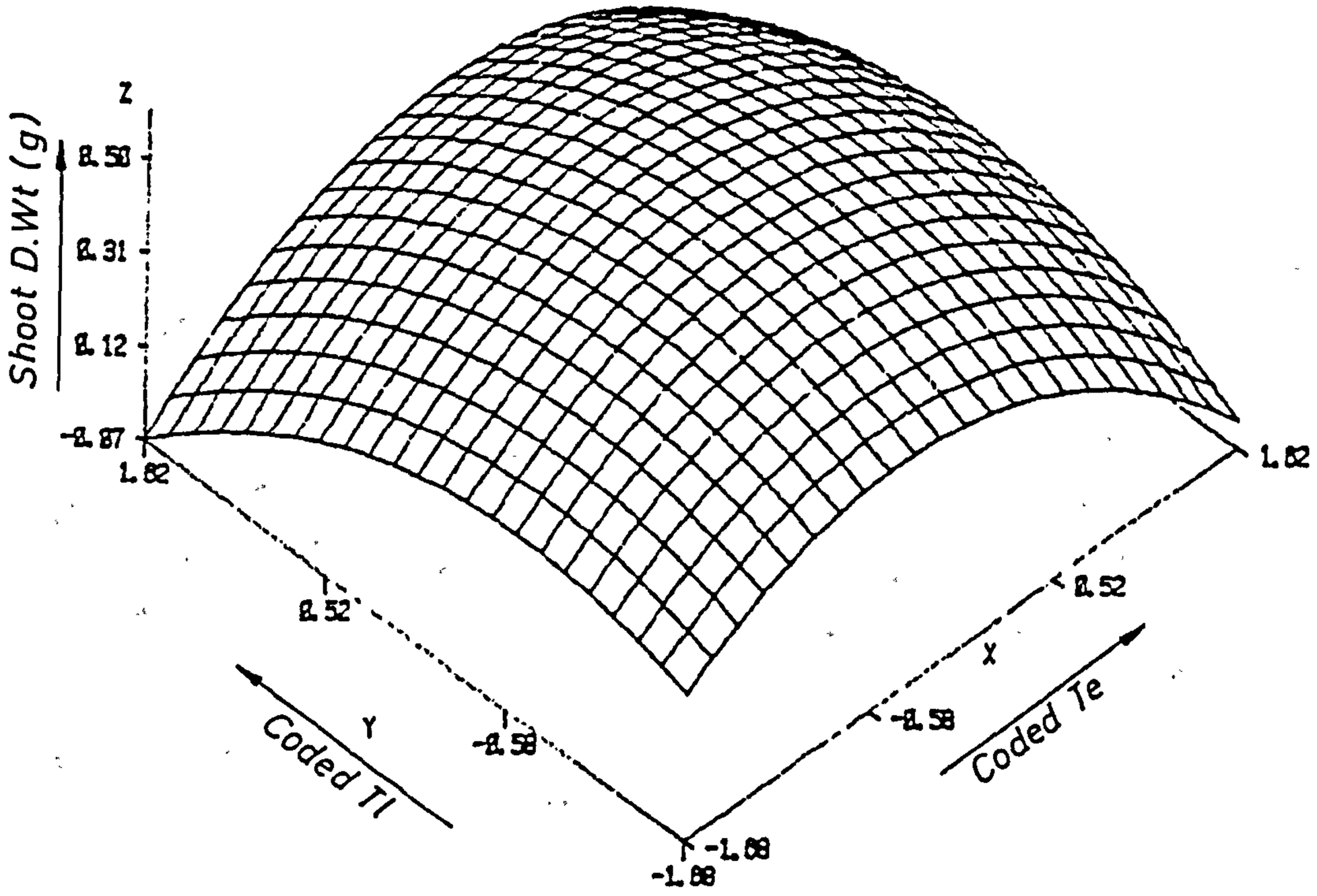
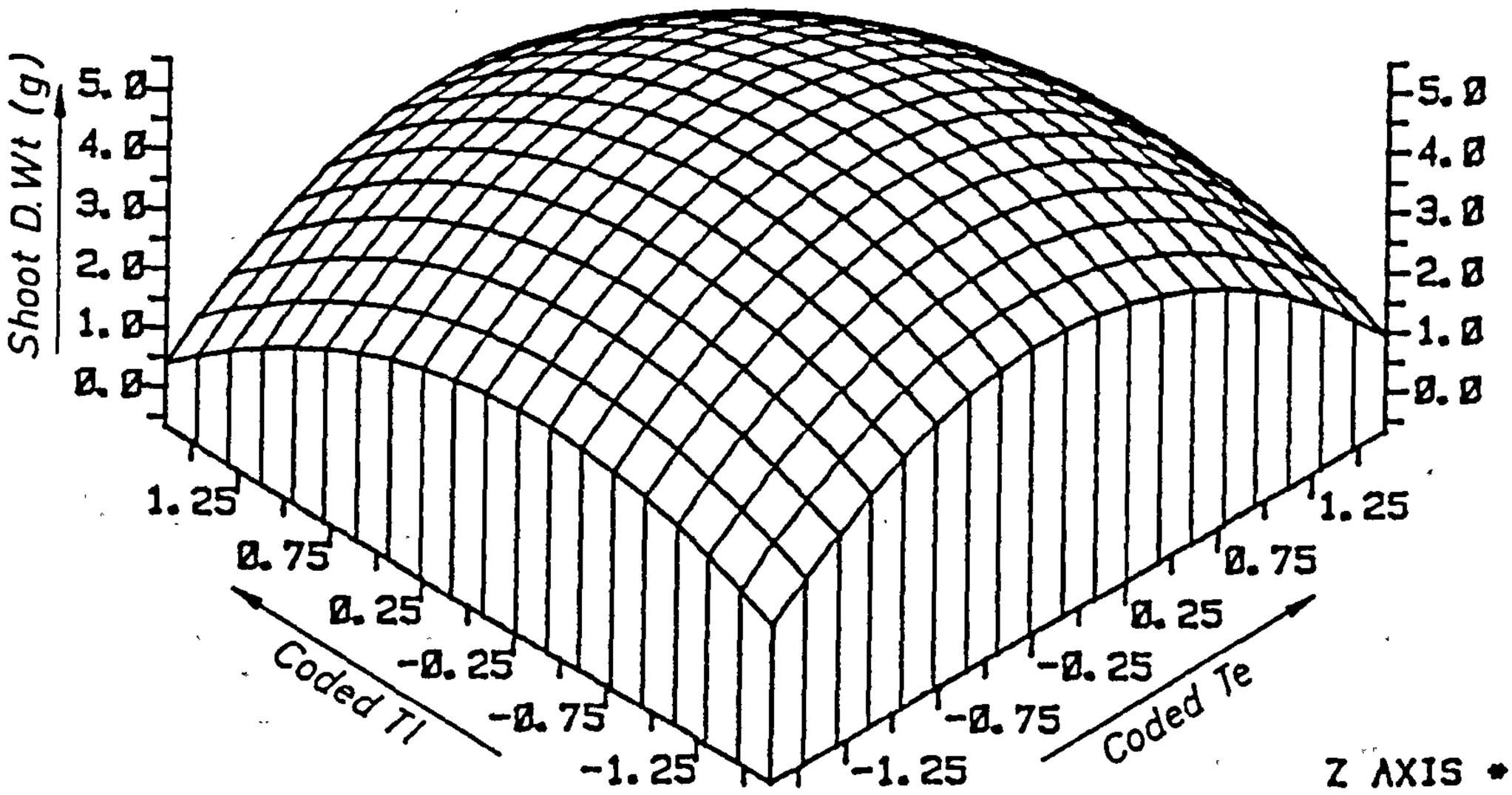


FIG. 6.14 RESPONSE SURFACE FOR SHOOT DRY WT. AT CODED $Se \doteq 0$

a) SAS

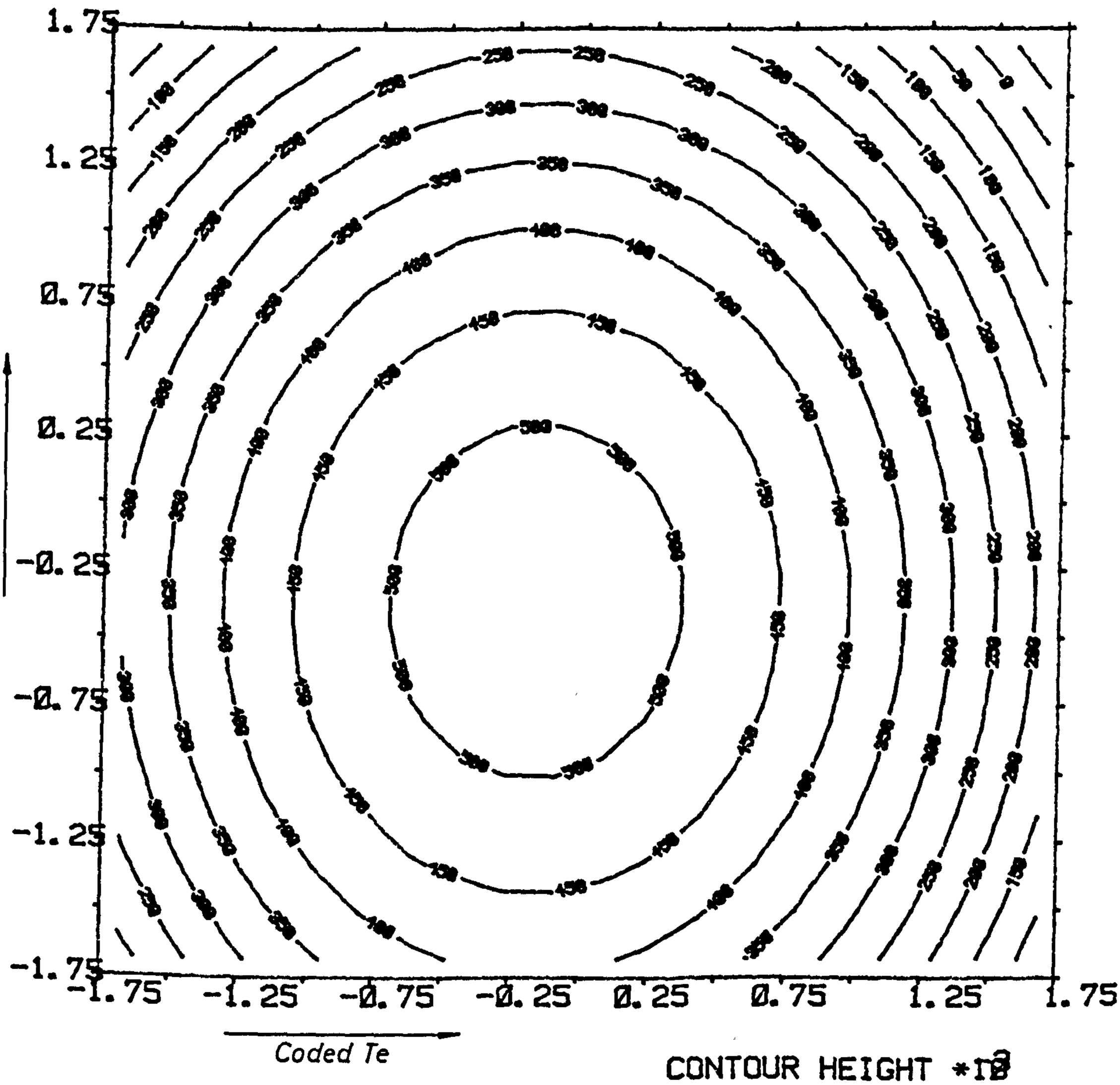


b) GINO



Te(VI), Tl(I), Dry Wt. Shoot

FIG.6.15 CONTOUR PLOT OF RESPONSE SURFACE FOR DRY WT. OF SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED Se = 0



Turning to the elemental analyses, initially the uptake of added elements by the plant, Figure 6.16 illustrates the concentration of Se in the tissue of the shoot. Notice in this case the pure error value is very low and most of the total error arises from the lack of fit of the model. In this case the R^2 value is 0.828, reflecting a reasonable fit of the data to the model being imposed on it. The probability of the regression values in terms of linear and quadratic terms are very high, being greater than 99.9% that they are factors in the model. But notice that the probability of the cross-product term is very small indeed, so once again no interaction terms are to be expected. This is borne out by a consideration of the parameter estimates, where only the linear and quadratic estimates for Se concentration are significant at the 95% level. Therefore, there seems little interaction between the Se(VI) uptake and the influence of increasing concentrations of Tl(I). As shown in Figures 6.16 and 6.18, the uptake of Se is virtually independent of the increasing Te(VI) and Tl(I) concentrations.

As commented previously, the non-interaction between the Se level in the tissue and the Tl(I) level in the nutrient solution is not surprising. However, a similar level of response is found in the case of increasing Te(VI) concentration where no interaction with the Se content is found; even in view of the similarity of these anions (chemically). Figure 6.20 depicts the response for Se level in tissue at Se = 0.1 μg/ml in solution with varying Te(VI) and Tl(I) concentrations. Clearly some form of interaction is hinted at because the Se content varies widely as the Te(VI) and Tl(I) concentrations are increased. However, no such interaction is hinted at in the PROB [18] where only the Se concentration of the nutrient solution is significant and highly so - as it

TABLE 6.14(a) : SELENIUM UPTAKE BY SHOOT (Se,Te,Tl)

RESPONSE SURFACE FOR VARIABLE Y2

RESPONSE MEAN	85.657
ROOT MSE	118.7283
R-SQUARE	0.8282581
COEF OF VARIATION	1.38609

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	356031.8	0.4338	8.42	0.0043
QUADRATIC	3	321807.1	0.3921	7.61	0.0061
CROSSPRODUCT	3	1987.377	0.0024	0.05	0.9857
TOTAL REGRESS	9	679826.3	0.8283	5.36	0.0075

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	140959.9	28191.98	33352.632	0.0001
PURE ERROR	5	4.22635	0.84527		
TOTAL ERROR	10	140964.1	14096.41		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	9.511233	48.41966	0.20	0.8482
X1	1	161.4328	32.14184	5.02	0.0005
X2	1	1.547403	32.14184	0.05	0.9626
X3	1	-5.46813	32.14184	-0.17	0.8683
X1*X1	1	143.8807	31.32526	4.59	0.0010
X2*X1	1	1.39375	41.9768	0.03	0.9742
X2*X2	1	-15.8698	31.32526	-0.51	0.6234
X3*X1	1	-11.0563	41.9768	-0.26	0.7976
X3*X2	1	-11.1463	41.9768	-0.27	0.7960
X3*X3	1	-16.3995	31.32526	-0.52	0.6120

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	653973.3	163493.3	11.60	0.0009
X2	4	4660.079	1165.02	0.08	0.9859
X3	4	6243.324	1560.831	0.11	0.9759

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE	
X1	-0.560474	= 0.082 µg Se/ml
X2	0.01855471	= 0.127 µg Te/ml
X3	0.0159087	= 0.0615 µg Tl/ml

PREDICTED VALUE AT OPTIMUM -35.7574

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
144.0761	0.9993851	0.005560564	-0.0346203
-10.6751	-0.0277339	0.7294908	-0.683428
-21.7896	0.02145495	0.6839681	0.7291964

SOLUTION WAS A SADDLE POINT

TABLE 6.14(b) SELENIUM UPTAKE BY SHOOT (Se,Te,Tl)

```
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aug. 25, 1986 *** Avon Universities, Bristol
storage available  50000

 -- read 'AdlySe' c1-c8
column          c1          c2          c3          c4          c5
c6
count           20          20          20          20          20
20
row
   1     -1.00000    -1.00000    -1.00000     18.1600      4.210
2.730
   2      1.00000    -1.00000    -1.00000     22.0800    166.660
3.030
   3     -1.00000     1.00000    -1.00000     16.2500      4.000
126.140
   4     -1.00000    -1.00000     1.00000     11.6600      2.730
3.100
   .  .  .

column          c7          c8
count           20          20
row
   1         1.310    0.329500
   2         2.740    0.562500
   3         1.550    0.383500
   4        39.840    0.305000
   .  .  .
 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c5 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    9.51 +  161. x1 +  1.55 x2
    -  5.47 x3 +  144. x4 +  1.39 x5
    -  11.1 x6 -  15.9 x7 -  11.1 x8
    -  16.4 x 9

                                        st. dev.    t-ratio =
       column      coefficient          of coef.    coef/s.d.
       --             9.51              48.42         0.20
x1     c1           161.43              32.14         5.02
x2     c2             1.55              32.14         0.05
x3     c3            -5.47              32.14        -0.17
x4     c11          143.88              31.33         4.59
x5     c12            1.39              41.98         0.03
x6     c13          -11.06              41.98        -0.26
x7     c22          -15.87              31.33        -0.51
x8     c23          -11.15              41.98        -0.27
x9     c33          -16.40              31.33        -0.52

the st. dev. of y about regression line is
s = 118.7

with (  20-10) =  10 degrees of freedom

r-squared = 82.8 percent
r-squared = 67.4 percent, adjusted for d.f.

analysis of variance

 due to      df          ss       ms=ss/df
regression    9      679826          75536
residual     10      140964          14096
total        19      820790

further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9      679826
c1            1      355591
c2            1          33
c3            1         408
c11           1      314995
c12           1          16
c13           1         978
c22           1        2949
c23           1         994
c33           1        3863

           x1          y    pred. y    st.dev.
row        c1         c5     value   pred. y   residual     st.res.
 9       1.68     919.1     686.8      92.5      232.3       3.12r

r denotes an obs. with a large st. res.

durbin-watson statistic = 3.06
 -- end
 -- stop

*** Minitab *** Statistics Dept * Penn State Univ. * release 81.1 *
storage available   50000
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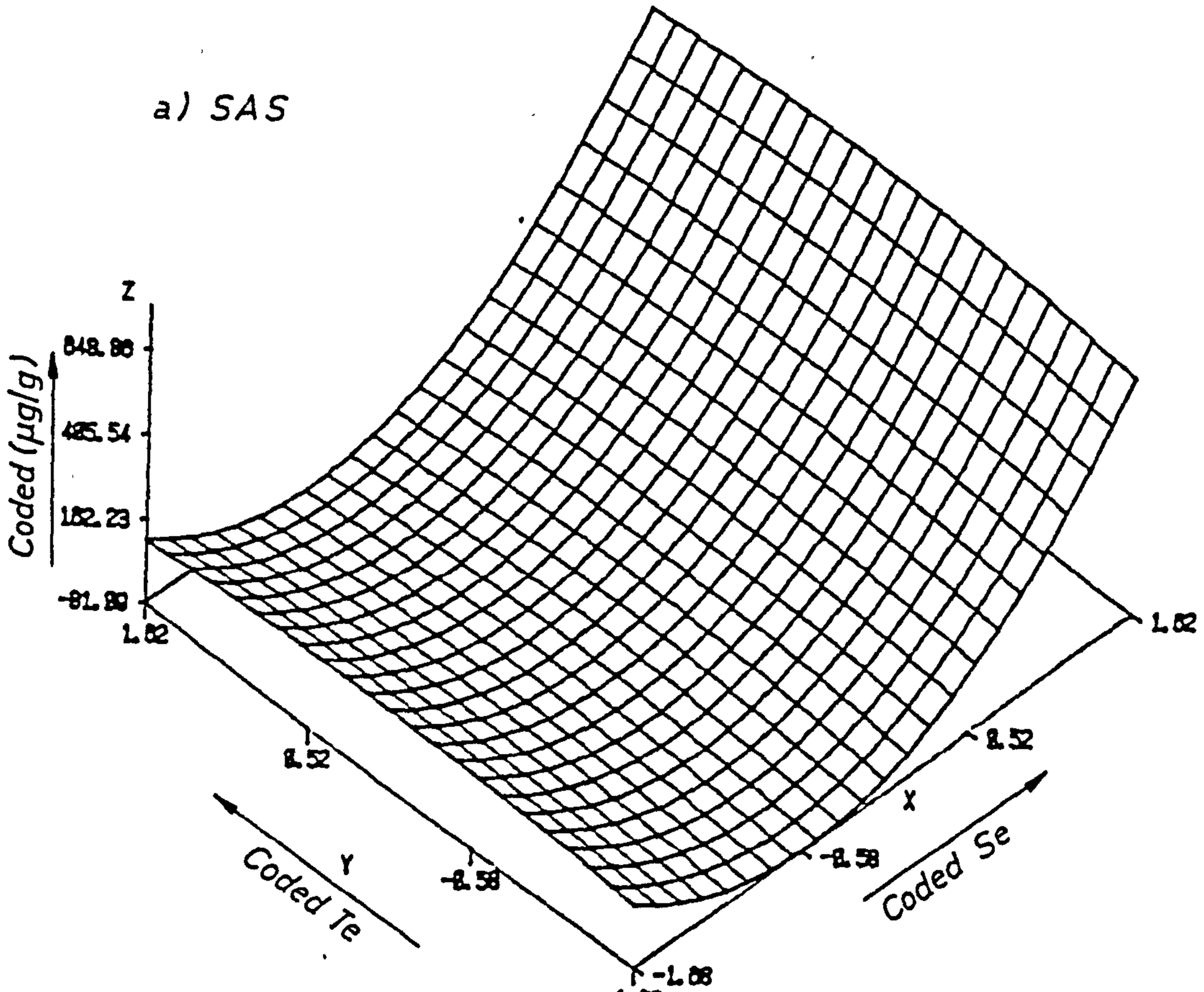
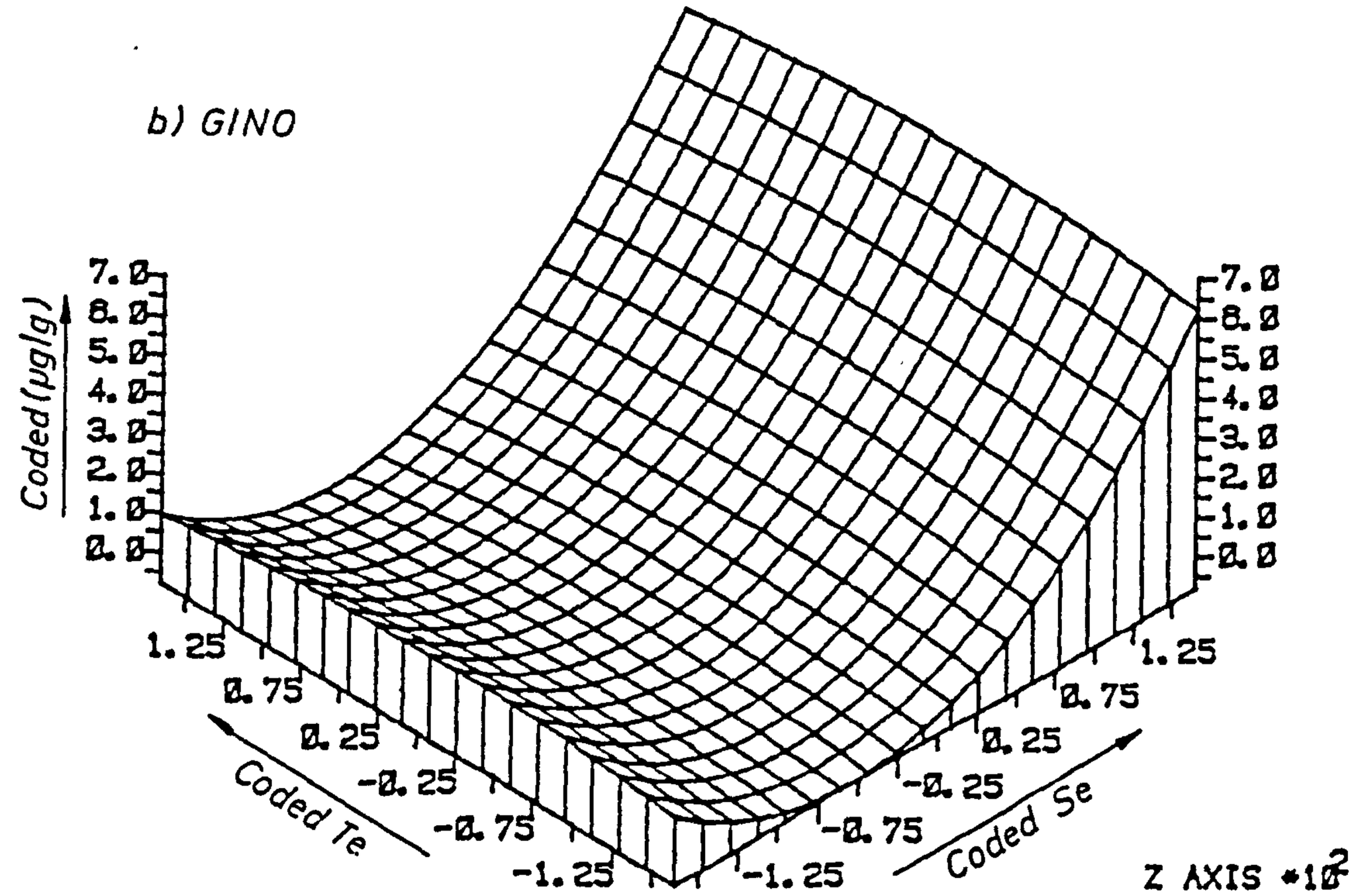
FIG. 6.16 RESPONSE SURFACE FOR SELENIUM UPTAKE BY SHOOT AT CODED TI = 0

a) SAS



b) GINO



Se(VI), Te(VI), Se uptake by Shoot

FIG. 6.17 CONTOUR PLOT OF RESPONSE SURFACE FOR SELENIUM UPTAKE BY SHOOT AT CODED TI = 0

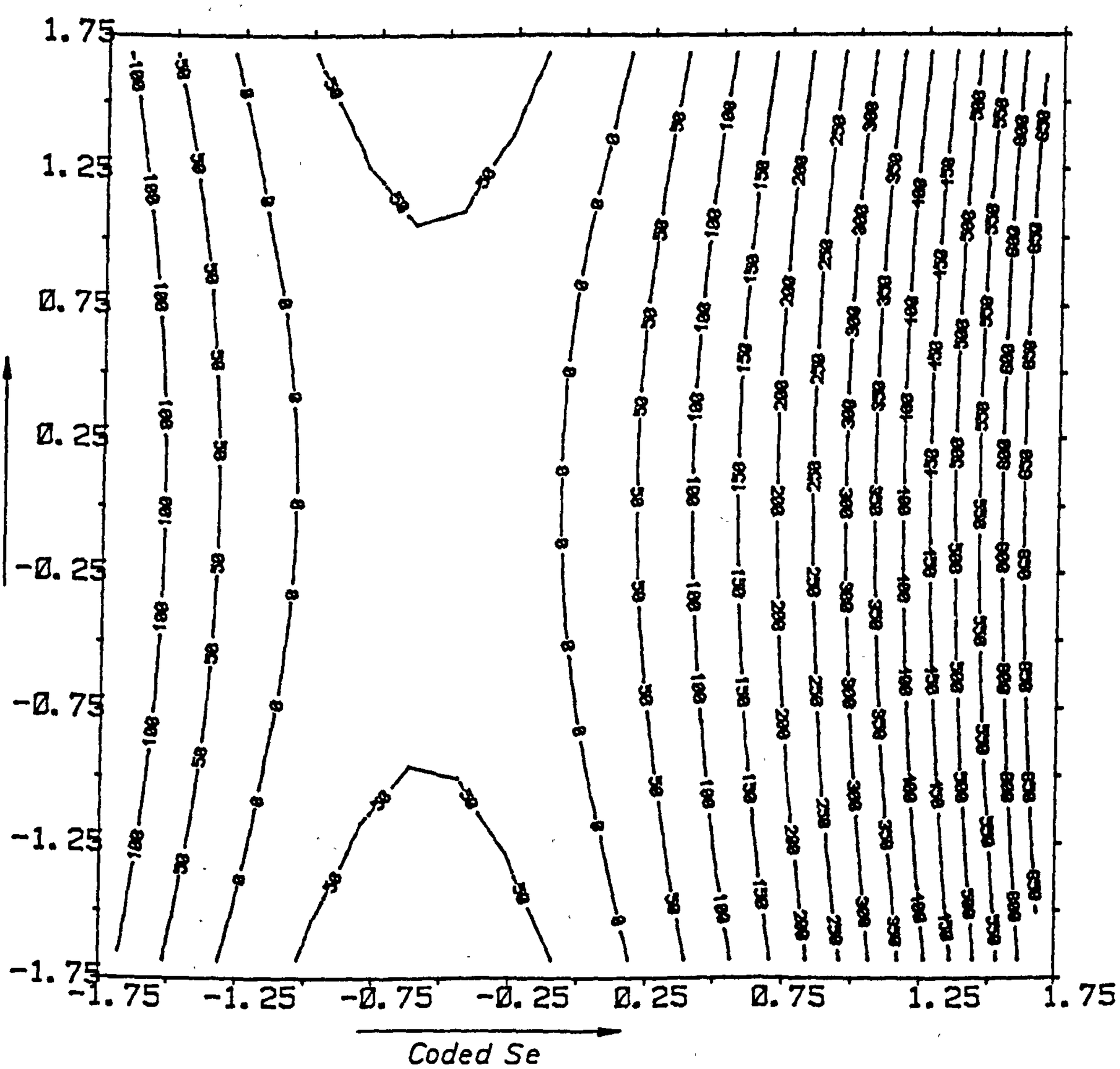
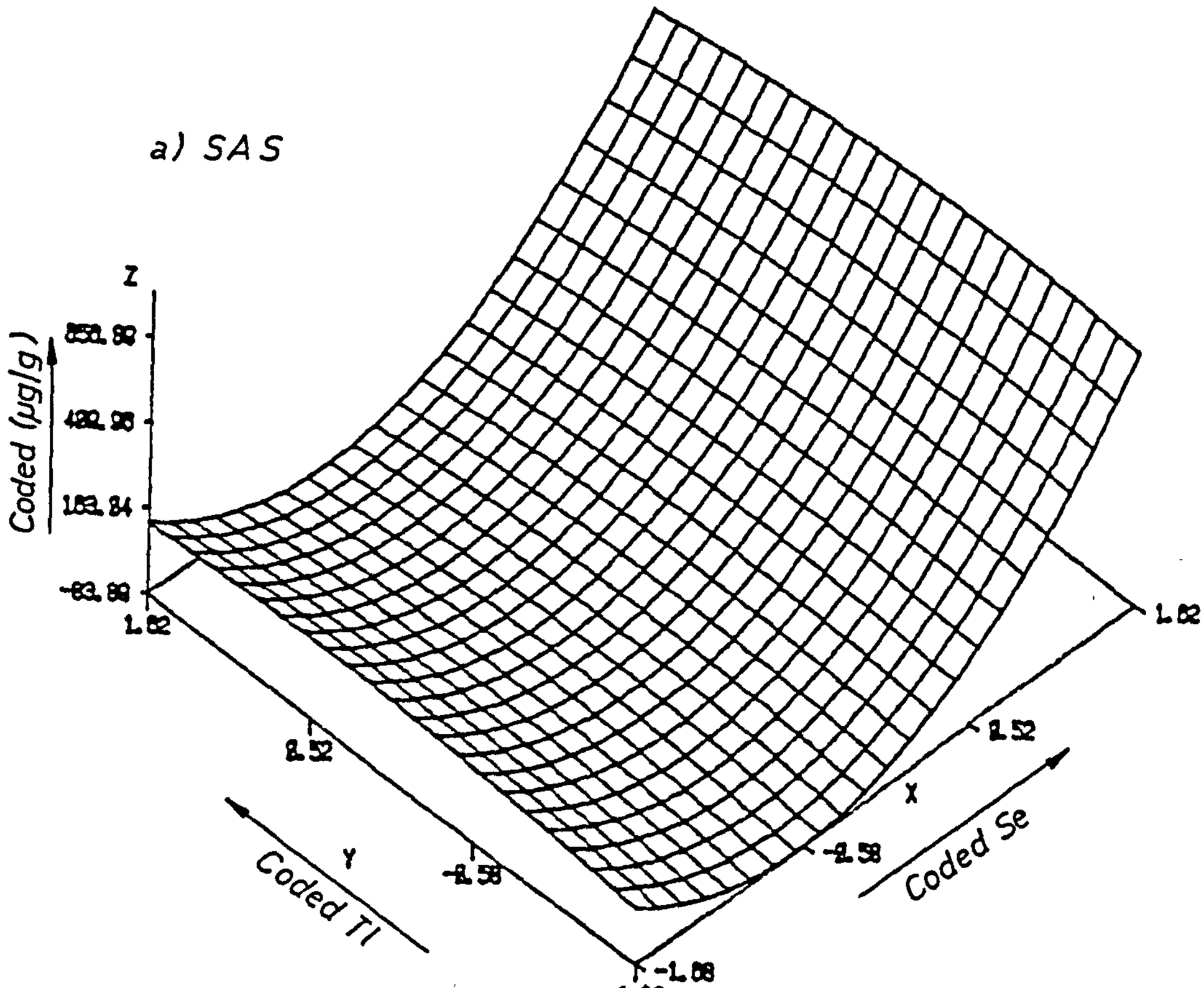
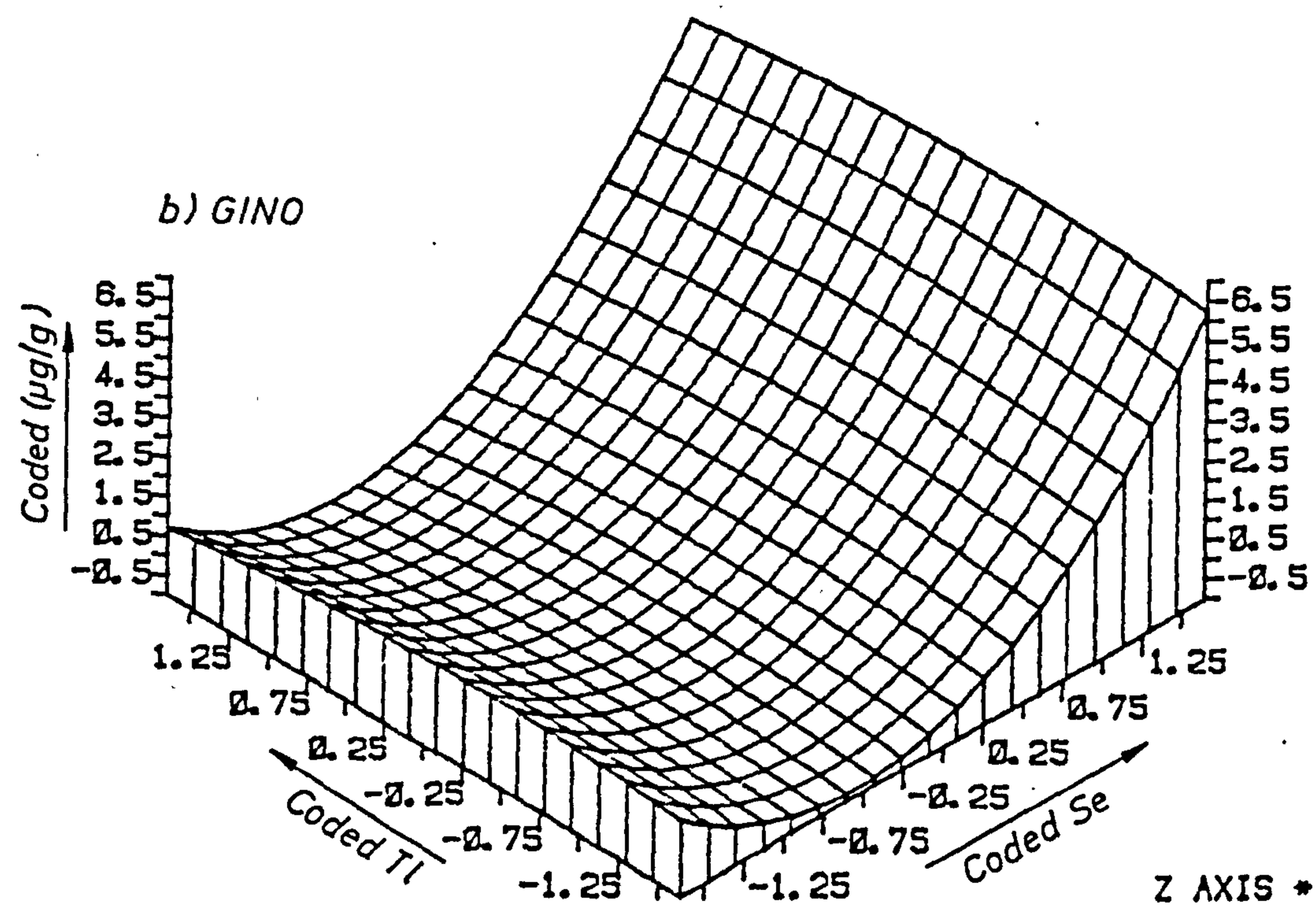


FIG. 6.18 RESPONSE SURFACE FOR SELENIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

a) SAS



b) GINO



Se(VI). Tl(I). Se uptake by Shoot

FIG. 6.19 CONTOUR PLOT OF RESPONSE SURFACE FOR SELENIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

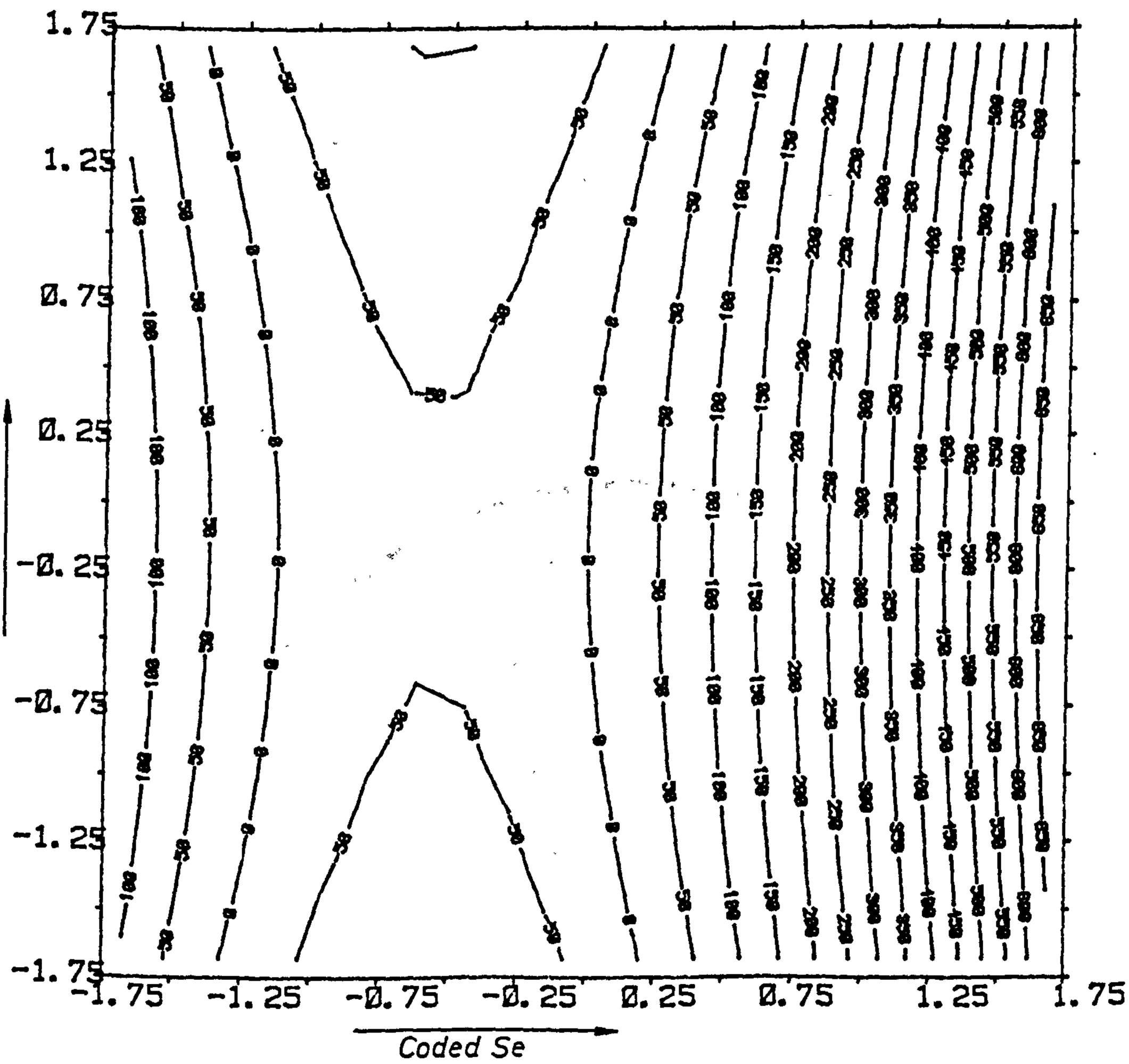
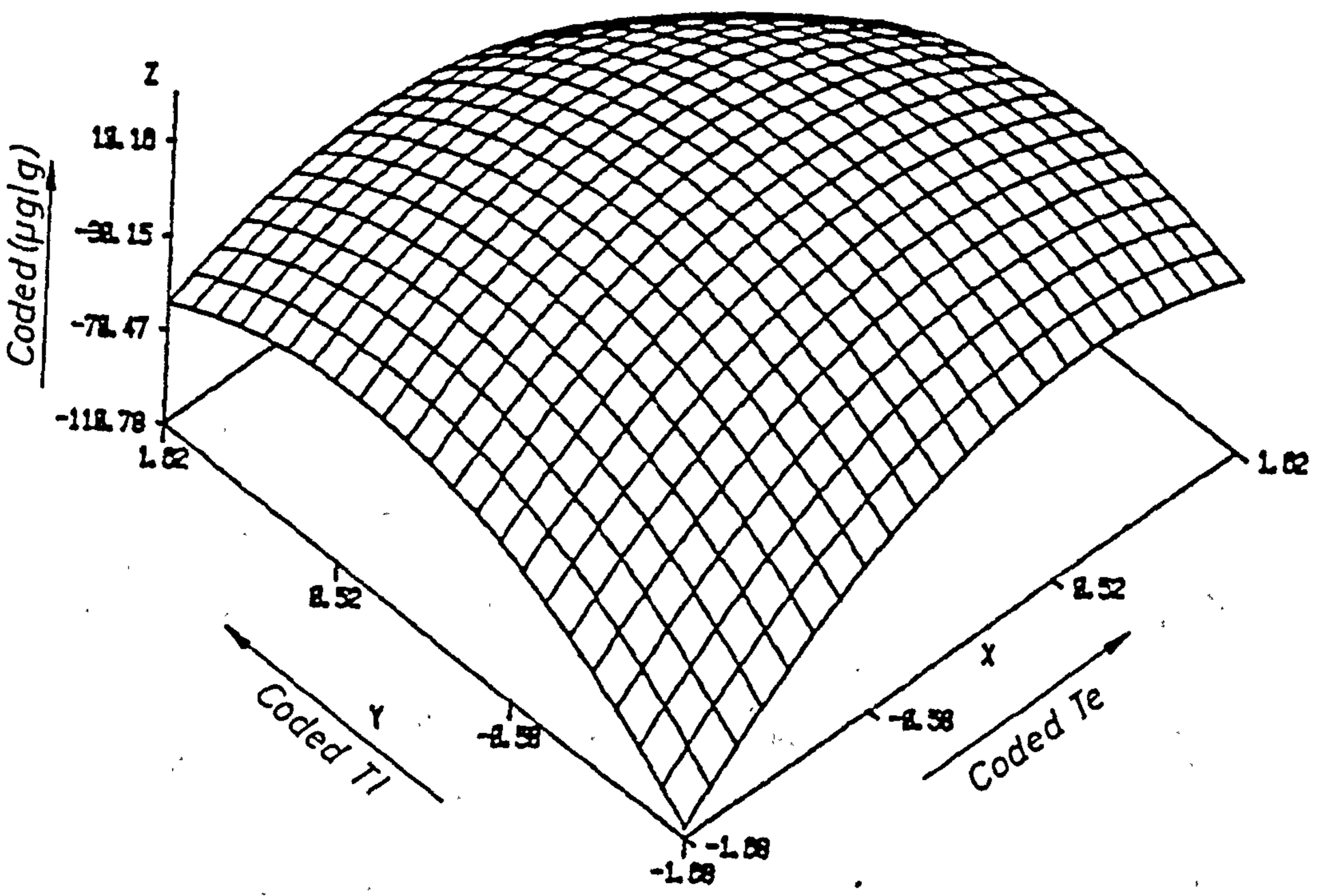
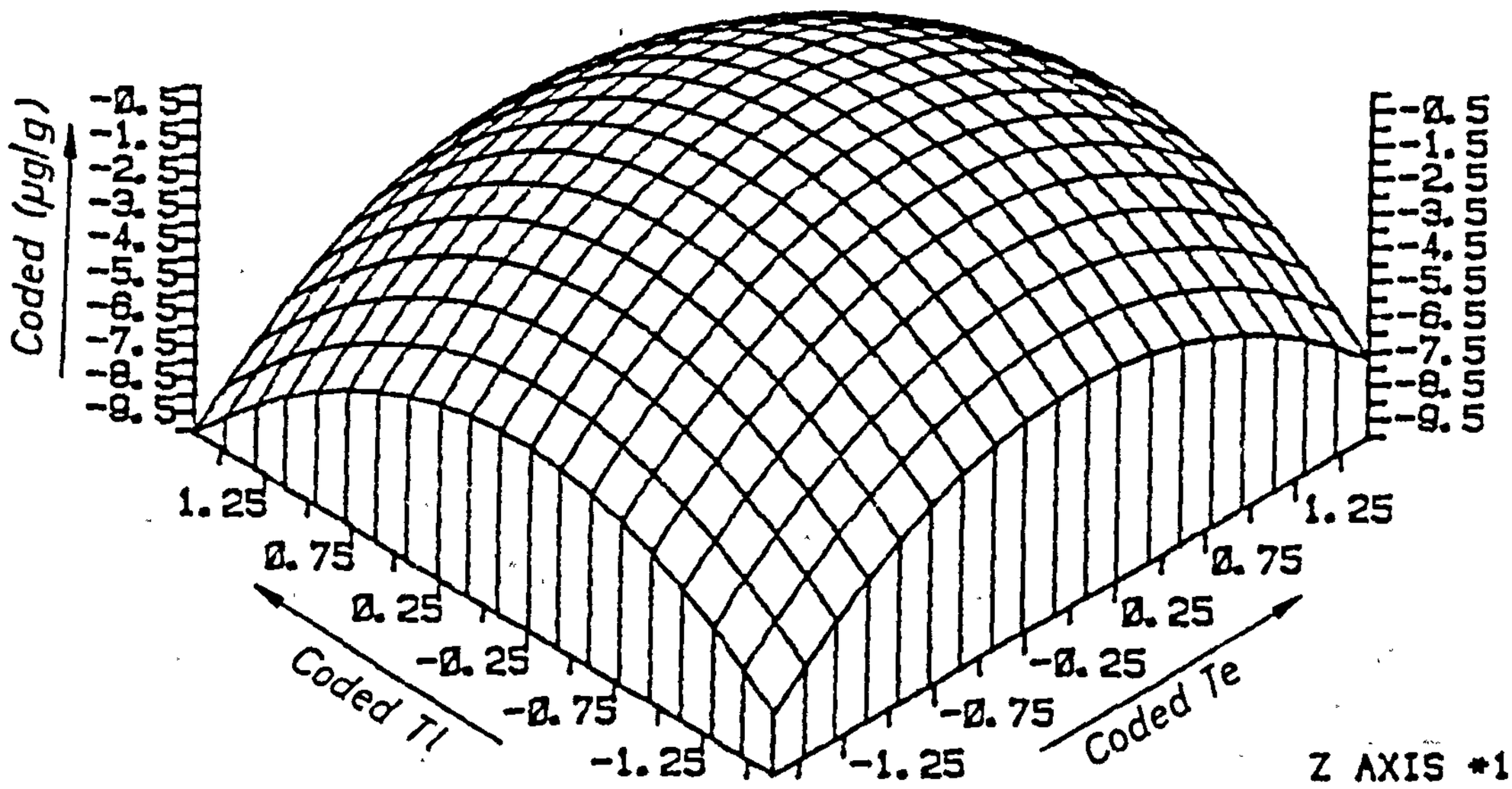


FIG. 6.20 RESPONSE SURFACE FOR SELENIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

a) SAS

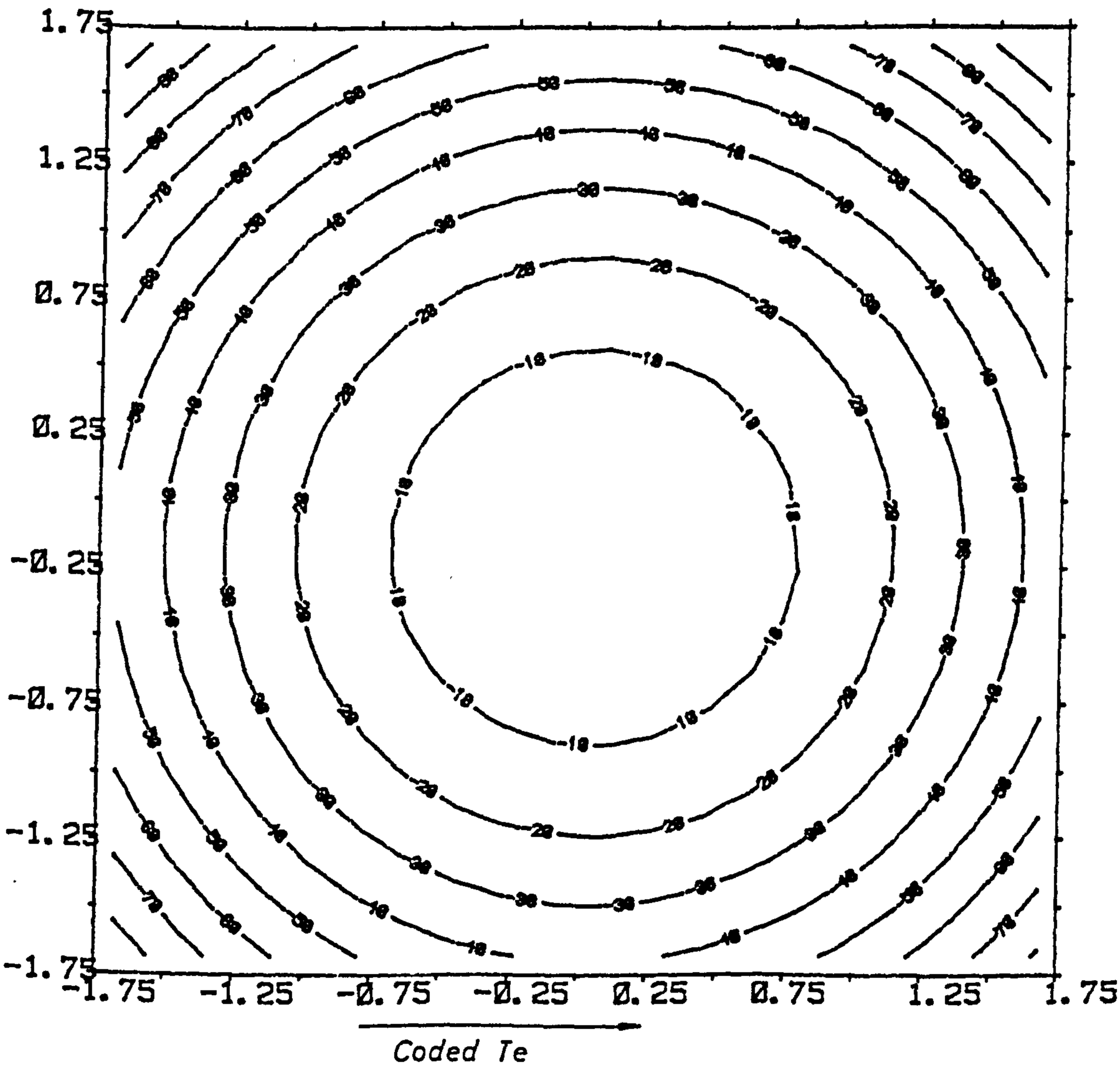


b) GINO



Te(VI), Tl(I), Se uptake by Shoot

FIG. 6.21 CONTOUR PLOT OF RESPONSE SURFACE FOR SELENIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0



should be - whilst the other two variables are not significant to any degree at all.

The responses are almost planar with respect to change - in either the Te(VI) or Tl(I) concentrations, i.e. no change across the Te(VI) or Tl(I) axes. In contrast the Se content increases dramatically as the Se concentration in the added medium is increased. Note the steepness of the Se content contours in Figures 6.17 and 6.21 once the concentration of Se(VI) in the nutrient solution rises above 0.10 µg/ml of Se(VI). The predicted value at the optimum is clearly in error because it is impossible to have a negative Se concentration in the tissue, obviously the value reflects the poorness of fit of the data to the model.

The Te uptake by the seedlings is similar to that just described for the Se uptake. The only variable of importance is the Te(VI) content of the nutrient solution, there being no effect due to the concentrations of Se(VI) or Tl(I) in the solution. An inspection of the PROB values [17] will confirm these facts; where only the linear Te(VI) and quadratic Te(VI)*Te(VI) are deemed significant. As expected they are significant at greater than 99.99% level, whilst, overall, the Te(VI) content is also greater than 99.99% significant [18].

The lack of fit of the model is deemed highly significant because of the extremely small value of the Type 1 SS for the pure error values. When an extremely low value of experimental error is recorded, because of the method of calculating the lack of fit (being the difference between Total Error and Pure Error), it will always return a highly significant value.

In a similar manner the Tl(I) content of the shoot tissue is only dependent on the Tl(I) concentration in the treatment solution. Very similar values for the probabilities and the lack of interaction between the Se(VI), Te(VI) and Tl(I) is to be noted. The probability of there being no effect of the Se(VI) and Te(VI) on the Tl(I) uptake is greater than 0.97 in each case, with an R^2 value of greater than 0.80 for the overall coefficient of determination.

TABLE 6.15 (a):

RESPONSE SURFACE FOR VARIABLE Y3 TELLURIUM UPTAKE BY SHOOT (Se, Te, Tl)

RESPONSE MEAN	73.08
ROOT MSE	156.4761
R-SQUARE	0.736164
COEF OF VARIATION	2.141161

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	317073.7	0.3417	4.32	0.0339
QUADRATIC	3	361685.5	0.3897	4.92	0.0236
CROSSPRODUCT	3	4422.651	0.0048	0.06	0.9796
TOTAL REGRESS	9	683181.9	0.7362	3.10	0.0463

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	244847.2	48969.45680	603.890	0.0001
PURE ERROR	5	0.35975	0.07195		
TOTAL ERROR	10	244847.6	24484.76		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	6.436917	63.81392	0.10	0.9216
X1	1	0.7624883	42.36082	0.02	0.9860
X2	1	152.0022	42.36082	3.59	0.0049
X3	1	-11.5082	42.36082	-0.27	0.7914
X1*X1	1	-26.308	41.28463	-0.64	0.5383
X2*X1	1	1.6475	55.32265	0.03	0.9768
X2*X2	1	148.9063	41.28463	3.61	0.0048
X3*X1	1	-10.17	55.32265	-0.18	0.8578
X3*X2	1	-21.135	55.32265	-0.38	0.7104
X3*X3	1	-24.9156	41.28463	-0.60	0.5598

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	10799.57	2699.892	0.11	0.9761
X2	4	637380.4	159345.1	6.51	0.0076
X3	4	15125.91	3781.477	0.15	0.9566

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE	
X1	0.001240093	= 0.102 µg/ml Se
X2	-0.511416	= 0.082 µg/ml Te
X3	-0.014289	= 0.0497 µg/ml Tl

PREDICTED VALUE AT OPTIMUM -32.3486

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
149.5537	0.006428868	0.9981388	-0.060644
-20.8954	-0.679928	0.04883232	0.7316508
-30.9756	0.7332504	0.03652992	0.6789768

SOLUTION WAS A SADDLE POINT

TABLE 6.15(b) Y3 TELLURIUM UPTAKE BY SHOOT (Se,Te,Tl)

```
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 -- read 'AdlyTe' c1-c8
```

column	c1	c2	c3	c4	c5	c6
count	20	20	20	20	20	20
row						
1	-1.00000	-1.00000	-1.00000	18.1600	4.210	2.730
2	1.00000	-1.00000	-1.00000	22.0800	166.660	3.030
3	-1.00000	1.00000	-1.00000	16.2500	4.000	126.140
4	-1.00000	-1.00000	1.00000	11.6600	2.730	3.100
. . .						

column	c7	c8
count	20	20
row		
1	1.310	0.329500
2	2.740	0.562500
3	1.550	0.383500
4	39.840	0.305000
. . .		

```
 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c6 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    6.44 + 0.762 x1 +  152. x2
    -  11.5 x3 -  26.3 x4 +  1.65 x5
    -  10.2 x6 +  149. x7 -  21.1 x8
    -  24.9 x 9
```

	column	coefficient	st. dev. of coef.	t-ratio = coef/s.d.
	--	6.44	63.81	0.10
x1	c1	0.76	42.36	0.02
x2	c2	152.00	42.36	3.59
x3	c3	-11.51	42.36	-0.27
x4	c11	-26.31	41.28	-0.64
x5	c12	1.65	55.32	0.03
x6	c13	-10.17	55.32	-0.18
x7	c22	148.91	41.28	3.61
x8	c23	-21.13	55.32	-0.38
x9	c33	-24.92	41.28	-0.60

```
the st. dev. of y about regression line is
s = 156.5

with (  20-10) =  10 degrees of freedom

r-squared = 73.6 percent
r-squared = 49.9 percent, adjusted for d.f.

analysis of variance
```

due to	df	ss	ms=ss/df
regression	9	683182	75909
residual	10	244848	24485
total	19	928029	

```
further analysis of variance
ss explained by each variable when entered in the order given
```

due to	df	ss
regression	9	683182
c1	1	8
c2	1	315259
c3	1	1807
c11	1	20462
c12	1	22
c13	1	827
c22	1	332305
c23	1	3574
c33	1	8918

row	x1 c1	y c6	pred. y value	st.dev. pred. y	residual	st.res.
11	0.00	990.1	682.1	121.9	308.0	3.14r

```
r denotes an obs. with a large st. res.

durbin-watson statistic = 2.38
 -- end
 -- stop

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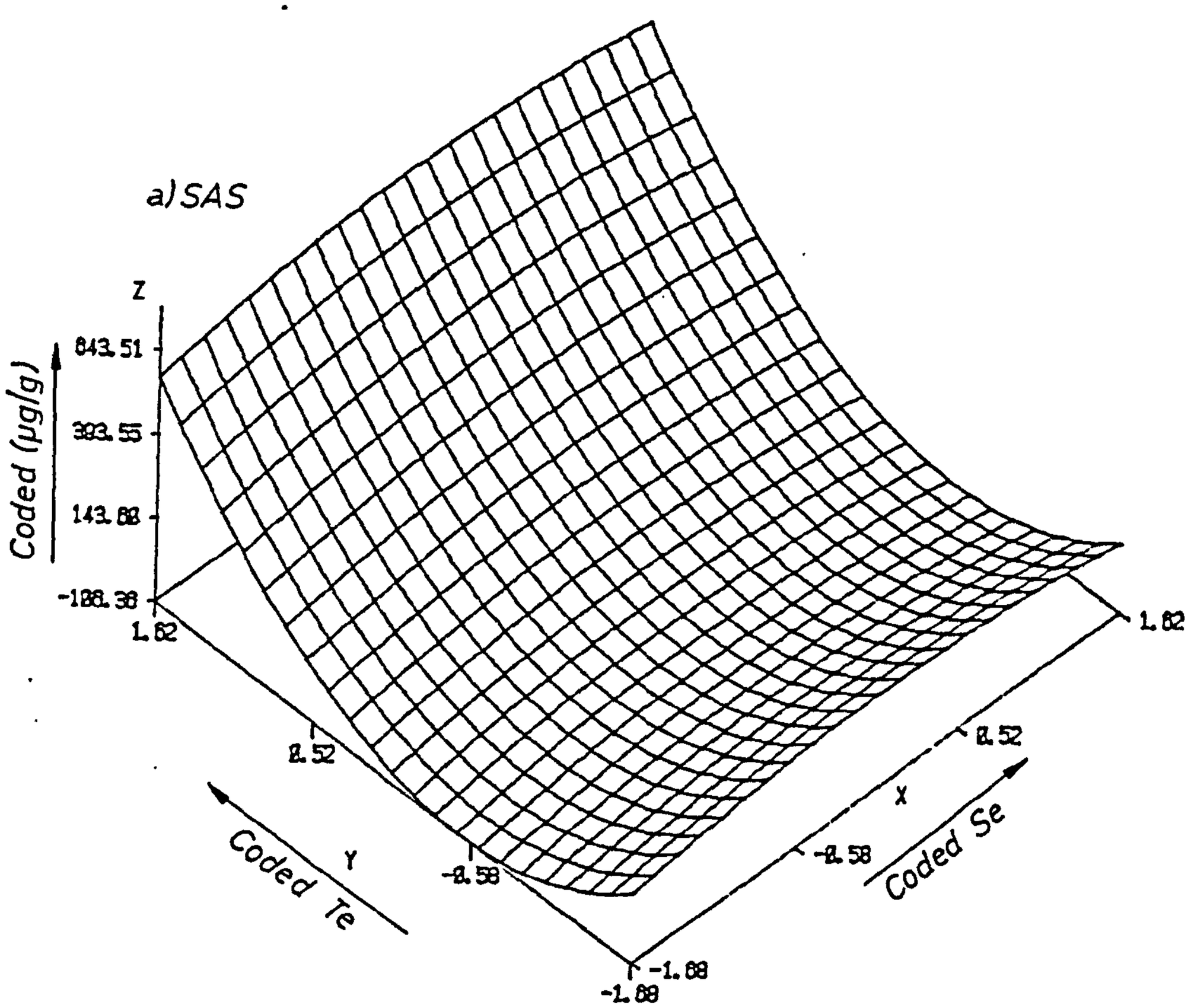
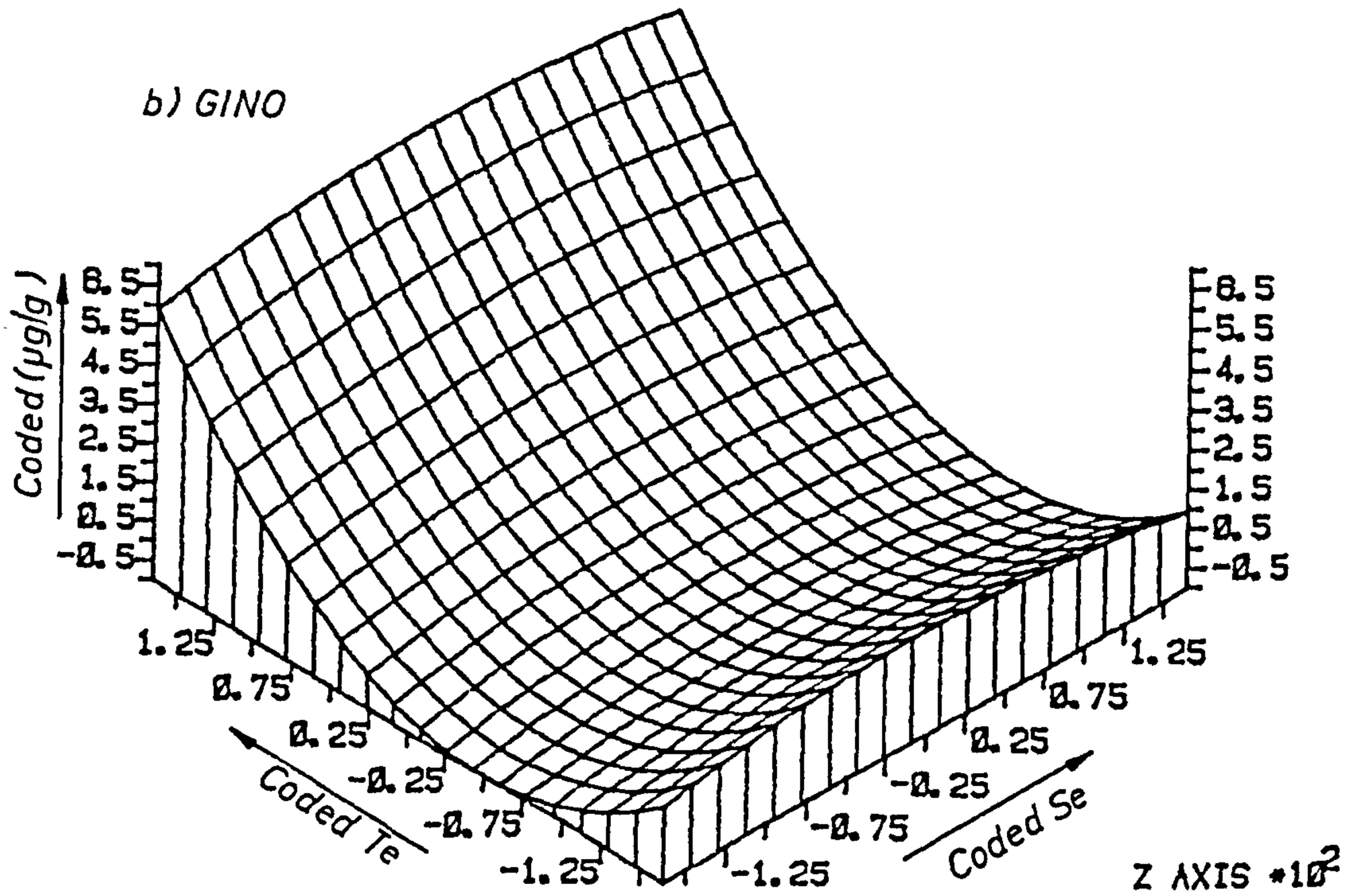
```

FIG. 6.22 RESPONSE SURFACE FOR TELLURIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0

a) SAS



b) GINO



Se(VI), Te(VI), Te uptake by Shoot

FIG. 6.23 CONTOUR PLOT OF RESPONSE SURFACE FOR TELLURIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Ti = 0

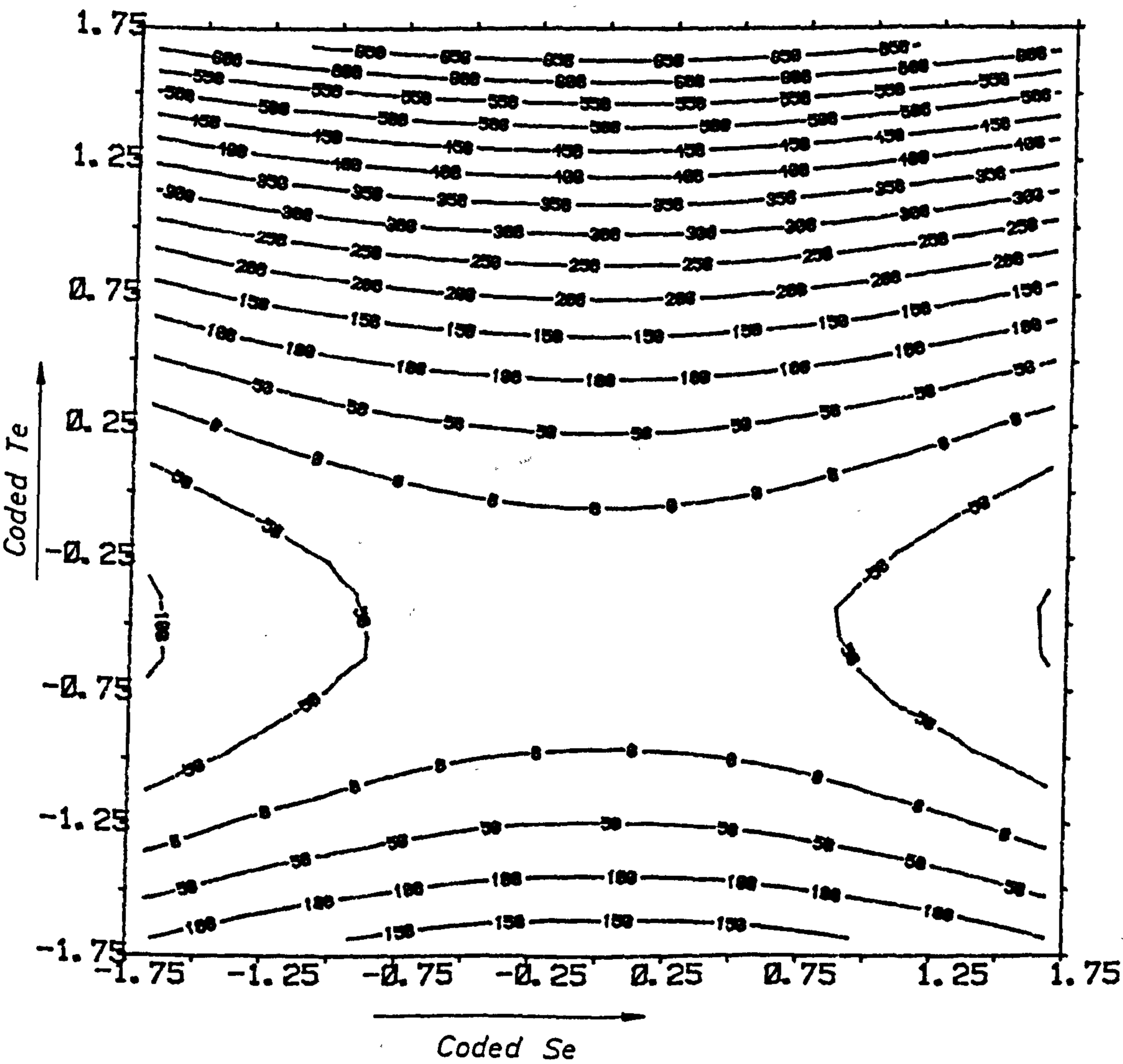
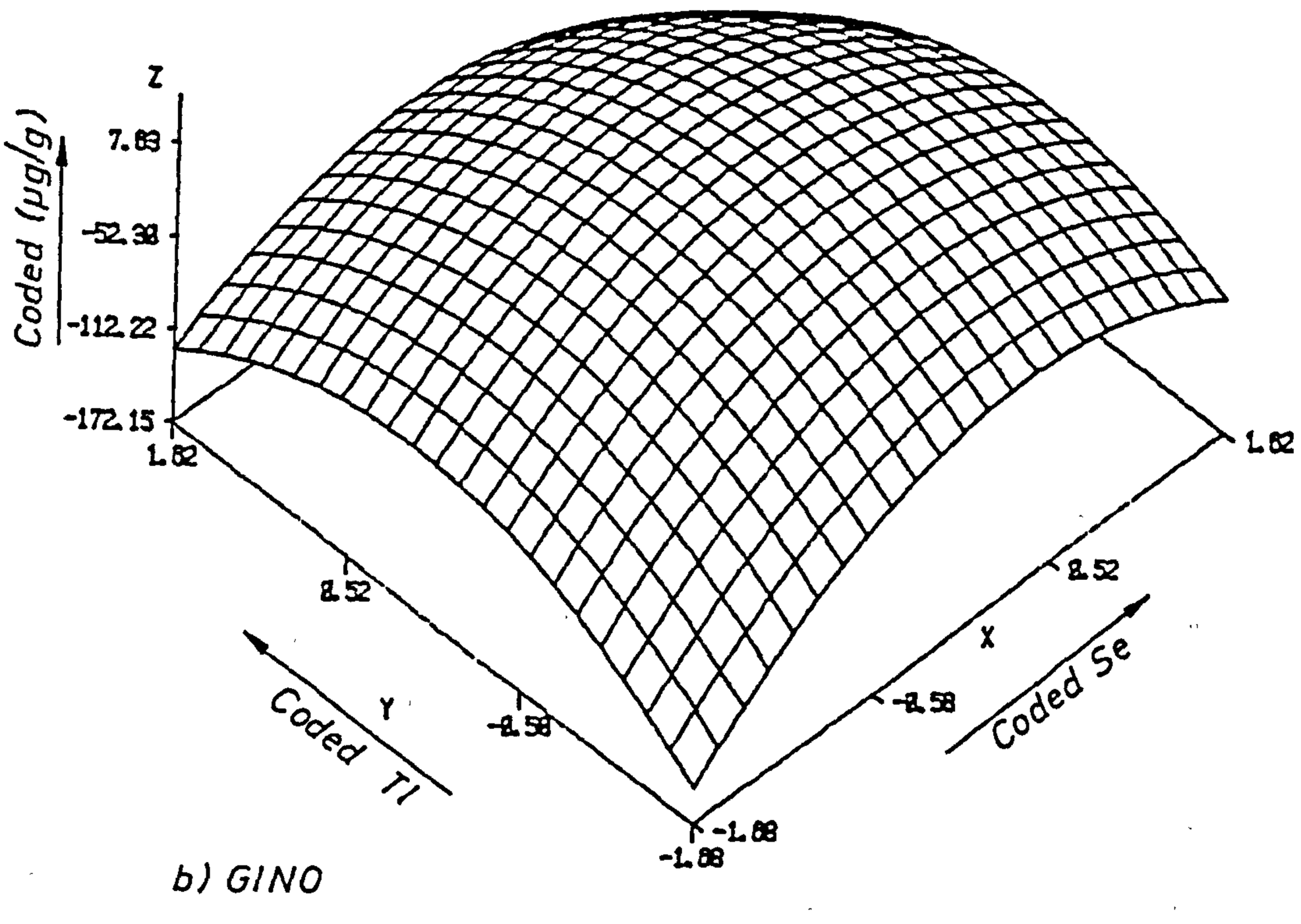
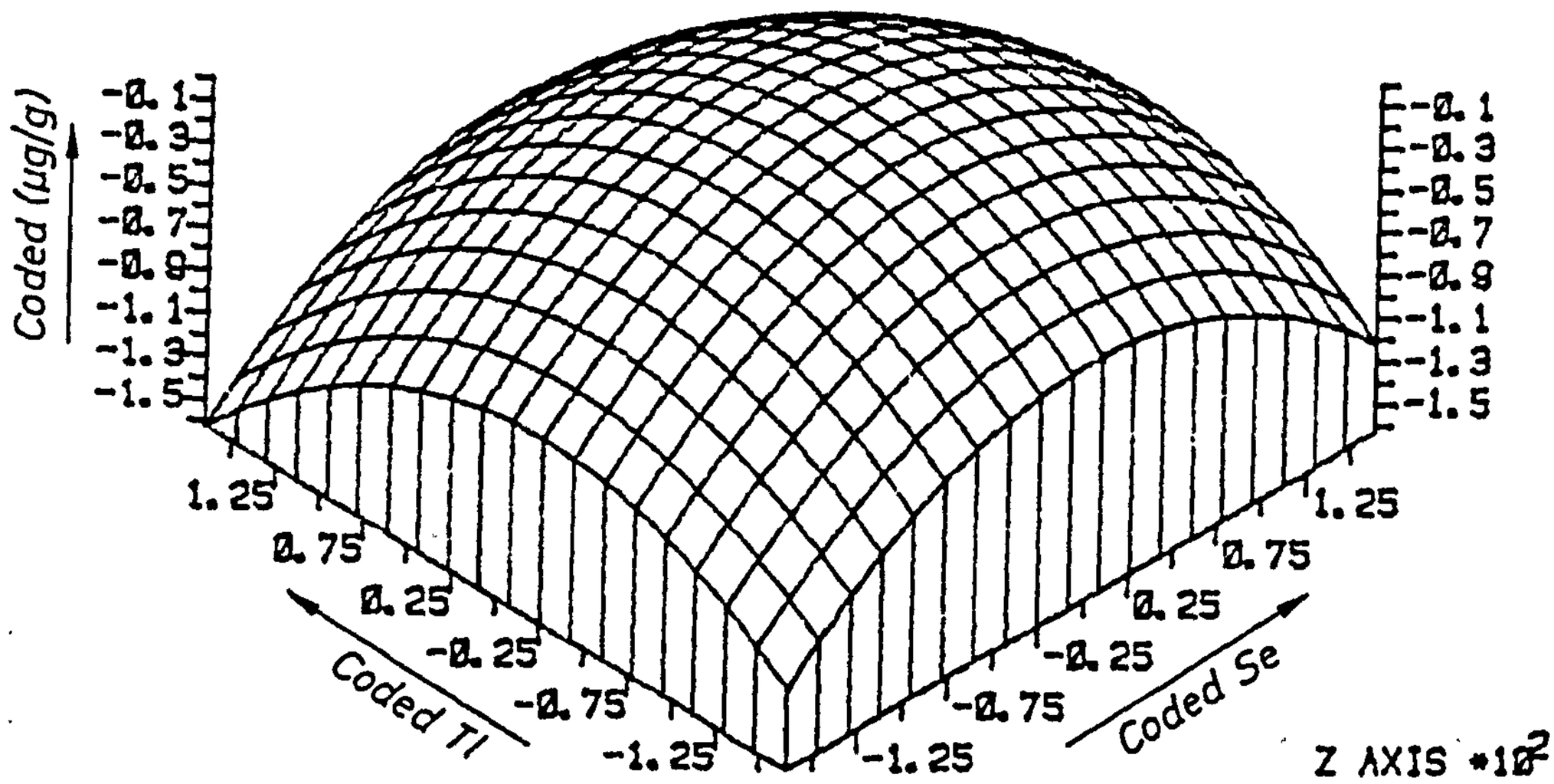


FIG. 6.24 RESPONSE SURFACE FOR TELLURIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

a) SAS



b) GINO



Se(VI), Tl(I), Te uptake by Shoot

FIG. 6.25 CONTOUR PLOT OF RESPONSE SURFACE FOR TELLURIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

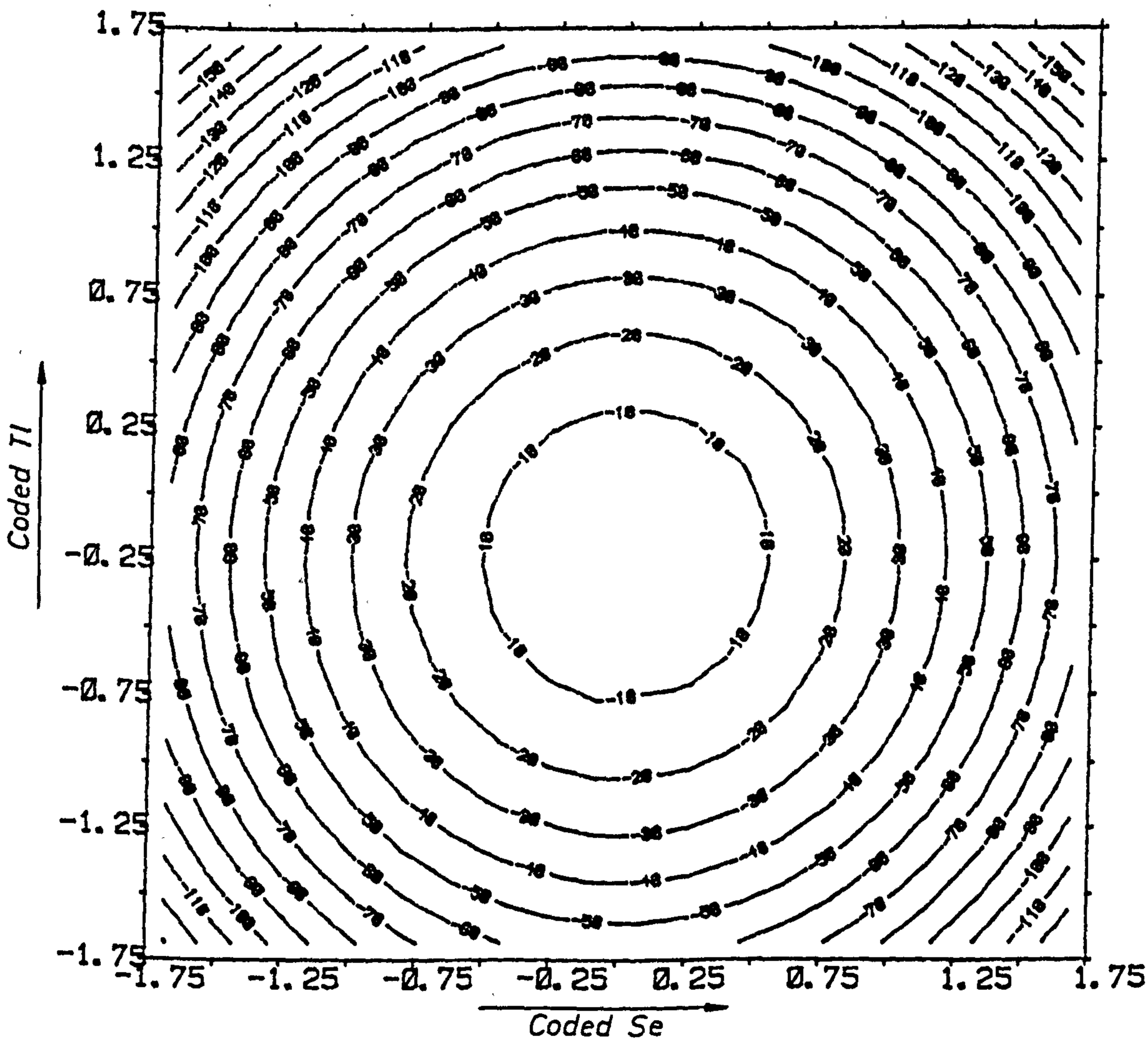
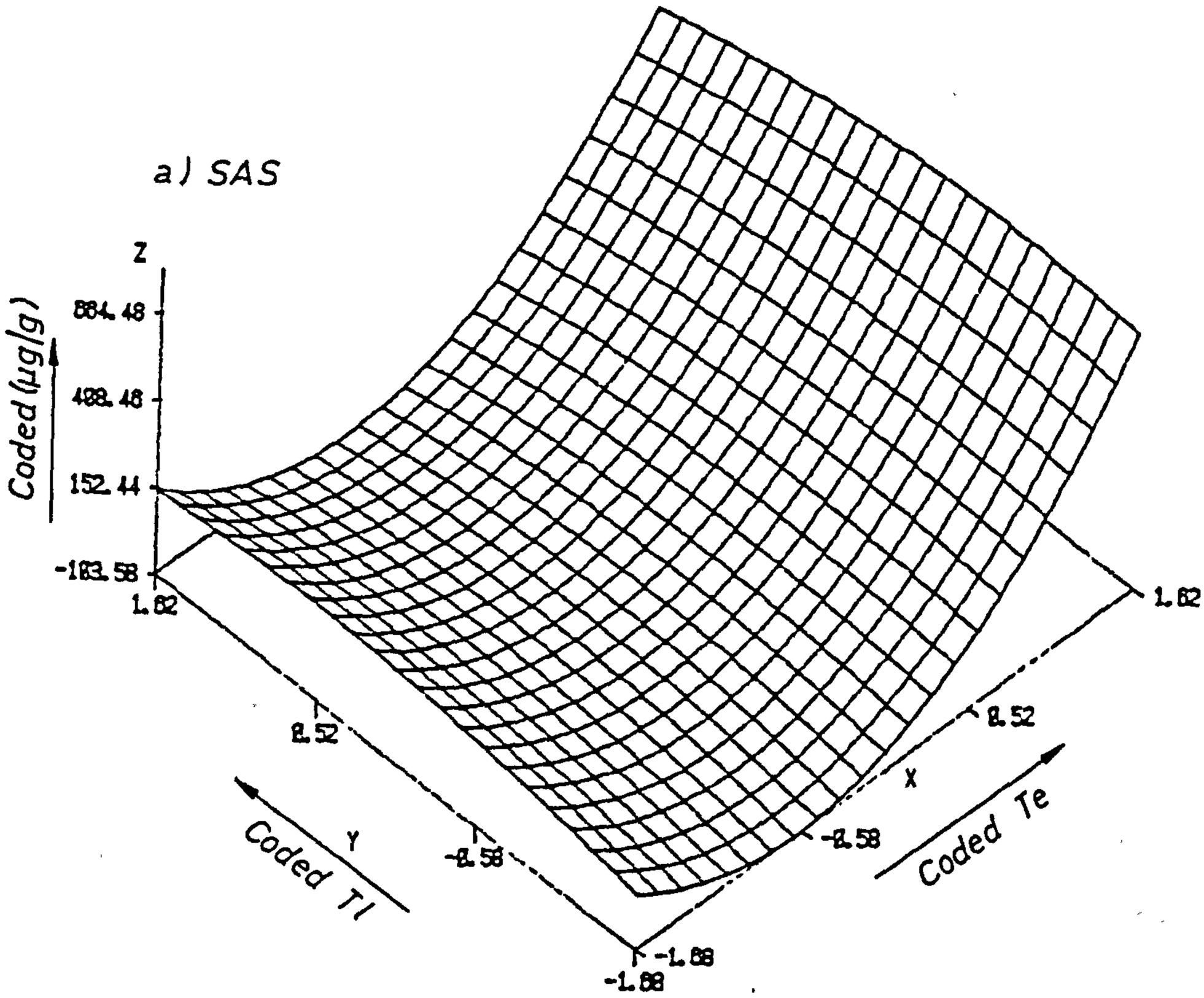
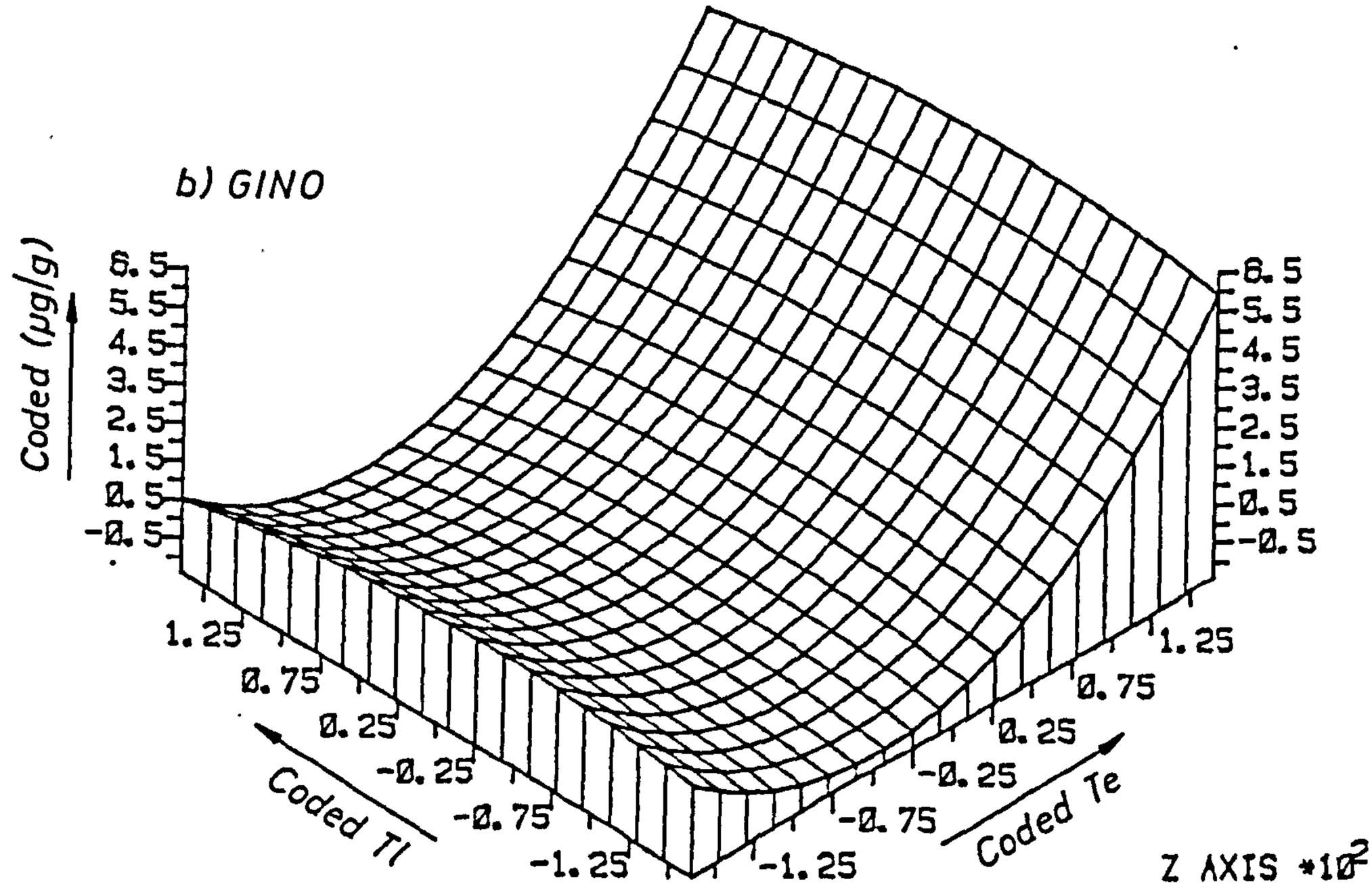


FIG. 6.26 RESPONSE SURFACE FOR TELLURIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

a) SAS



b) GINO



Te(VI), Tl(I), Te uptake by Shoot

FIG. 6.27 CONTOUR PLOT OF RESPONSE SURFACE FOR TELLURIUM UPTAKE BY SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED Se = 0

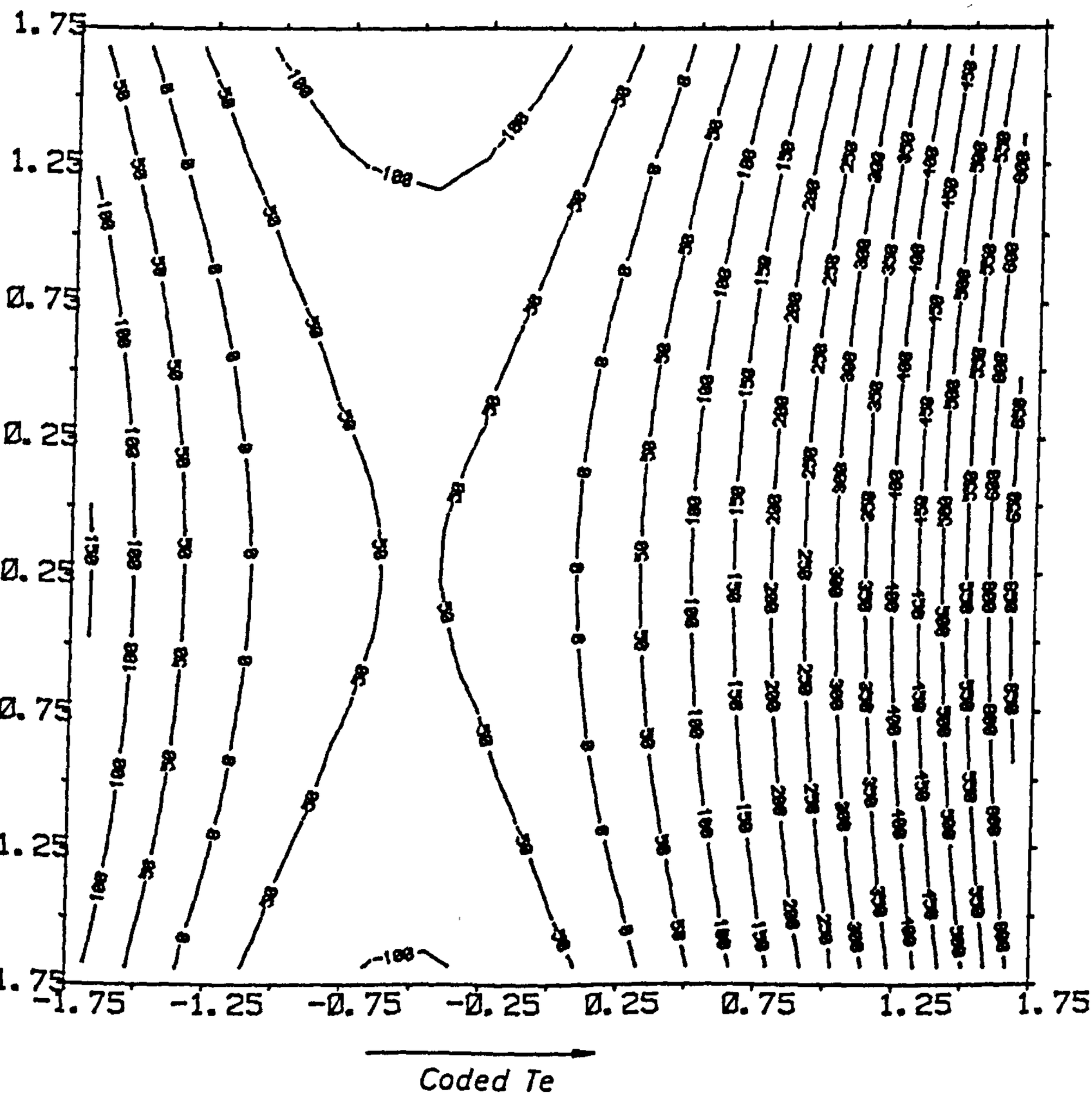


TABLE 6.16(a)

RESPONSE SURFACE FOR VARIABLE Y4 THALLIUM UPTAKE BY SHOOT (Se, Te, Tl)

RESPONSE MEAN	18.644
ROOT MSE	27.70529
R-SQUARE	0.8065814
COEF OF VARIATION	1.486017

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	16288.84	0.4105	7.07	0.0078
QUADRATIC	3	15629.28	0.3938	6.79	0.0089
CROSSPRODUCT	3	91.13645	0.0023	0.04	0.9888
TOTAL REGRESS	9	32009.26	0.8066	4.63	0.0126

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	7673.576	1534.715	3399.976	0.0001
PURE ERROR	5	2.25695	0.45139		
TOTAL ERROR	10	7875.833	787.5833		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	2.659672	11.29875	0.24	0.8187
X1	1	0.4335718	7.500309	0.06	0.9550
X2	1	-2.26231	7.500309	-0.30	0.7691
X3	1	34.4742	7.500309	4.60	0.0010
X1*X1	1	-4.23617	7.309762	-0.58	0.5751
X2*X1	1	-0.4425	9.795301	-0.05	0.9649
X2*X2	1	-3.85174	7.309762	-0.53	0.6097
X3*X1	1	0.455	9.795301	0.05	0.9639
X3*X2	1	-3.315	9.795301	-0.34	0.7420
X3*X3	1	31.51709	7.309762	4.31	0.0015

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	263.577	65.89426	0.09	0.9849
X2	4	372.4396	93.1099	0.12	0.9716
X3	4	30575.6	7643.9	9.96	0.0016

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE	
X1	0.0246764	= 0.136 µg/ml Se
X2	-0.0583442	= 0.098 µg/ml Te
X3	-0.550159	= 0.040 µg/ml Tl

PREDICTED VALUE AT OPTIMUM -6.75213

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
31.59618	0.006630582	-0.046748	0.9988847
-3.82263	-0.452047	0.8908739	0.04469373
-4.34436	0.8919696	0.4518389	0.01522526

SOLUTION WAS A SADDLE POINT

TABLE 6.16(b) Y4 THALLIUM UPTAKE BY SHOOT (Se, Te, Tl)

```
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 -- read 'AdlyTl' c1-c8
```

column	c1	c2	c3	c4	c5	c6
count	20	20	20	20	20	20
row						
1	-1.00000	-1.00000	-1.00000	18.1600	4.210	2.730
2	1.00000	-1.00000	-1.00000	22.0800	166.660	3.030
3	-1.00000	1.00000	-1.00000	16.2500	4.000	126.140
4	-1.00000	-1.00000	1.00000	11.6600	2.730	3.100
. . .						

column	c7	c8
count	20	20
row		
1	1.310	0.329500
2	2.740	0.562500
3	1.550	0.383500
4	39.840	0.305000
. . .		

```
 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c7 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    2.66 + 0.434 x1 -  2.26 x2
     +  34.5 x3 -  4.24 x4 - 0.442 x5
     + 0.455 x6 -  3.85 x7 -  3.31 x8
     +  31.5 x 9
```

	column	coefficient	st. dev. of coef.	t-ratio = coef/s.d.
	--	2.66	11.30	0.24
x1	c1	0.434	7.500	0.06
x2	c2	-2.262	7.500	-0.30
x3	c3	34.474	7.500	4.60
x4	c11	-4.236	7.310	-0.58
x5	c12	-0.442	9.795	-0.05
x6	c13	0.455	9.795	0.05
x7	c22	-3.852	7.310	-0.53
x8	c23	-3.315	9.795	-0.34
x9	c33	31.517	7.310	4.31

```
the st. dev. of y about regression line is
s = 27.71

with (  20-10) =  10 degrees of freedom

r-squared = 80.7 percent
r-squared = 63.3 percent, adjusted for d.f.

analysis of variance
```

due to	df	ss	ms=ss/df
regression	9	32009.3	3556.6
residual	10	7675.8	767.6
total	19	39685.1	

```
further analysis of variance
ss explained by each variable when entered in the order given
```

due to	df	ss
regression	9	32009.3
c1	1	2.6
c2	1	69.8
c3	1	16216.4
c11	1	658.9
c12	1	1.6
c13	1	1.7
c22	1	700.8
c23	1	87.9
c33	1	14269.6

row	x1 c1	y c7	pred. y value	st.dev. pred. y	residual	st.res.
13	0.00	204.02	149.53	21.58	54.49	3.14r

```
r denotes an obs. with a large st. res.

durbin-watson statistic = 2.02
 -- end
 -- stop

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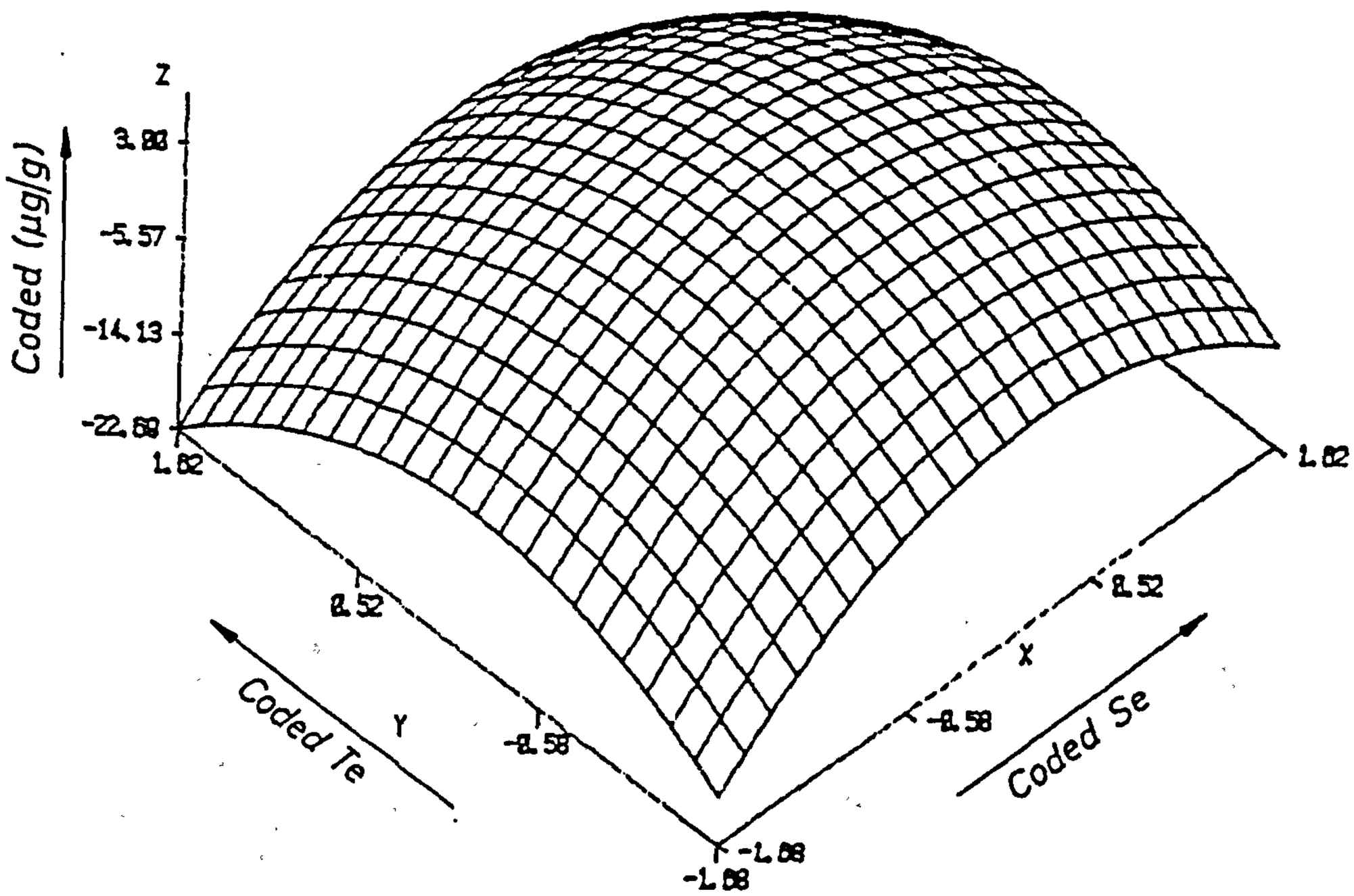
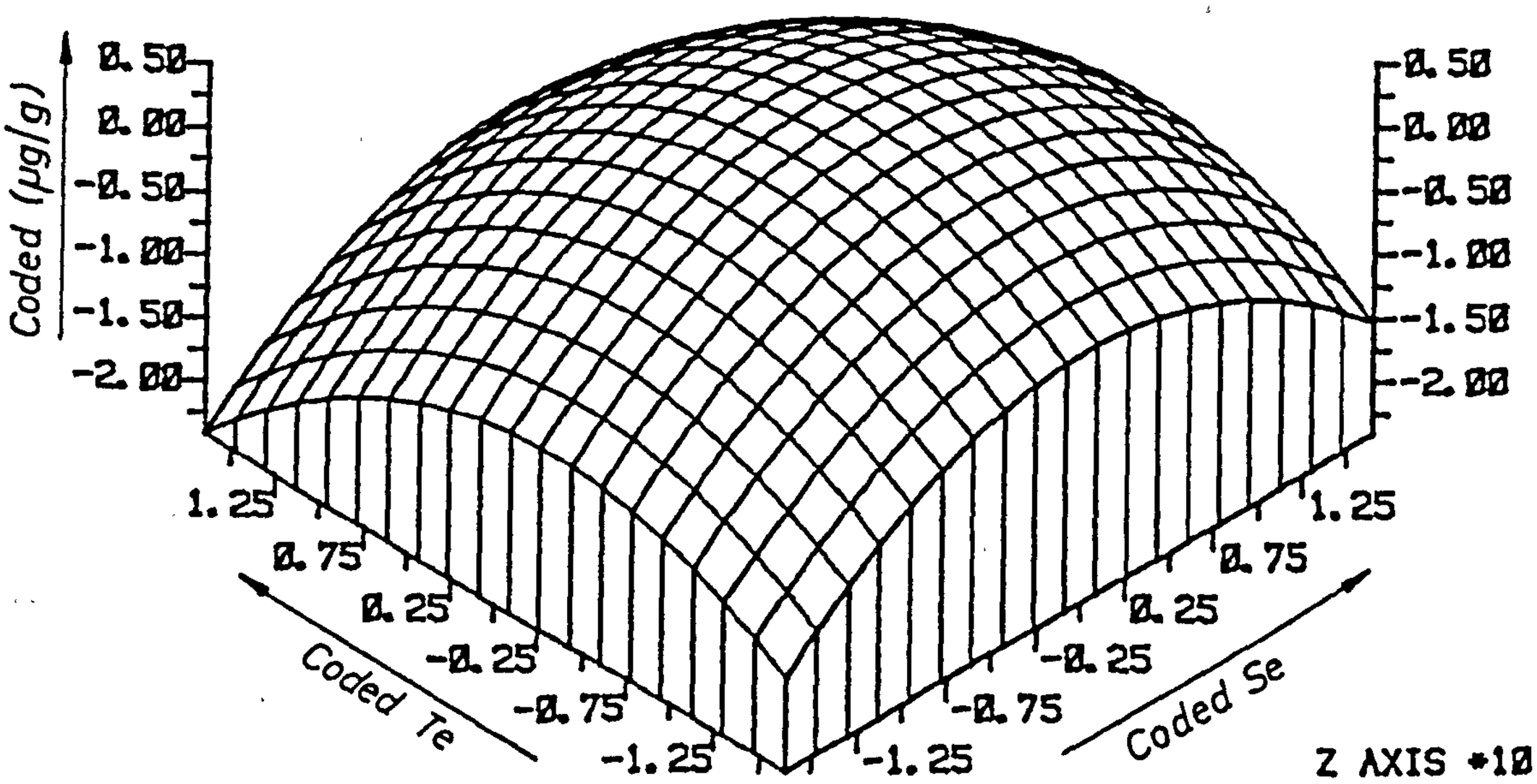
storage available   50000
```

FIG. 6.28 RESPONSE SURFACE FOR THALLIUM UPTAKE BY SHOOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0

(a) SAS



(b) GINO



Se(VI), Te(VI), Tl uptake by Shoot

FIG. 6.29 CONTOUR PLOT OF RESPONSE SURFACE FOR THALLIUM UPTAKE BY SHOOT OF LOLIUM PERENNE AT CODED TI = 0

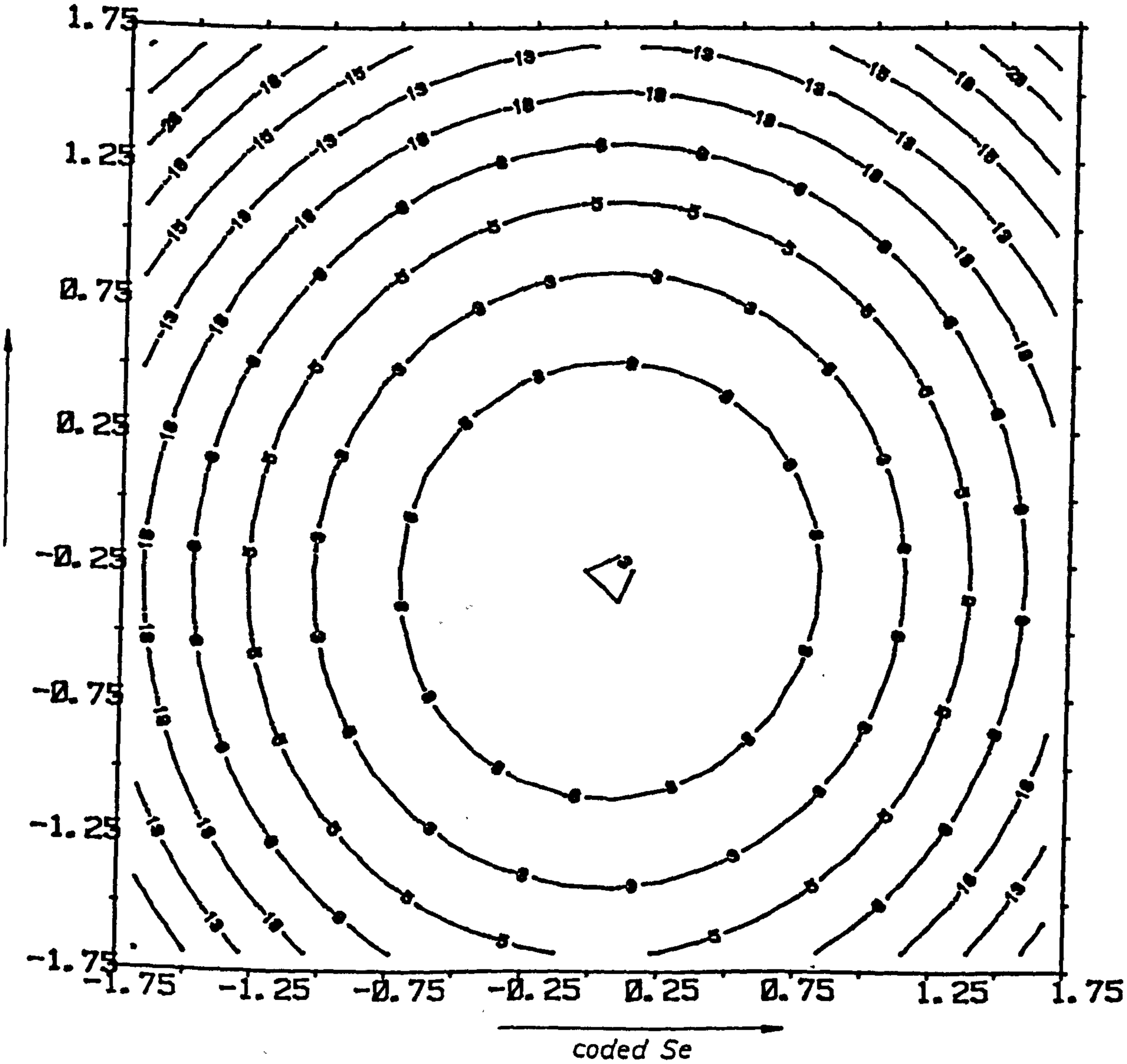
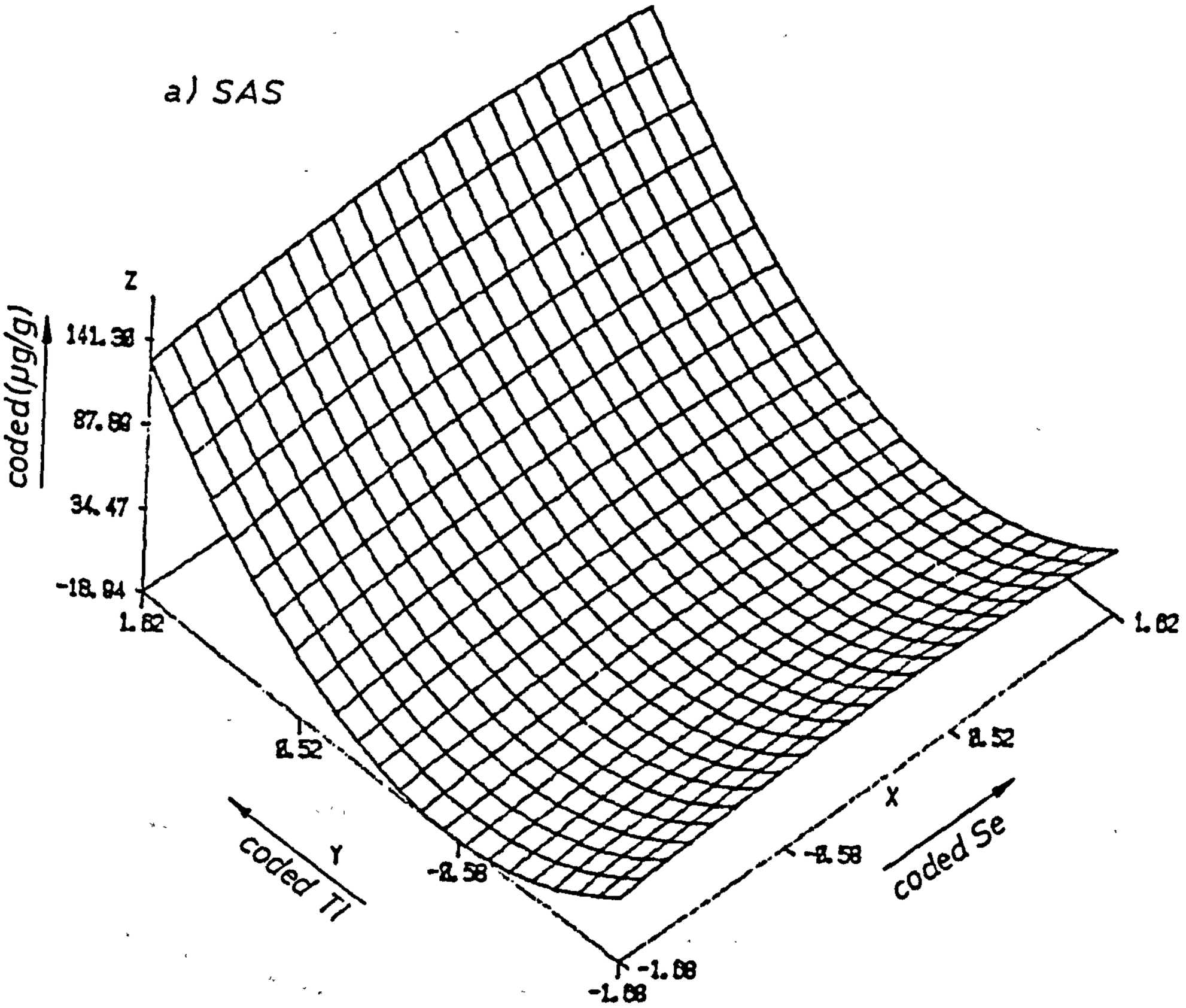
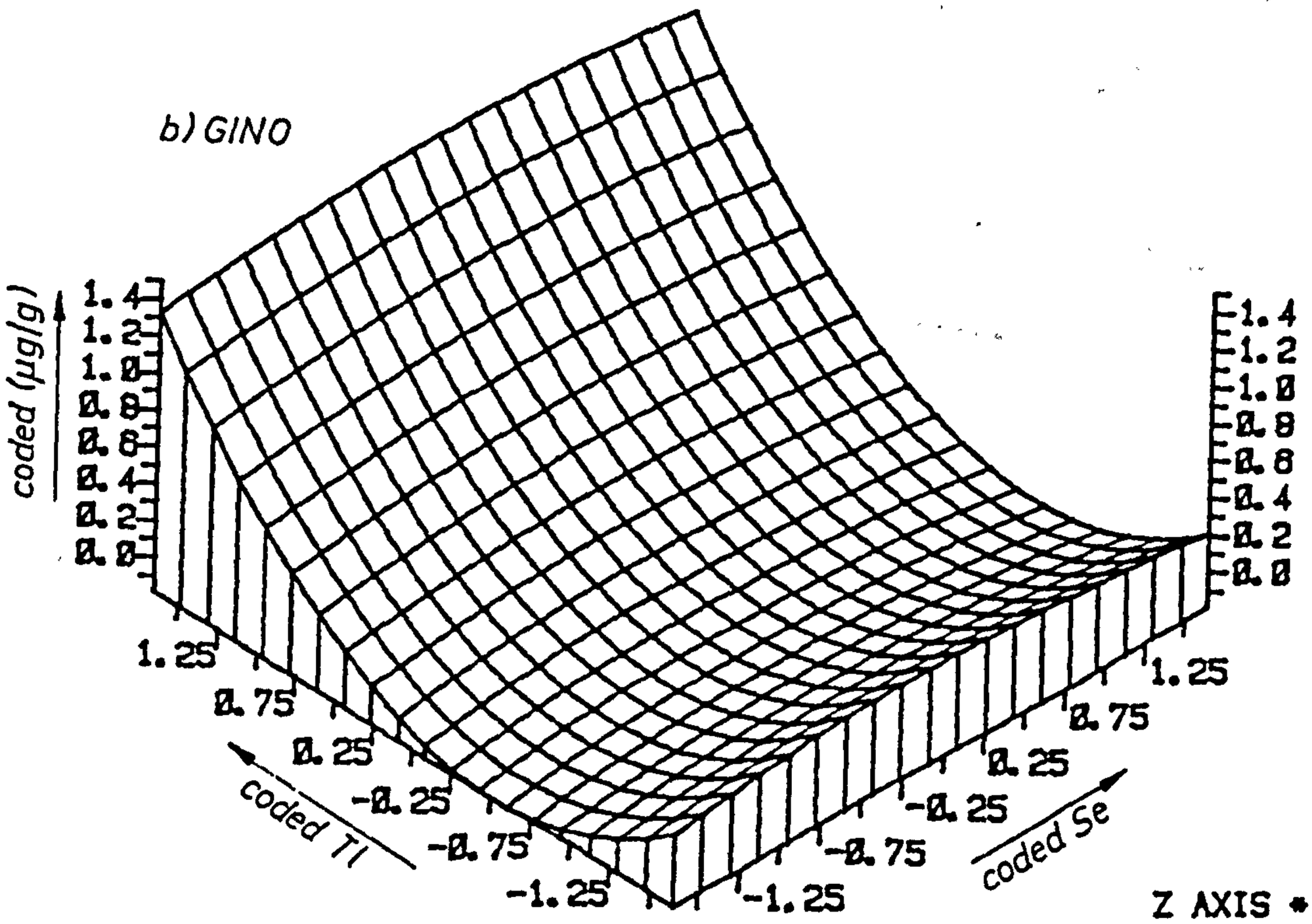


FIG. 6.30 RESPONSE SURFACE FOR THALLIUM UPTAKE BY SHOOT SEEDLINGS AT CODED Te = 0

a) SAS



b) GINO



Se(VI), Tl(I), Tl uptake by Shoot

FIG. 6.31 CONTOUR PLOT OF RESPONSE SURFACE FOR THALLIUM UPTAKE BY SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED Te = 0

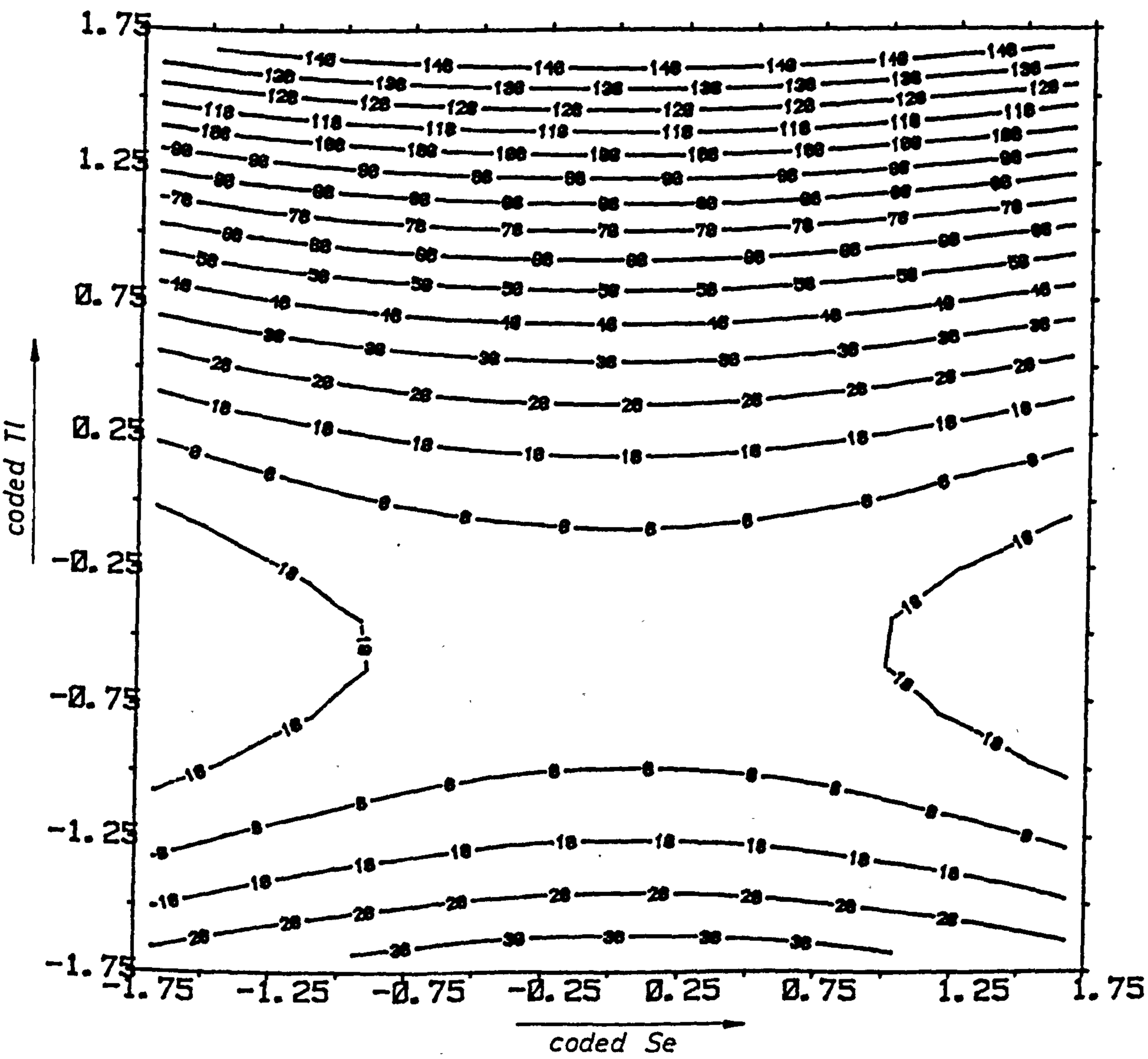
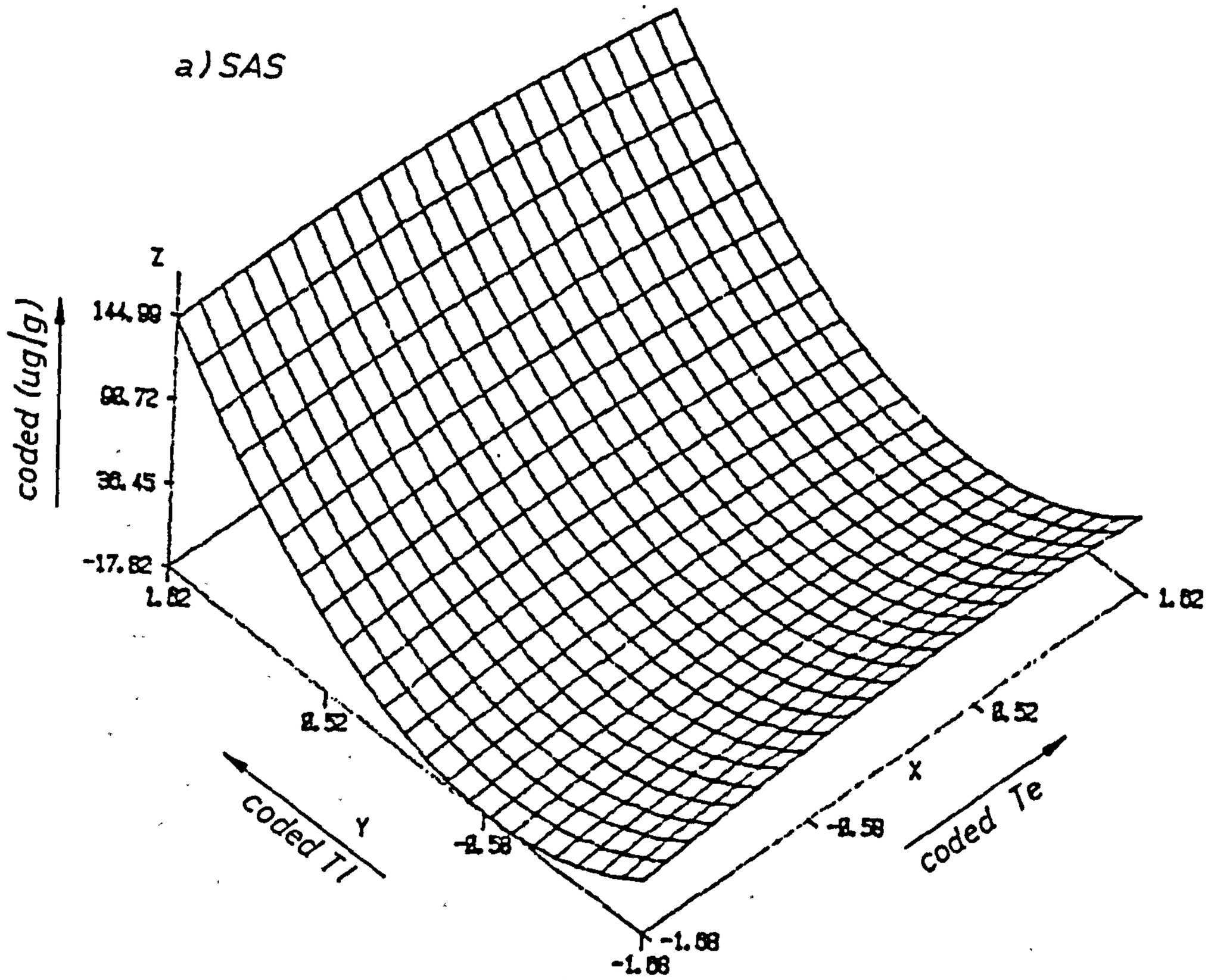
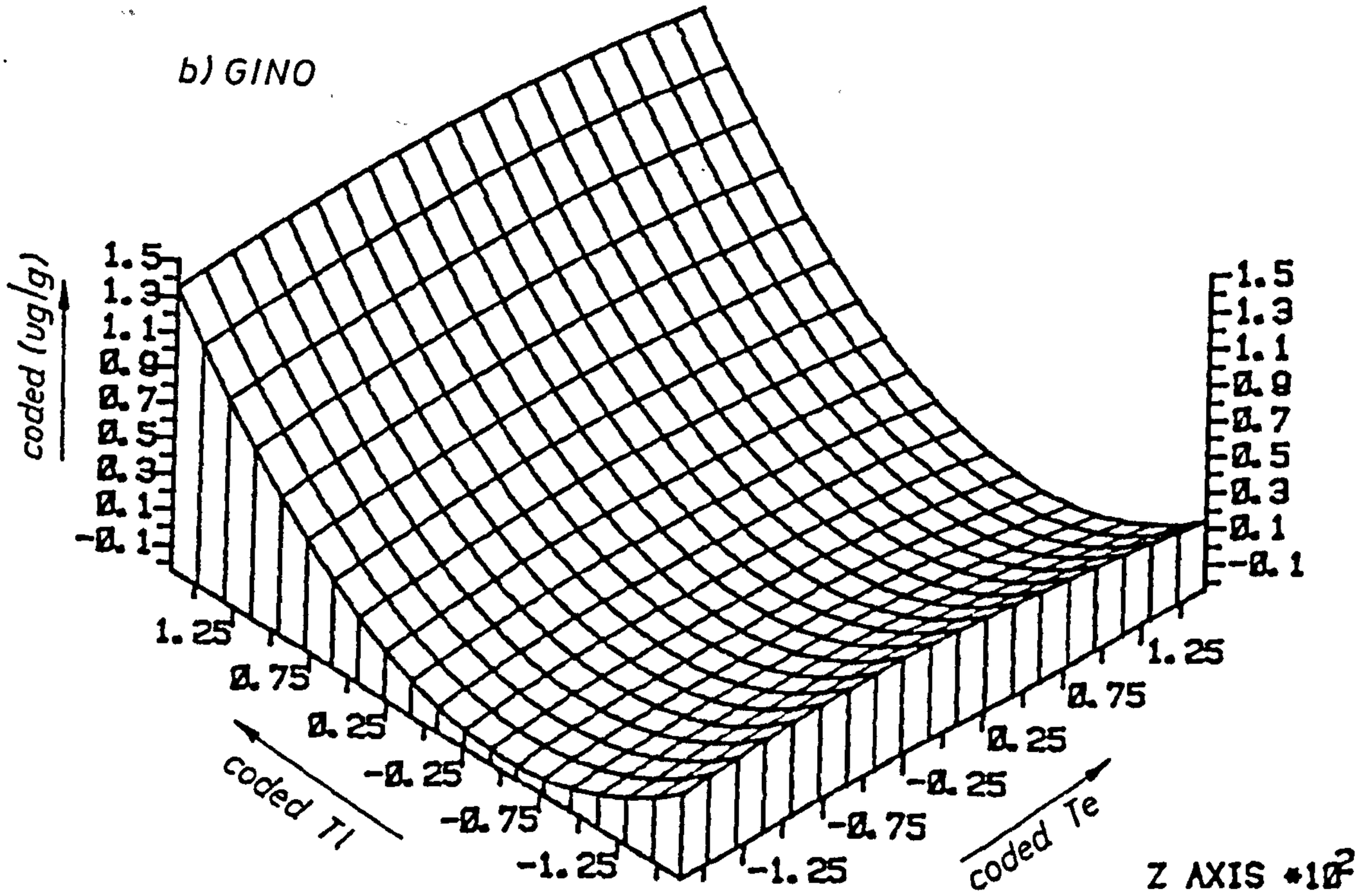


FIG. 6.32 RESPONSE SURFACE FOR THALLIUM UPTAKE BY SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED Se=0

a) SAS

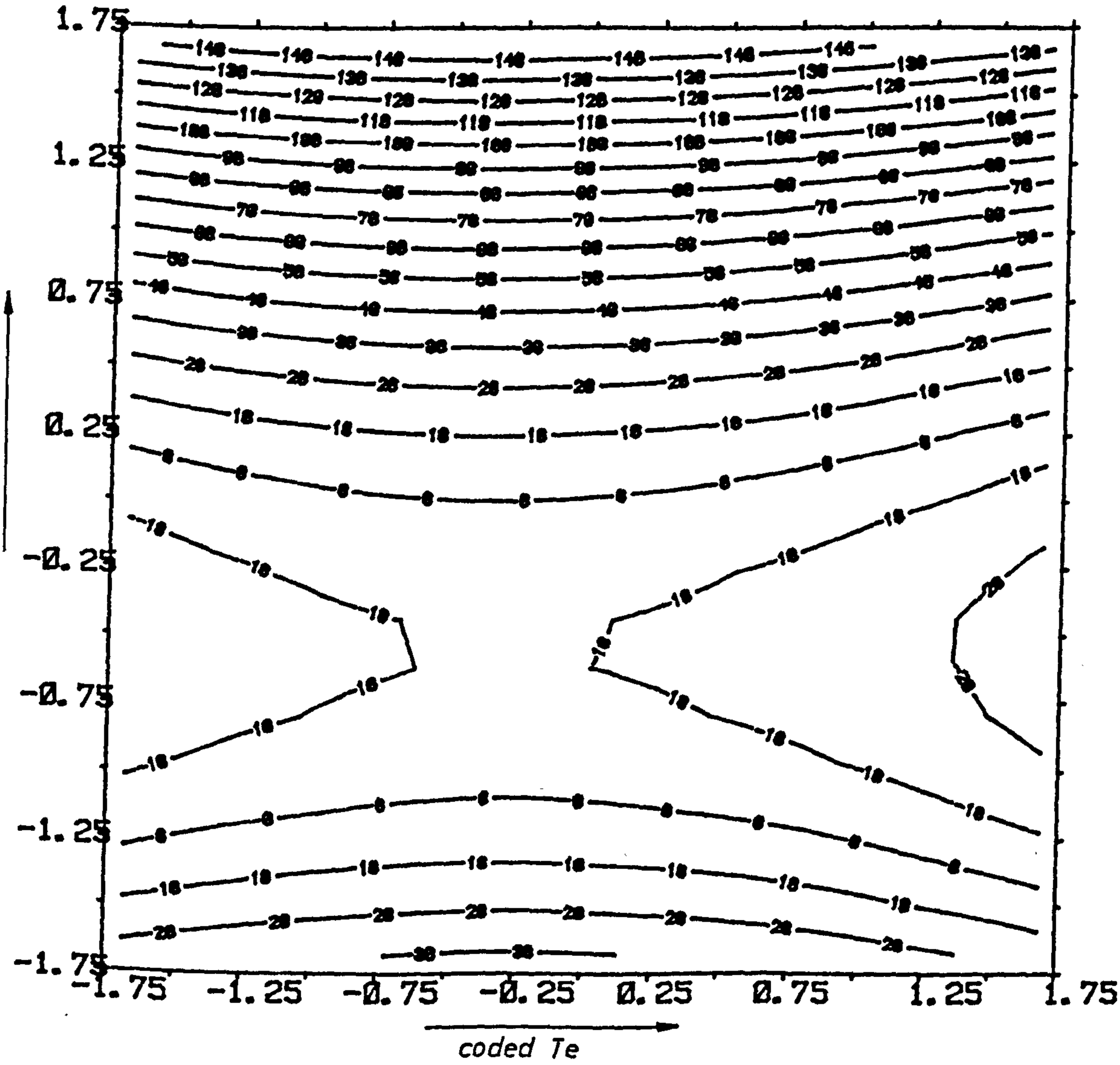


b) GINO



Te(VI), Tl(I), Tl uptake by Shoot

FIG. 6.33 CONTOUR PLOT OF RESPONSE SURFACE FOR THALLIUM UPTAKE BY SHOOT OF LOLIUM PERENNE SEEDLINGS AT CODED Se = 0



Since the plant material was divided into roots and shoots on harvesting, a set of data similar to that describing the shoots was also available for the roots and has been processed in a similar manner. However, because of the differing biological functions of roots and shoots, differences in behaviour towards toxic metals and hence in the form of the response observed is to be expected.

For roots in the same experiment, Table 6.17(a) describes the response variable root length (response Y_1, Table 6.7), in terms of the three experimental variables, X_1, X_2 and X_3 which represent the concentration of Se(VI), Te(VI) and Tl(I) respectively. In this case, R^2 (the coefficient of determination) reaches a value of 0.9120, which is due to the model rather than to random error. Thus the model used involving linear, quadratic and cross-terms appears to be a good descriptor for the root length versus metal concentration.

With the very significant probability terms [10], it is obvious that the linear and quadratic terms are important but not the cross-product term. The coefficient of variation is quite high, almost one quarter of the total error, and this is reflected in the Type 1 SS term [11]. In this case there is not a significant difference between the LACK OF FIT and PURE ERROR, so the probability value is not significant (0.1415, term [12]). That shows that the effect in the model of the lack of fit term is not significant when viewed in terms of the pure error term. In other words, the biological availability causing variance in the six (0,0,0) pots renders the possibility of a highly significant fit of the model to the data (i.e. a very small lack of fit) unlikely.

Examination of the PROB values [17] seems to confirm that Se(VI) and Tl(I) have a significant effect on root length, whilst Te(VI) does not. Notice that this is also carried on into the X1*X1, X2*X2 and

X3*X3 terms which are significant, while in the Cross-Product terms none are significant. In this case the root length results are similar to the shoot length ones in terms of Cross-Product but in the root length case all the QUADRATIC terms are significant while for shoot length only two terms are significant (elements X2*X2 and X3*X3).

Examination of the response surfaces confirms these findings; in Figures 6.34, 6.36 and 6.38 where in each case increasing Te(VI) concentration has only a small effect on growth, with Se(VI) having a greater effect, while Tl(I) has a very marked effect on growth. For confirmation, Figures 6.35, 6.37 and 6.39 illustrate the corresponding contour diagrams.

The factor test [18] produces a greater than 95% probability that all the experimental variables have an effect on the root length response. The critical factor values for Se(VI), Te(VI) and Tl(I) are 0.06905 µg Se/ml, 0.09752 µg Te/ml and 0.0341 µg Tl/ml respectively. The values reflect the relative needs or toxicity towards the Lolium perenne seedlings of these variables. The lower value for the Tl(I) reflects the extremely toxic nature of this metal to the plant growth.

The predicted value for root length at optimum is 13 cm.

TABLE 6:17(a):

RESPONSE SURFACE FOR VARIABLE Y1 ROOT LENGTH (Se,Te,Tl)

RESPONSE MEAN	6.5285
ROOT MSE	1.980556
R-SQUARE	0.9120189
COEF OF VARIATION	0.3033707

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	168.7692	0.3785	14.34	0.0006
QUADRATIC	3	211.2582	0.4738	17.95	0.0002
CROSSPRODUCT	3	26.59244	0.0596	2.26	0.1439
TOTAL REGRESS	9	406.8198	0.9120	11.52	0.0003

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	28.90367	5.780735	2.800	0.1415
PURE ERROR	5	10.32233	2.064467		
TOTAL ERROR	10	39.22601	3.922601		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	11.12802	0.8077082	13.78	0.0001
X1	1	-1.40493	0.5361712	-2.62	0.0256
X2	1	-0.835395	0.5361712	-1.56	0.1503
X3	1	-3.11401	0.5361712	-5.81	0.0002
X1*X1	1	-1.28236	0.5225496	-2.45	0.0340
X2*X1	1	-1.25625	0.7002322	-1.79	0.1030
X2*X2	1	-3.20272	0.5225496	-6.13	0.0001
X3*X1	1	1.04875	0.7002322	1.50	0.1651
X3*X2	1	0.80375	0.7002322	1.15	0.2778
X3*X3	1	-2.25671	0.5225496	-4.32	0.0015

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	71.98021	17.99505	4.59	0.0231
X2	4	174.6678	43.66696	11.13	0.0011
X3	4	219.4409	54.86022	13.99	0.0004

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE
X1	-0.864373
X2	-0.0709181
X3	-0.908067

PREDICTED VALUE AT OPTIMUM 13.19275

EIGENVALUES EIGENVECTORS

	X1	X2	X3
-0.966853	0.9274049	-0.204207	0.3134003
-2.16399	-0.193989	0.4537978	0.8697333
-3.61095	0.3198258	0.867391	-0.38124

SOLUTION WAS A MAXIMUM

TABLE 6.17(b) RESPONSE SURFACE FOR ROOT LENGTH (Se,Te,Tl)

```
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 -- read 'EmanR' c1-c8
column        c1           c2           c3           c4           c5
c6
count         20           20           20           20           20
20
row
   1    -1.00000     -1.00000     -1.00000       9.5000       6.580
3.150
   2     1.00000     -1.00000     -1.00000      10.6600      46.450
2.600
   3    -1.00000      1.00000     -1.00000      11.0600       4.230
25.380
   4    -1.00000     -1.00000      1.00000       0.5000       4.620
6.100
   .  .  .

column        c7           c8
count         20           20
row
   1       5.810     0.111000
   2      10.560     0.144000
   3       6.500     0.131000
   4      76.200     0.082000
   .  .  .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c4 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    11.1 -  1.40 x1 - 0.835 x2
    -  3.11 x3 -  1.28 x4 -  1.26 x5
    +  1.05 x6 -  3.20 x7 + 0.804 x8
    -  2.26 x 9

                                        st. dev.     t-ratio =
       column      coefficient          of coef.     coef/s.d.
       --             11.1280            0.8077        13.78
x1     c1             -1.4049            0.5362        -2.62
x2     c2             -0.8354            0.5362        -1.56
x3     c3             -3.1140            0.5362        -5.81
x4     c11            -1.2824            0.5225        -2.45
x5     c12            -1.2562            0.7002        -1.79
x6     c13             1.0487            0.7002         1.50
x7     c22            -3.2027            0.5225        -6.13
x8     c23             0.8037            0.7002         1.15
x9     c33            -2.2567            0.5225        -4.32

the st. dev. of y about regression line is
s = 1.981

with (  20-10) =  10 degrees of freedom

r-squared = 91.2 percent
r-squared = 83.3 percent, adjusted for d.f.

analysis of variance

 due to      df          ss        ms=ss/df
regression    9      406.620          45.180
residual     10       39.226           3.923
total        19      445.846

further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9      406.620
c1            1       26.933
c2            1        9.523
c3            1      132.314
c11           1        9.210
c12           1       12.625
c13           1        8.799
c22           1      128.888
c23           1        5.168
c33           1       73.160

              x1           y     pred. y     st.dev.
row           c1          c4      value    pred. y    residual     st.res.
  2         1.00      10.660      7.942      1.621       2.718       2.39r
  7        -1.00       0.500      2.853      1.621      -2.353      -2.07r

r denotes an obs. with a large st. res.

durbin-watson statistic = 2.06
 --
 -- Q
* error * name not found in dictionary

 --
 --
 --
 -- end
 -- stop

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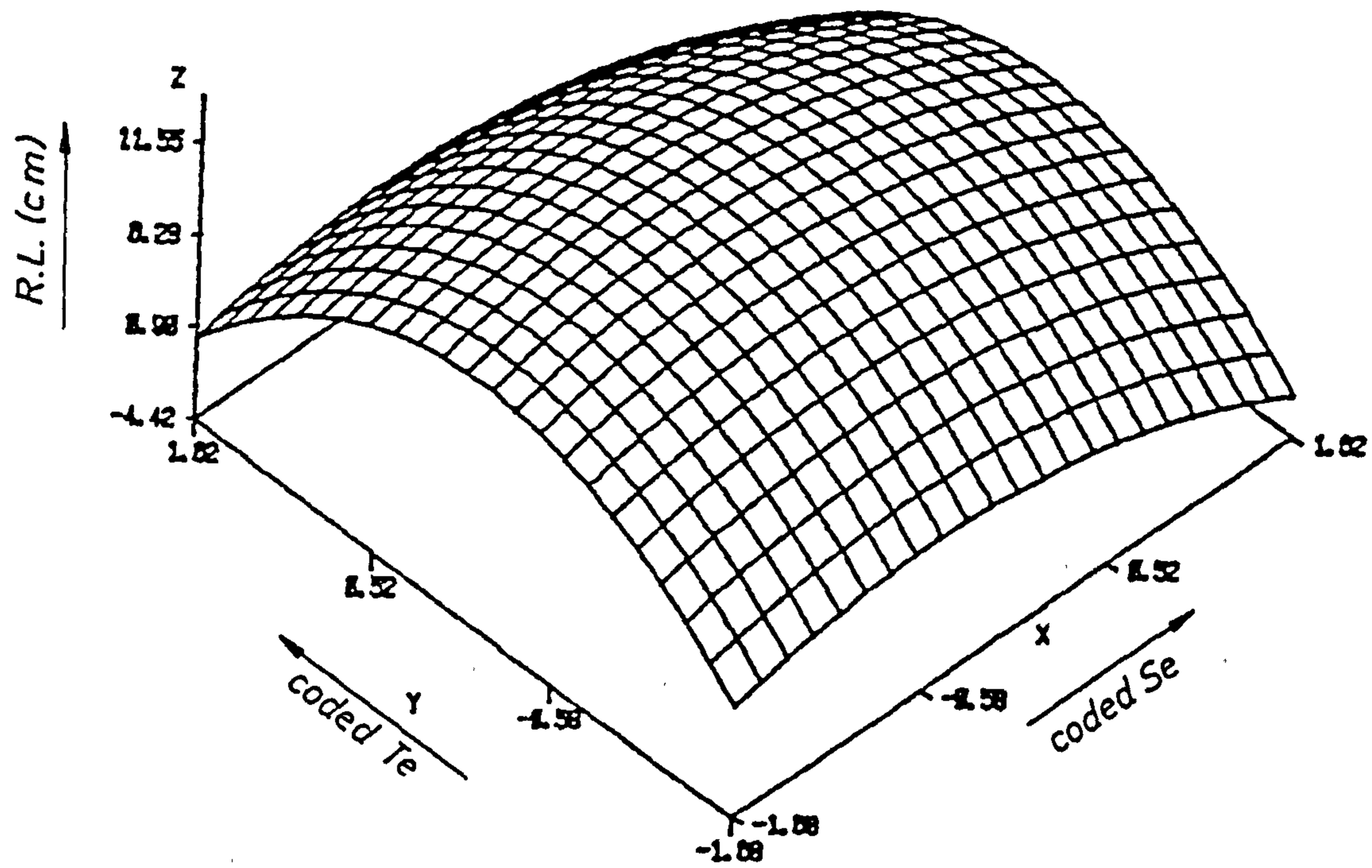
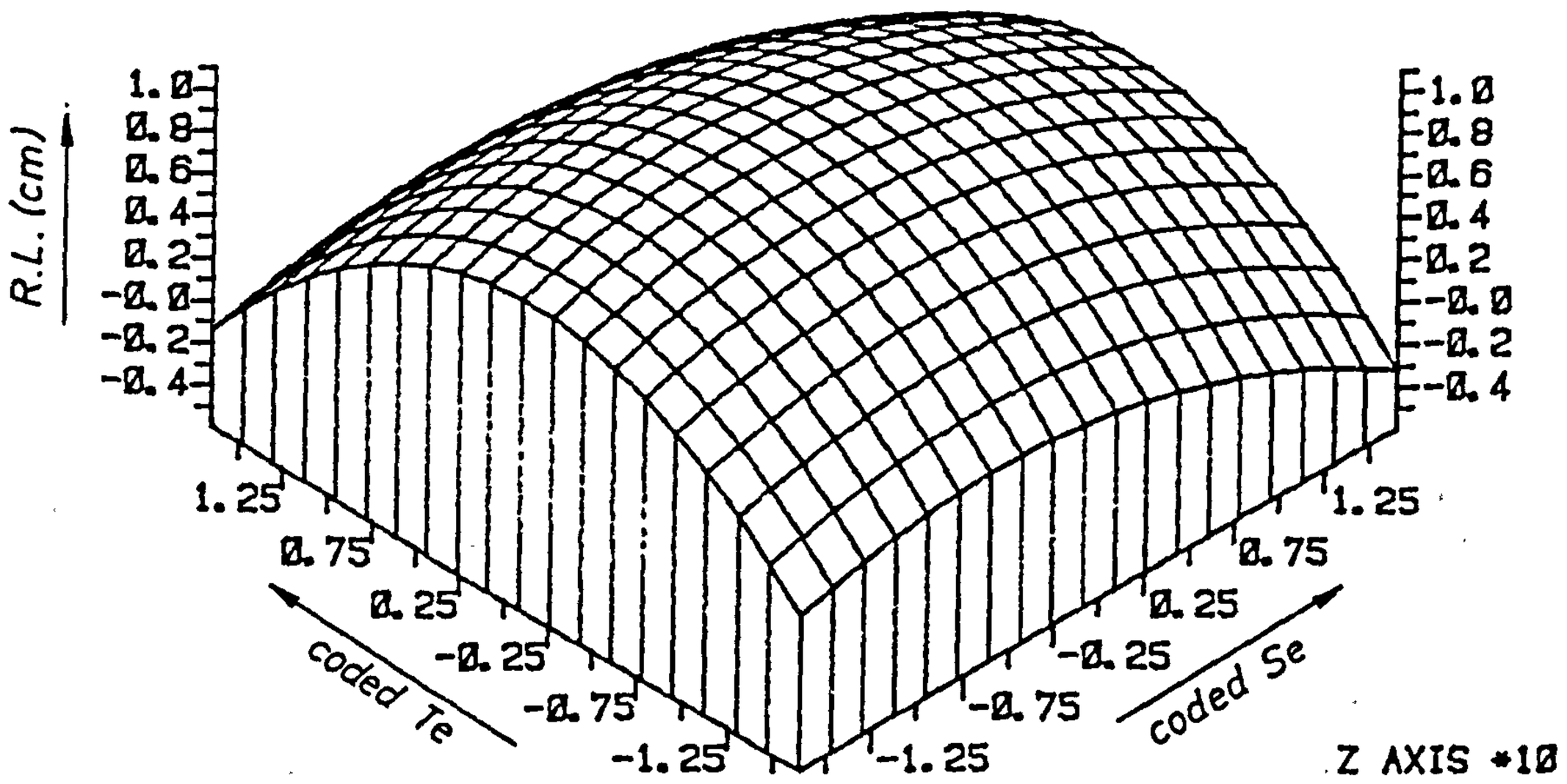
storage available   50000
```

FIG. 6.34 RESPONSE SURFACE FOR ROOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED TI =0

a) SAS



b) GINO



Se(VI), Te(VI), Root length

FIG. 6.35 CONTOUR PLOT OF RESPONSE SURFACE FOR ROOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0

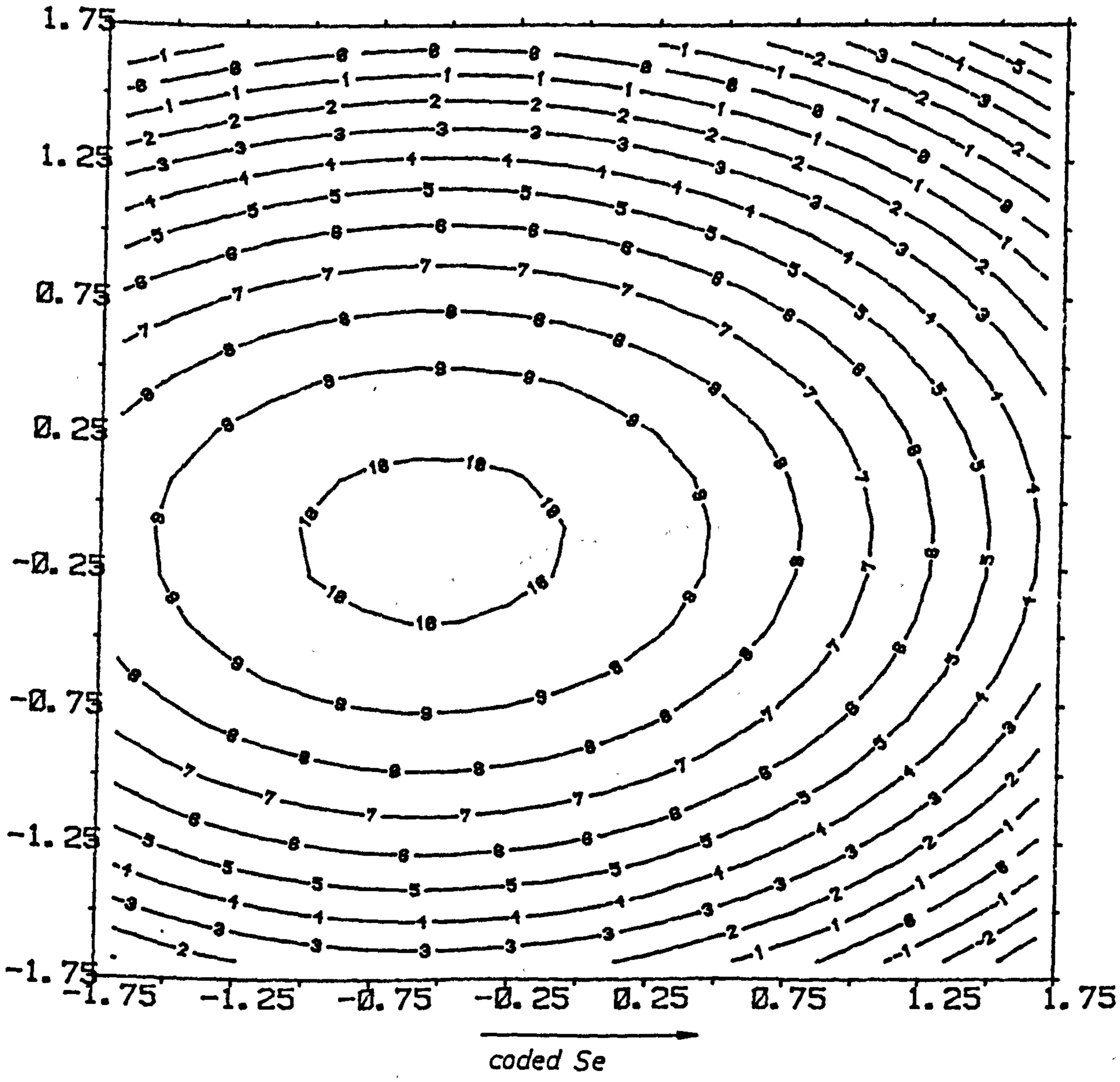
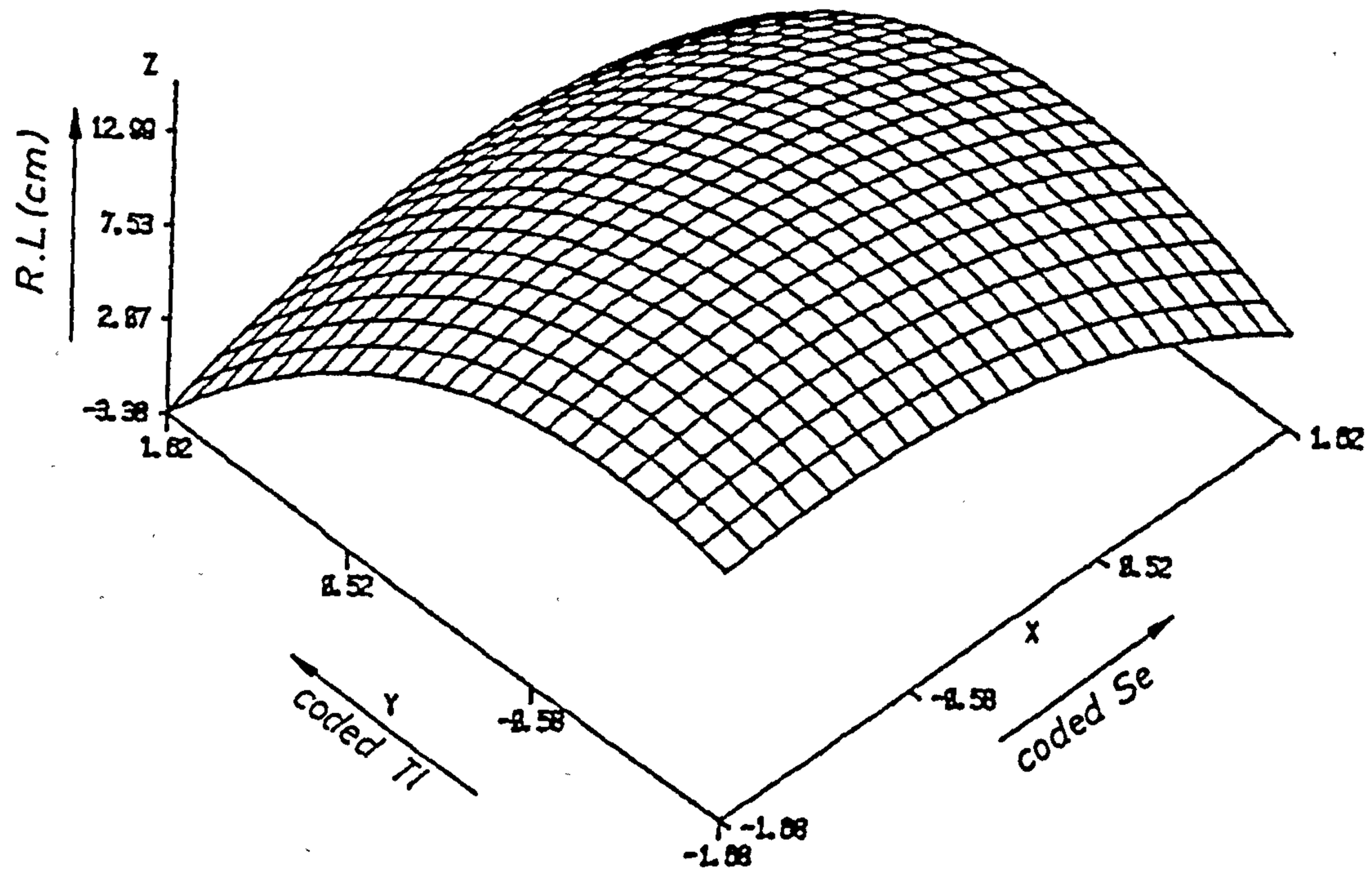
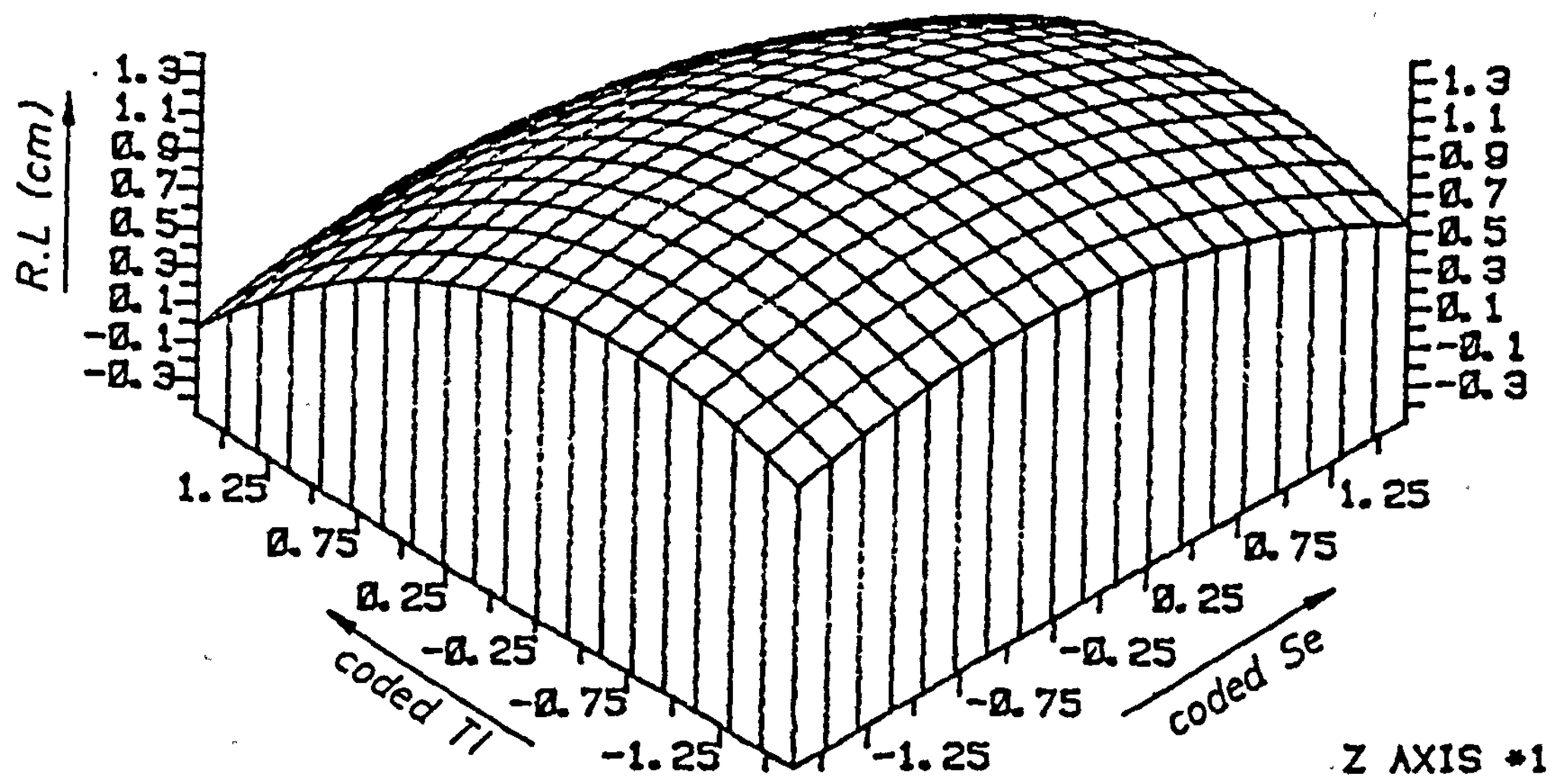


FIG. 6.36 RESPONSE FOR ROOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

a) SAS



b) GINO



Se(VI) , Tl(I) , Root length

FIG. 6.37 CONTOUR PLOT OF RESPONSE SURFACE FOR ROOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

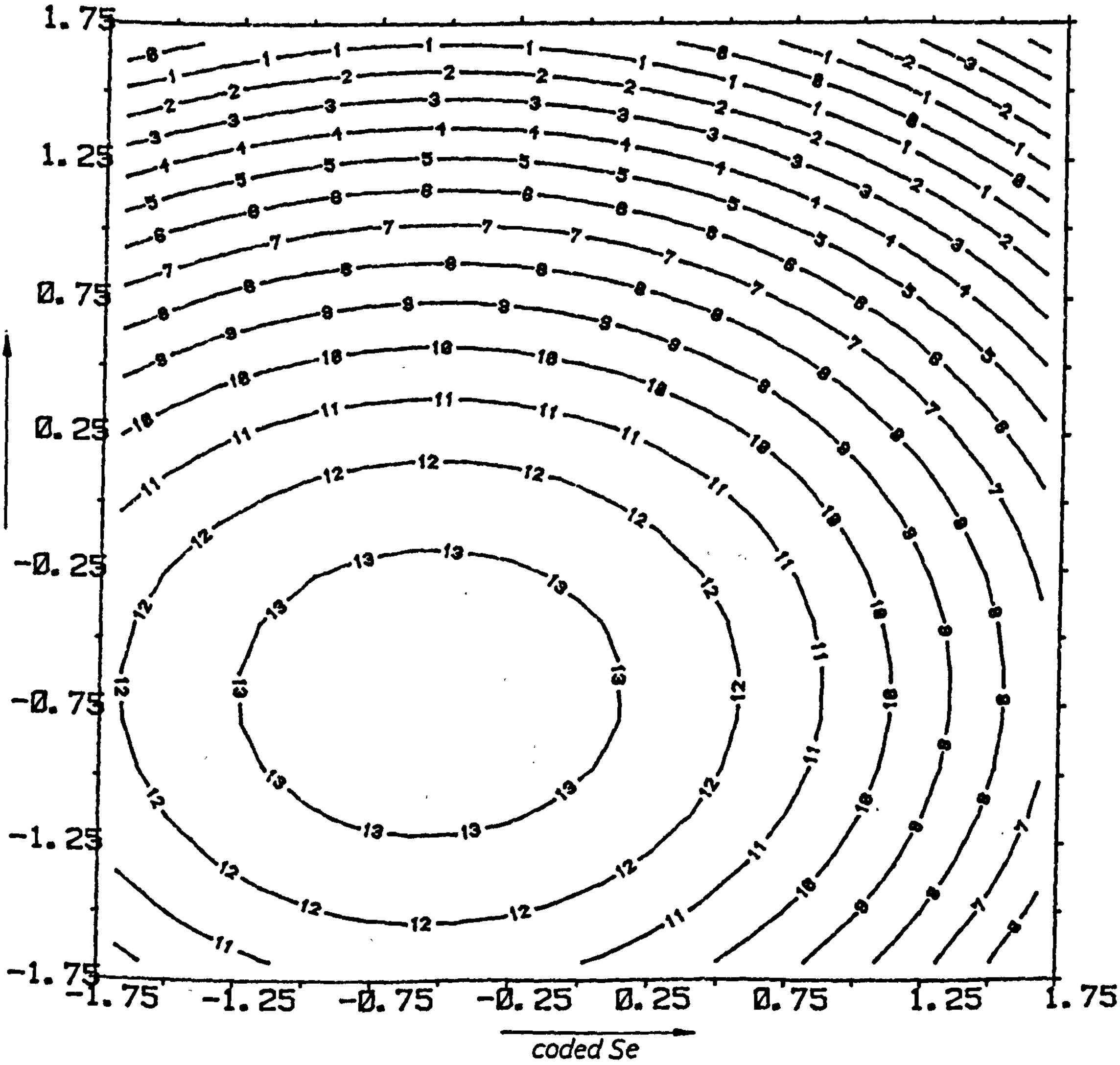
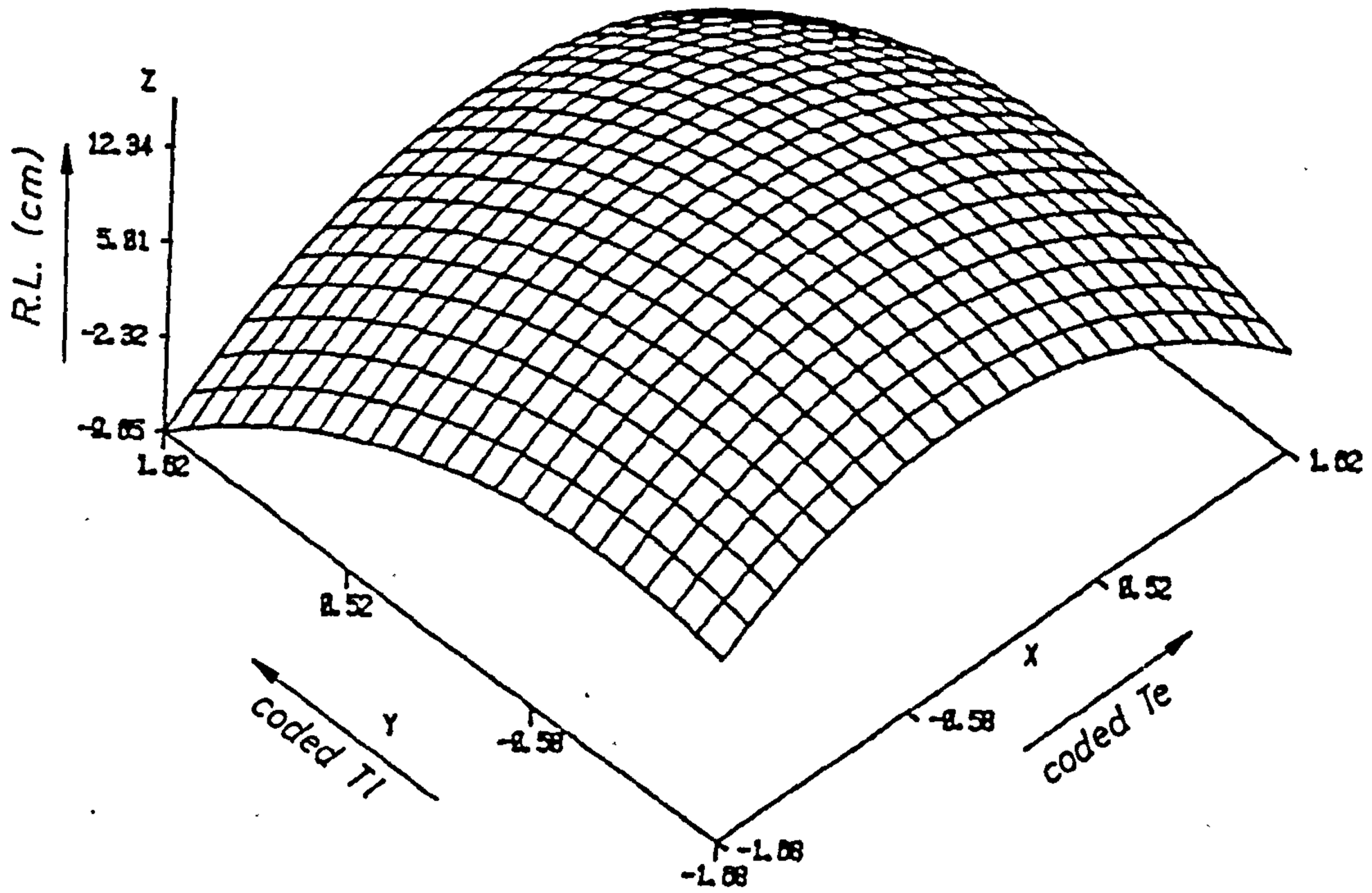
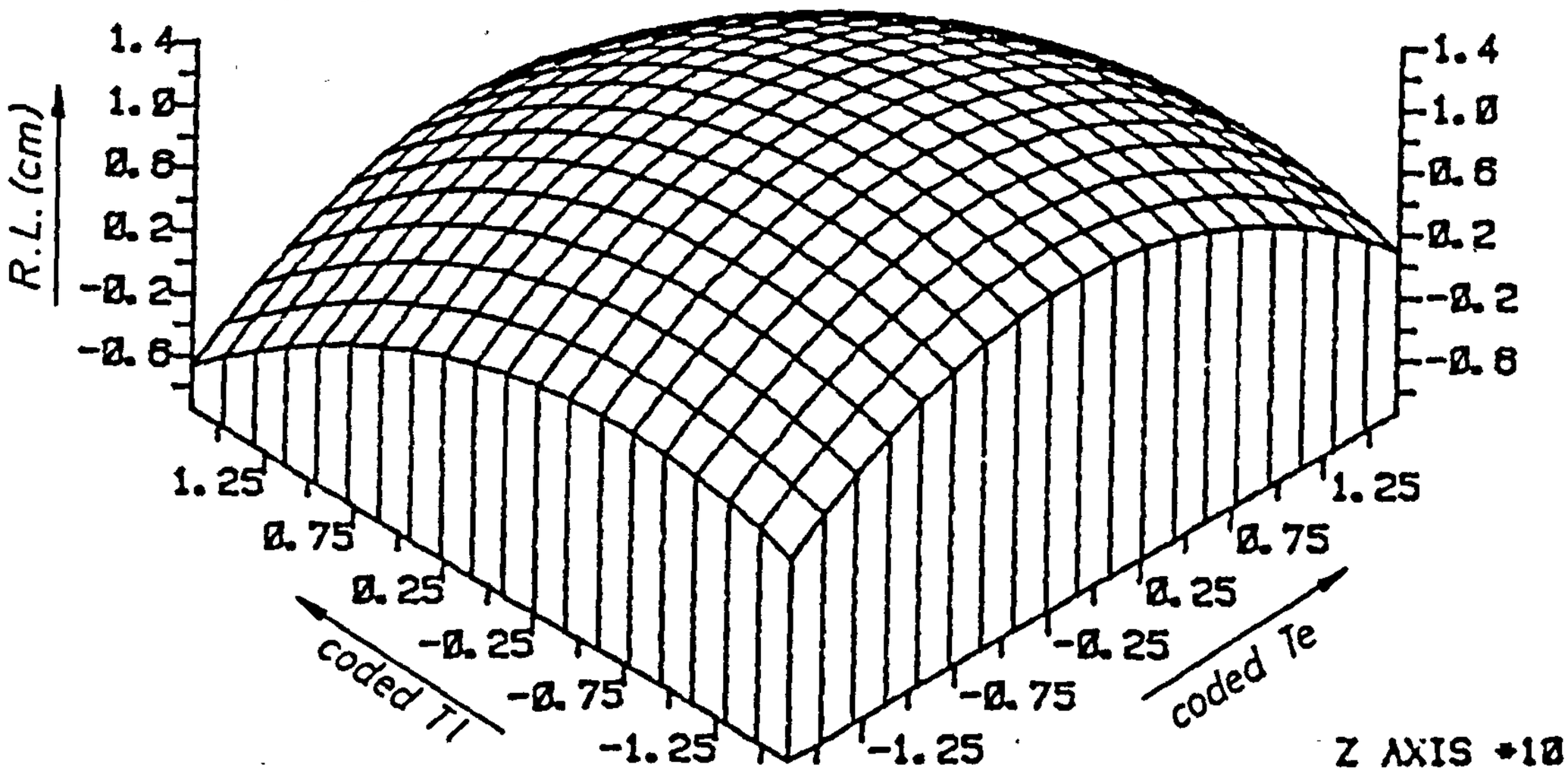


FIG. 6.38 RESPONSE SURFACE FOR ROOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

a) SAS

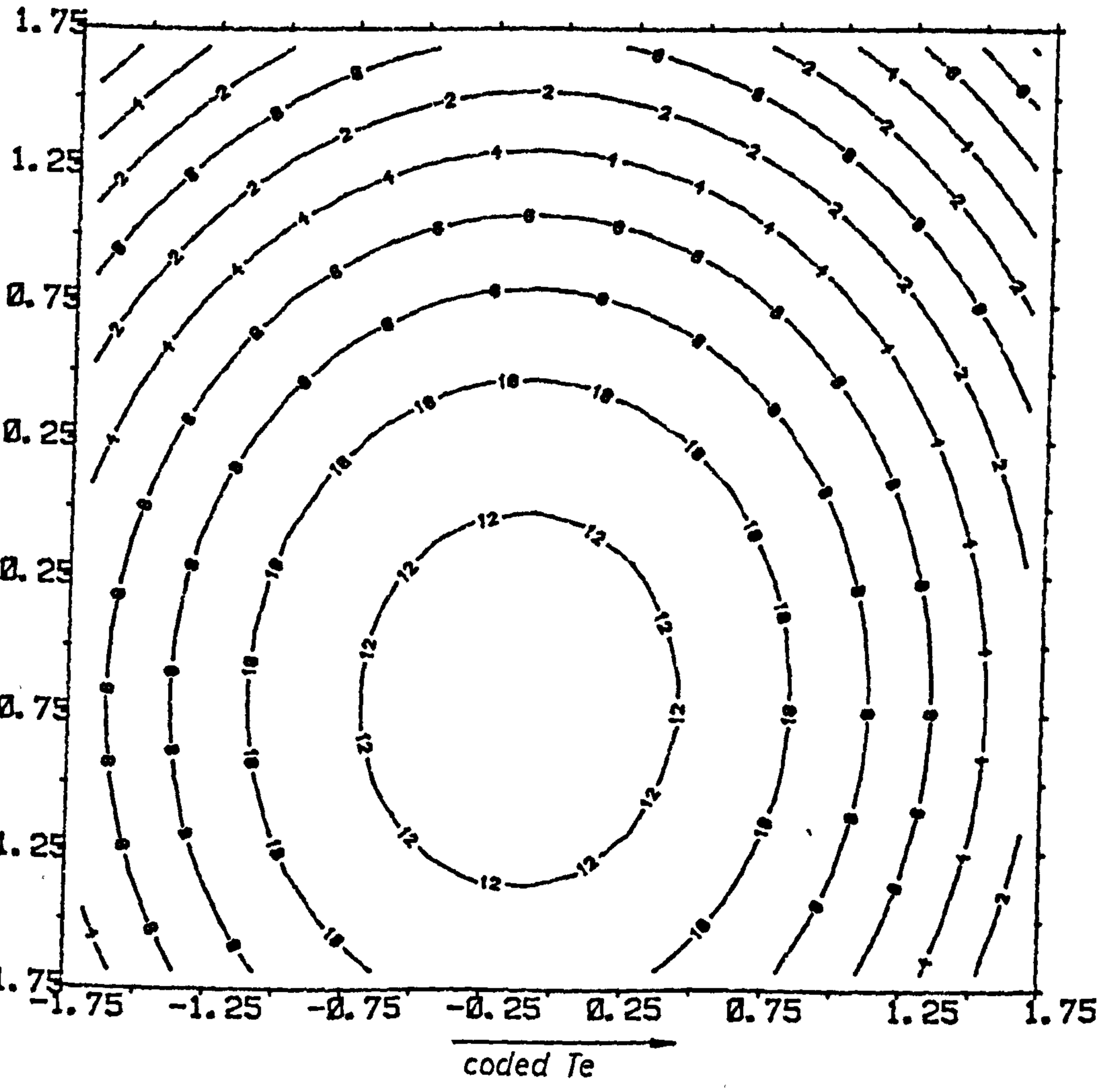


b) GINO



Te(VI) , Tl(I) , Root length

FIG. 6.39 CONTOUR PLOT OF RESPONSE SURFACE FOR ROOT LENGTH SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0



For the dry weight of the roots as a response variable (response Y9, Table 6.7), Table 6.18(a) presents the regression statistics for this response. In this case, the fit of R^2 is only 0.6845, being almost 23% lower quality fit than for the root length response, but allowing an R value of about 0.827, showing the extreme sensitivity of the R^2 value to slight changes in the fit of the model.

For the regression characteristics [10] only the linear factors appear to be of any significance, with the Quadratic and Cross-Product terms having non-significant values. The Cross-Product term is particularly interesting in that it has a value approaching unity, meaning the absence of a Cross-Product term. Such parameter values should be reflected in the shapes of the response surface. In a similar manner, the Lack of Fit term and Pure Error terms are of almost equal magnitude reflecting the inability of the fitted equation to model the data.

Because of this large degree of variation, it would be unexpected to find significant probability levels for most of the variables in the Linear, Quadratic or Cross-Product terms. Examination of the PROB values [17] seems to confirm this proposal, certainly Se(VI) and Te(VI) in linear combination are not significant, whilst Tl(I) has a significant value (0.0023). However, none of the Quadratic nor the Cross-Product terms are significant. The response surfaces confirm these findings; in Figures 6.40, 6.42 and 6.44 the effect of changing Se and Te concentrations are almost negligible, while the effect of Tl is obvious and reflects the toxicity of this metal to plant growth. The critical factor values for Se(VI), Te(VI) and Tl(I) are 7.812 μg/ml, 0.0724 μg/ml and < 0.0005 μg/ml respectively. When a comparison of these results is made with the root length parameters completely different results are noticed.

Thus the factors affecting the function of elongation are probably very different to those involving cell growth in general. Here the optimum value for Se is approximately 10 times that for elongation, while the Te(VI) values remains unchanged. But the major change is in the Tl(I) value which decreases by a factor of almost 60, supporting the hypothesis of the extremely toxic nature of Tl(I) towards root growth. Indeed the value is so low as to be outside the zone of direct experimentation; being the first case we have noted in the work being carried out in these laboratories. It is for this reasosn that the experiment results should be viewed with caution because Tl(I) is so toxic to root growth that a new experiment involving much lower levels of Tl(I) and noting the response with respect to dry weight of roots produced should be carried out. In other words, the dry weight response factor is an extremely sensitive indicator of exposure of roots to Tl(I).

Figures 6.41, 6.43 and 6.45 show the corresponding contour diagrams.

TABLE 6.18(a) ROOT DRY WEIGHT (Se, Te, TI)

RESPONSE SURFACE FOR VARIABLE Y9

RESPONSE MEAN	0.110315
ROOT MSE	0.01800799
R-SQUARE	0.6844979
COEF OF VARIATION	0.1632415

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	0.005508331	0.5359	5.66	0.0157
QUADRATIC	3	0.001348573	0.1312	1.39	0.3031
CROSSPRODUCT	3	0.0001786838	0.0174	0.18	0.9051
TOTAL REGRESS	9	0.007035588	0.6845	2.41	0.0934

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	0.001640203	0.0003280405	1.023	0.4902
PURE ERROR	5	0.001602675	0.000320535		
TOTAL ERROR	10	0.003242878	0.0003242878		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	0.1190009	0.007344001	16.20	0.0001
X1	1	-0.00113479	0.004875079	-0.23	0.8206
X2	1	-0.00329943	0.004875079	-0.68	0.5139
X3	1	-0.0197869	0.004875079	-4.06	0.0023
X1*X1	1	0.0002559175	0.004751226	0.05	0.9581
X2*X1	1	-0.0041625	0.006366786	-0.65	0.5280
X2*X2	1	-0.00909782	0.004751226	-1.91	0.0845
X3*X1	1	0.0011625	0.006366786	0.18	0.8588
X3*X2	1	0.0019125	0.006366786	0.30	0.7700
X3*X3	1	-0.00388949	0.004751226	-0.82	0.4321

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	0.0001679345	.00004198361	0.13	0.9682
X2	4	0.001505446	0.0003763616	1.16	0.3841
X3	4	0.005599615	0.001399904	4.32	0.0276

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE
X1	1.50396
X2	-0.789515
X3	-2.51299

PREDICTED VALUE AT OPTIMUM 0.1443121

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
0.0007284331	0.9766061	-0.198896	0.08173767
-0.00372845	-0.0431015	0.1913409	0.9805768
-0.00973138	0.2106722	0.9611603	-0.178292

SOLUTION WAS A SADDLE POINT

TABLE 6.18(b): Y9 ROOT DRY WEIGHT (Se, Te, Tl)

```
Minitab release 81.1 *** copyright - Penn State Univ. 1981
aug. 21, 1986 *** Avon Universities, Bristol
storage available  50000

 -- read 'EmanDryWt' c1-c8
olumn        c1           c2           c3           c4           c5
c6
count        20           20           20           20           20
20
row
  1     -1.00000     -1.00000     -1.00000       9.5000       6.580
3.150
  2      1.00000     -1.00000     -1.00000      10.6600      46.450
2.600
  3     -1.00000      1.00000     -1.00000      11.0600       4.230
25.380
  4     -1.00000     -1.00000      1.00000       0.5000       4.620
6.100
  .  .  .

column        c7           c8
count         20           20
row
  1        5.810     0.111000
  2       10.560     0.144000
  3        6.500     0.131000
  4       76.200     0.082000
  .  .  .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c8 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =   0.119 -0.0011 x1 -0.0033 x2
    -0.0198 x3 +0.0003 x4 -0.0042 x5
    +0.0012 x6 -0.0091 x7 +0.0019 x8
    -0.0039 x 9

                                    st. dev.    t-ratio =
      column     coefficient       of coef.    coef/s.d.
      --          0.119001       0.007344        16.20
x1    c1         -0.001135       0.004875        -0.23
x2    c2         -0.003299       0.004875        -0.68
x3    c3         -0.019787       0.004875        -4.06
x4    c11         0.000256       0.004751         0.05
x5    c12        -0.004162       0.006367        -0.65
x6    c13         0.001163       0.006367         0.18
x7    c22        -0.009098       0.004751        -1.91
x8    c23         0.001912       0.006367         0.30
x9    c33        -0.003889       0.004751        -0.82

the st. dev. of y about regression line is
s = 0.01801

with (  20-10) =  10 degrees of freedom

r-squared = 68.4 percent
r-squared = 40.1 percent, adjusted for d.f.

analysis of variance

 due to      df          ss        ms=ss/df
regression    9     0.0070356      0.0007817
residual     10     0.0032429      0.0003243
total        19     0.0102785

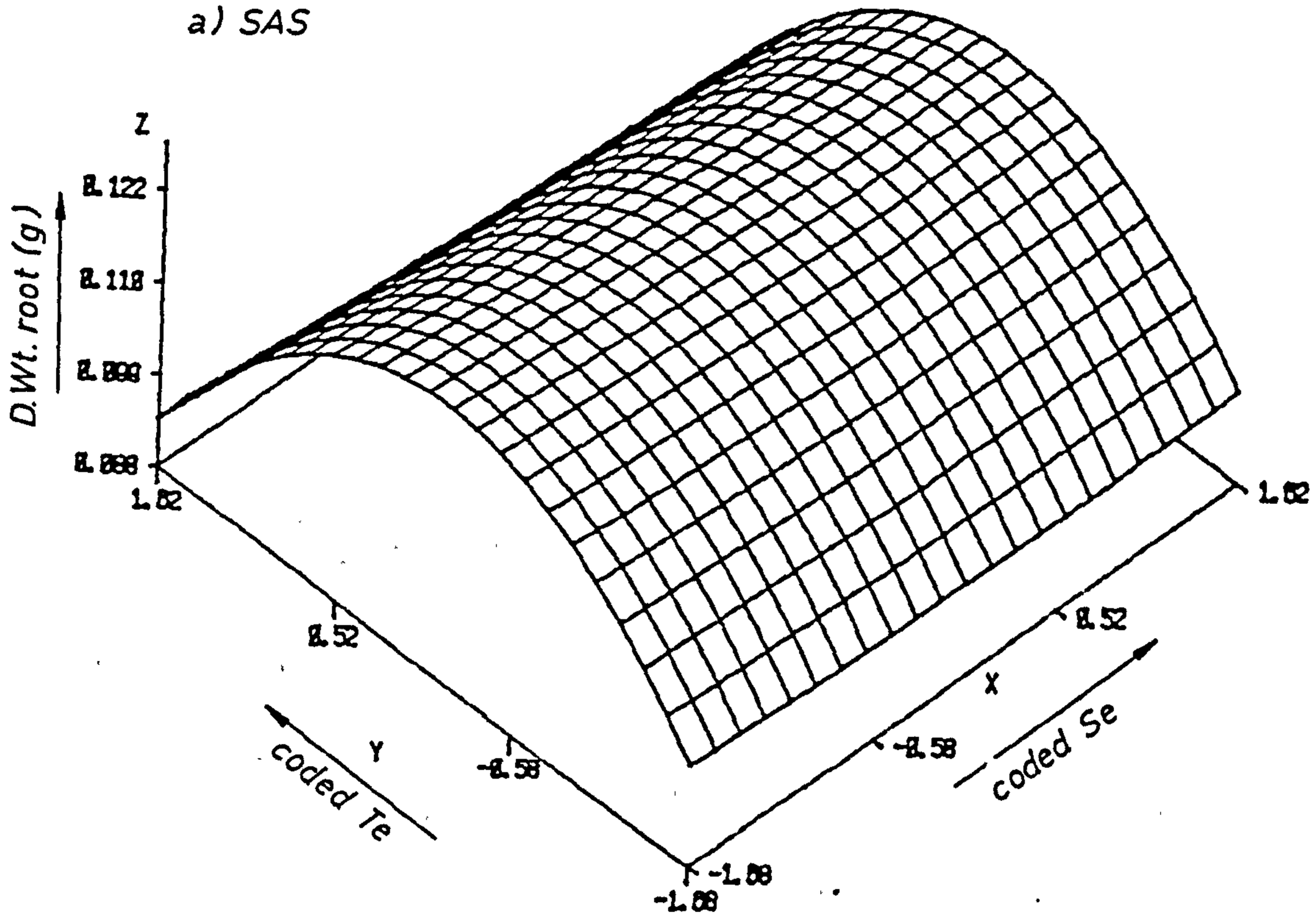
further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9     0.0070356
c1            1     0.0000176
c2            1     0.0001485
c3            1     0.0053422
c11           1     0.0000294
c12           1     0.0001386
c13           1     0.0000108
c22           1     0.0011018
c23           1     0.0000293
c33           1     0.0002173


durbin-watson statistic = 1.52
 -- end
 -- stop

*** Minitab *** Statistics Dept * Penn State Univ. * release 81.1 *
storage available    50000
```

FIG. 6.40 RESPONSE SURFACE FOR DRY WT. ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0



Se(VI), Te(VI) , Dry Wt. Root

FIG. 6.41 CONTOUR PLOT OF RESPONSE SURFACE FOR DRY WT. ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI =0

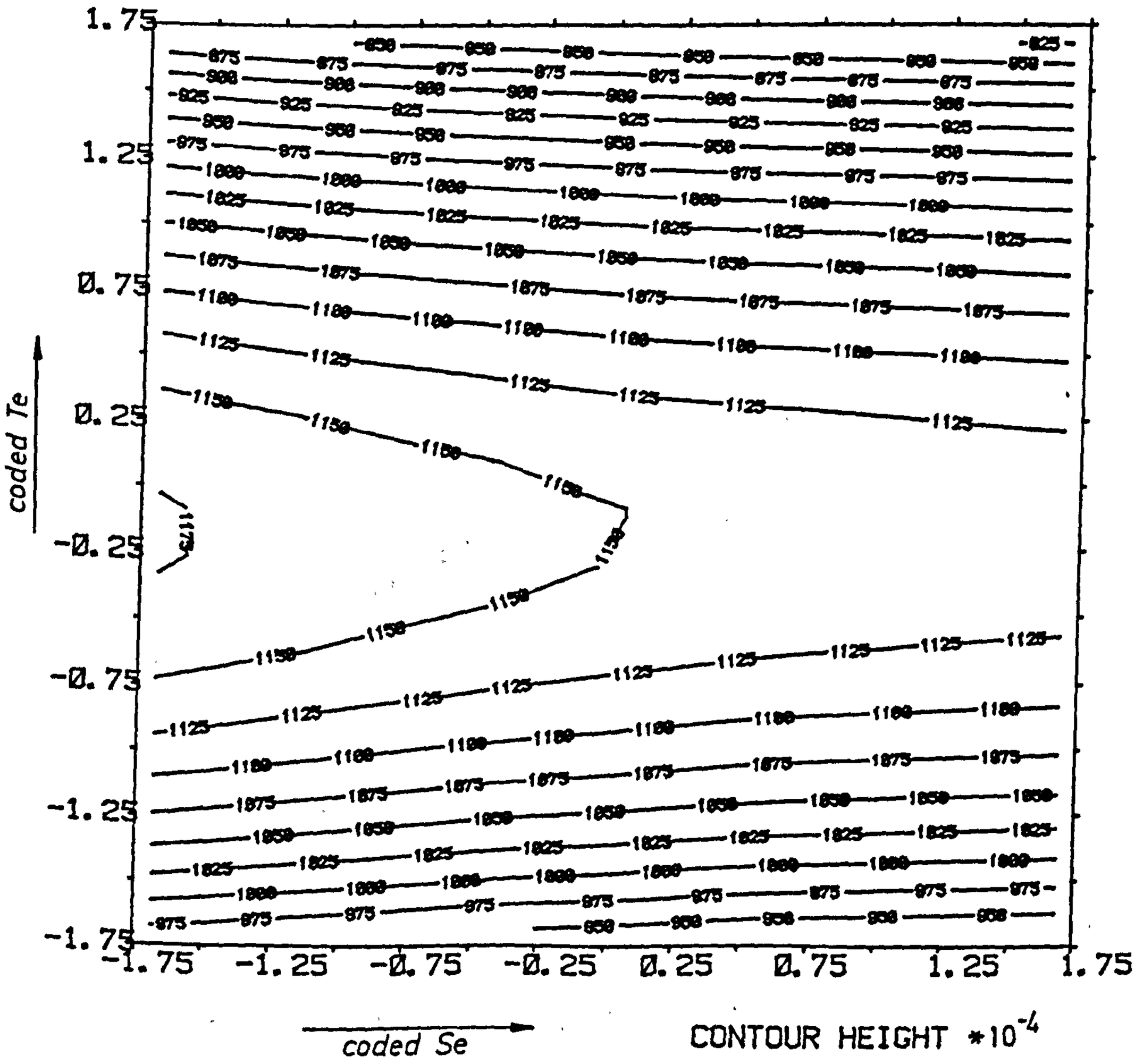
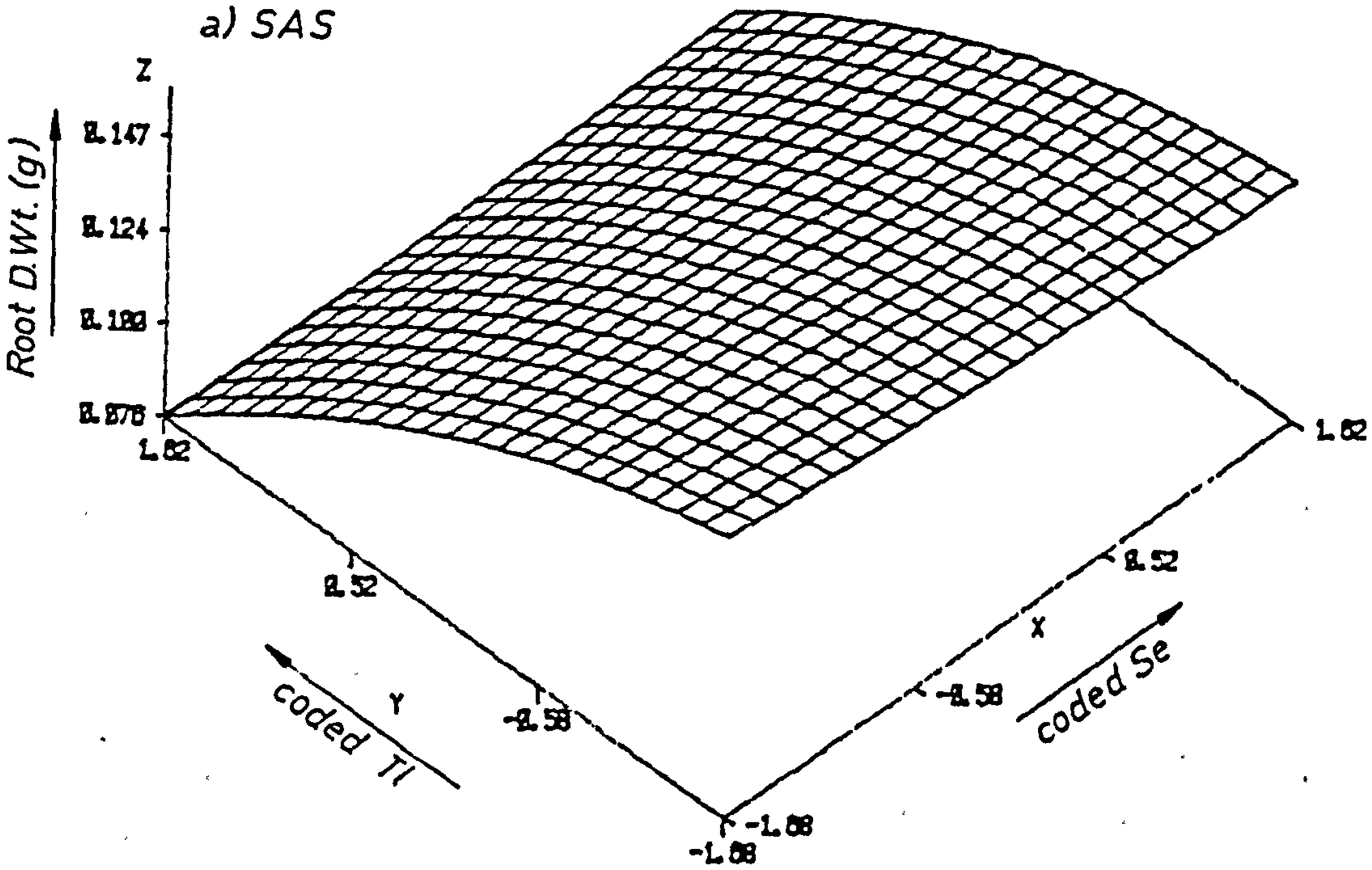


FIG. 6.42 RESPONSE SURFACE FOR DRY WT. ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te=0



Se(VI) , Tl(I) , Dry Wt. Root

FIG.6.43 CONTOUR PLOT OF RESPONSE SURFACE FOR DRY WT. OF ROOT OF LOLIUM PERENNE AT Te = 0

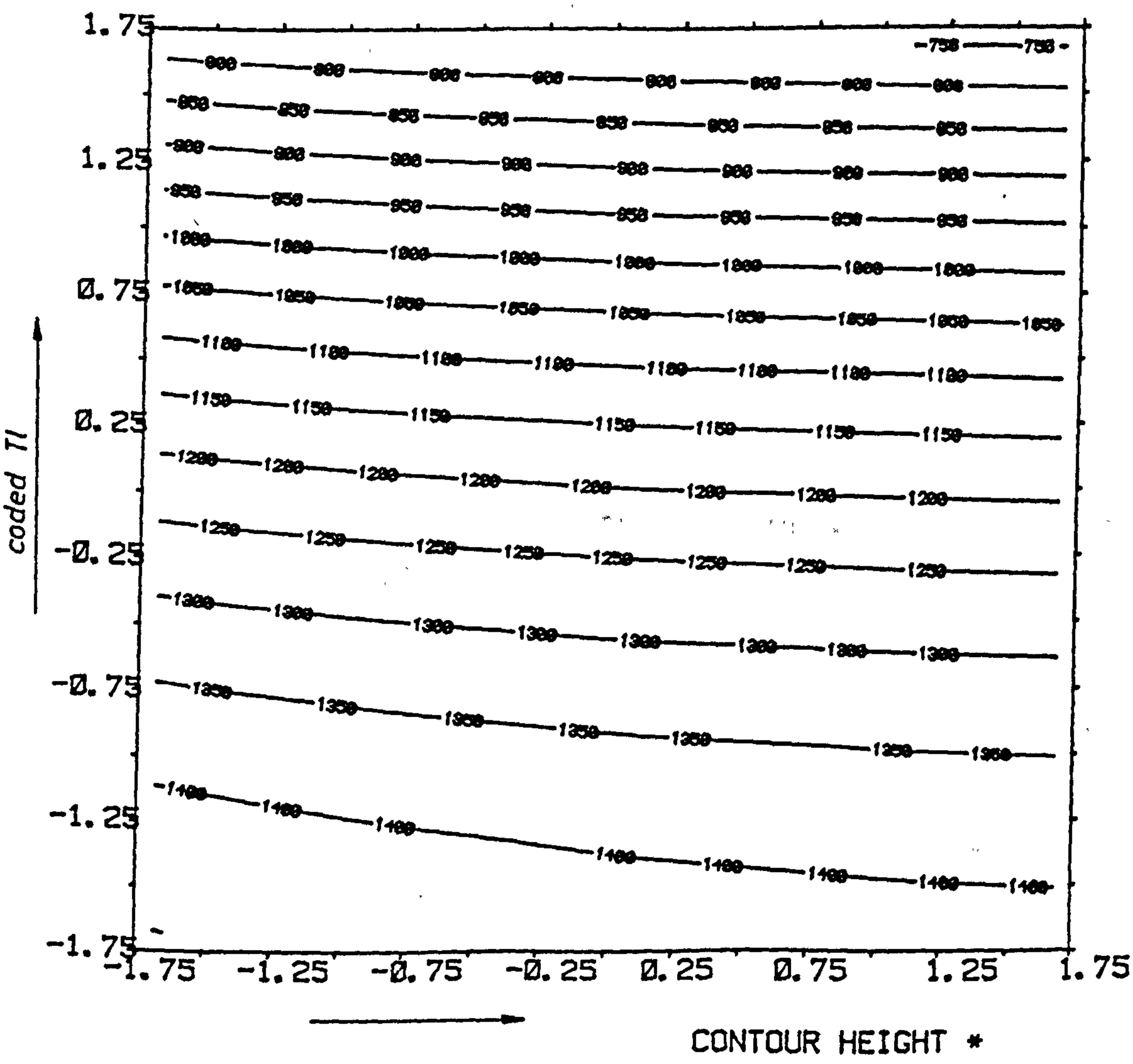
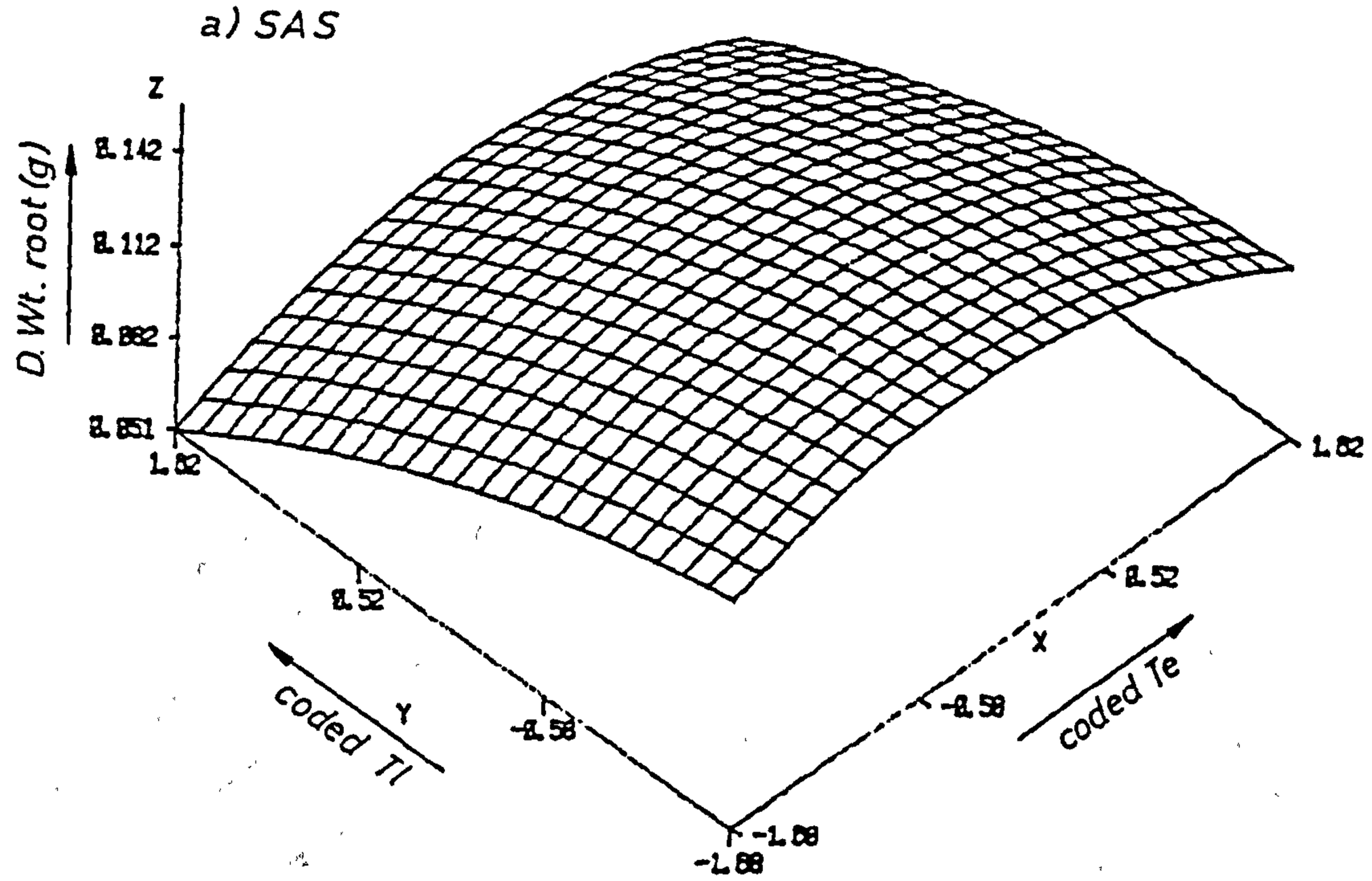
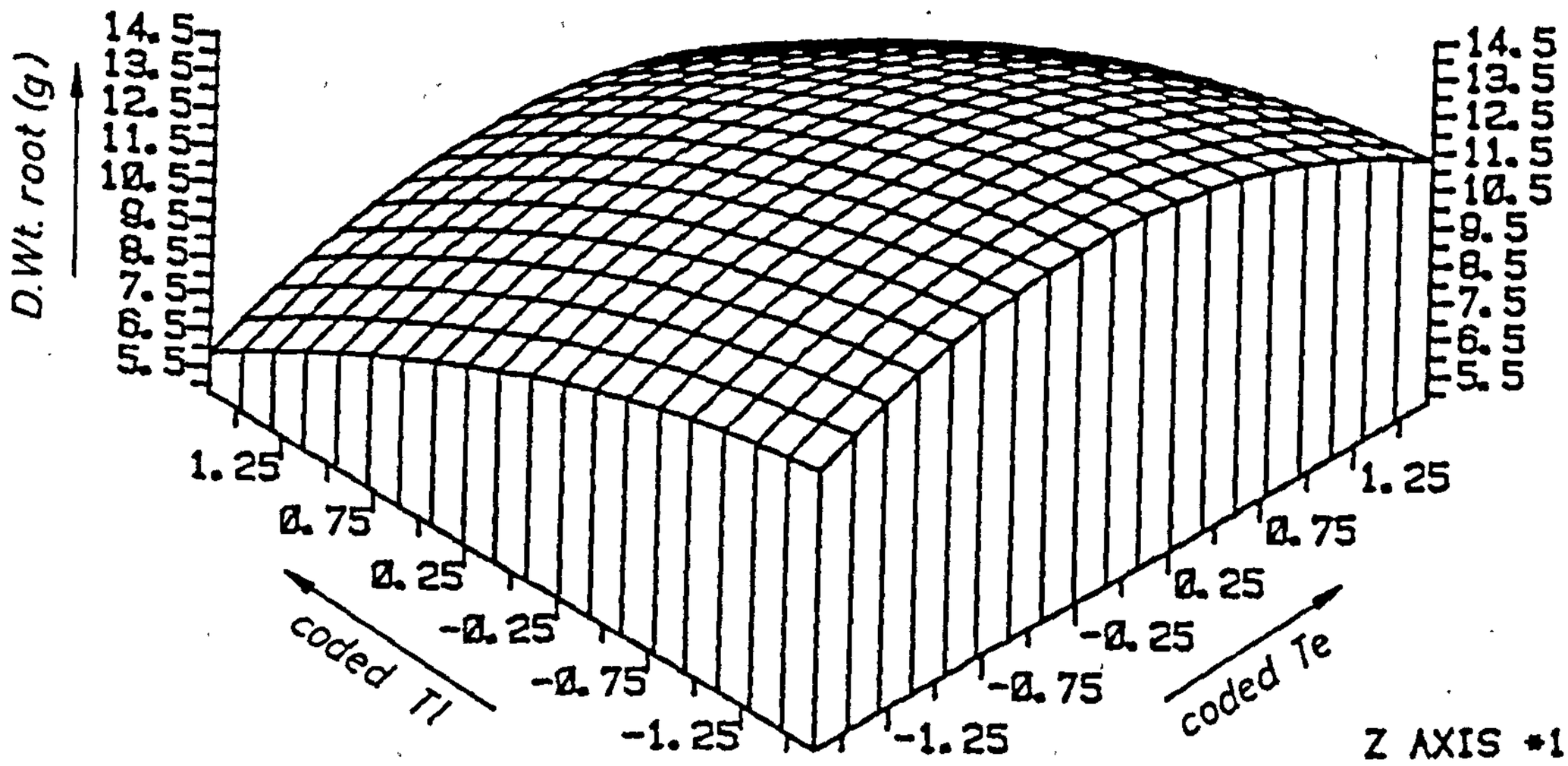


FIG. 6.44 RESPONSE SURFACE FOR DRY WT. ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

a) SAS

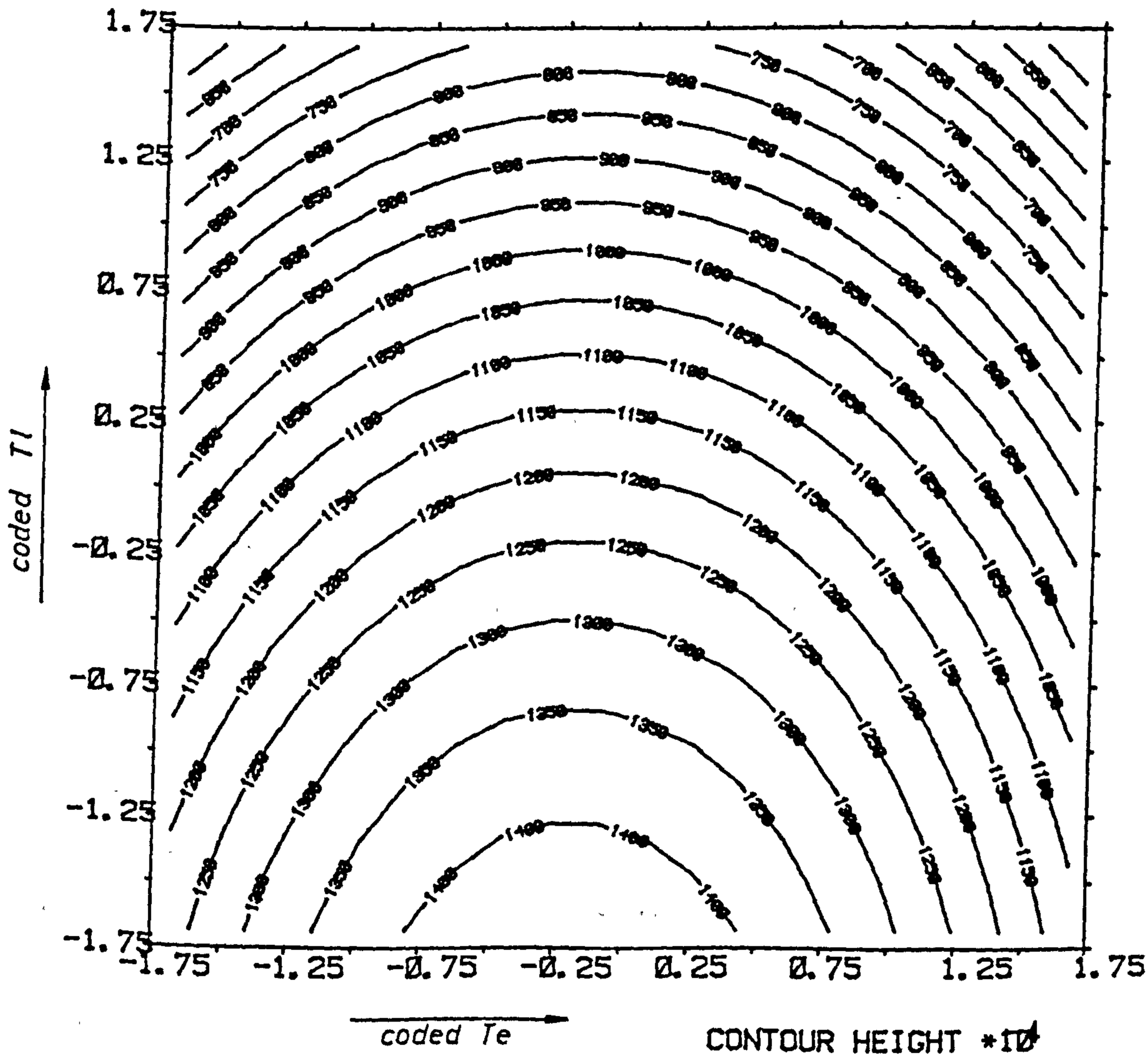


b) GINO



T*(VI) , T1(I) , Dry Wt. Root

FIG. 6.45 CONTOUR PLOT OF RESPONSE SURFACE FOR DRY Wt. ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0



A number of elemental analyses were undertaken on the plant tissue, especially important is the uptake of the 'spiked' element from the nutrient solution into the plant. Considering selenium first with uptake by the roots as a response variable (Reponse Y2, Table 6.7), where Table 6.19(a) represents the data treatment values. In this case, the R^2 value of 0.9719 is extremely high, in that 97.19% of the variation is due to the model rather than random error. Notice that the pure error value is very low and most of the total error arises from the lack of fit of the model. The probability of the regression values in terms of linear and quadratic terms is also very high, being greater than 99.9% that they are factors in the model. But notice that the probability of the Cross-product term is small, so once again slight interaction terms are to be expected. Therefore, there seems little interaction between the Se(VI) uptake and the influence of increasing concentrations of Tl(I). As shown in Figures 6.46 and 6.48, the uptake of Se is virtually independent of either the increasing Te(VI) and Tl(I) concentrations. As commented previously for the shoot uptake situation, the non-interaction between the Se level in the tissue and the Tl(I) level in the nutrient solution is not surprising. So, a similar level of response is found in the case of increasing Te(VI) concentration where no interaction with the Se content is found; even in view of the similarity of these anions.

Obviously, no such interaction is hinted at in the PROB [18], where only the Se concentration of the nutrient solution is sigificant and highly so (as it should be), whilst the other two variables (X2 and X3) are not significant to any degree at all.

Clearly all the responses are almost planar with respect to

change - in either the Te(VI) or Tl(I) concentration, i.e. no change acorss the Te(VI) or Tl(I) axes. In contrast, the Se content increases dramatically as the Se concentration in the added medium is increased. Figures 6.47 and 6.49 show the steepness of the Se content contours, once the concentration of Se(VI) in the nutrient solution rises above 0.1 ug Se/ml.

Figure 6.50 shows the response for Se uptake by the roots with varying Tl(I) and Te(VI) concentrations at a constant Se concentration of 0.1 µg/ml. Obviously, the Se content increases markedly with rise in Tl(I) concentration, while for the Te(VI) case the increase is much less marked. Clearly, however, the solution for the minimum is much in error because a negative Se concentration level is not possible, and so this reflects the lack of fit of the data towards the imposed model.

TABLE 6.19(a) SELENIUM UPTAKE BY ROOT (Se, Te, Tl)

RESPONSE SURFACE FOR VARIABLE Y2

RESPONSE MEAN	19.021
ROOT MSE	6.562276
R-SQUARE	0.9719045
COEF OF VARIATION	0.3450016

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	8914.184	0.5816	69.00	0.0001
QUADRATIC	3	5806.639	0.3788	44.95	0.0001
CROSSPRODUCT	3	176.0574	0.0115	1.36	0.3096
TOTAL REGRESS	9	14896.88	0.9719	38.44	0.0001

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	425.0548	85.01092	76.174	0.0001
PURE ERROR	5	5.580083	1.116017		
TOTAL ERROR	10	430.6347	43.06347		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	3.807411	2.676221	1.42	0.1853
X1	1	25.48467	1.776523	14.35	0.0001
X2	1	-1.38418	1.776523	-0.78	0.4539
X3	1	1.385055	1.776523	0.78	0.4537
X1*X1	1	20.06602	1.73139	11.59	0.0001
X2*X1	1	-3.21875	2.320115	-1.39	0.1955
X2*X2	1	1.420543	1.73139	0.82	0.4311
X3*X1	1	2.53375	2.320115	1.09	0.3004
X3*X2	1	-2.28625	2.320115	-0.99	0.3477
X3*X3	1	0.8128037	1.73139	0.47	0.6488

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	14760.29	3690.071	85.81	0.0001
X2	4	179.8298	44.9574	1.04	0.4320
X3	4	128.8434	32.21084	0.75	0.5813

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE
X1	-0.653282
X2	0.905645
X3	1.439734

PREDICTED VALUE AT OPTIMUM -4.14666

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
20.29908	0.9935882	-0.0889297	0.06981409
2.066502	0.1130526	0.7884059	-0.604678
-0.066117	-0.00126805	0.6086934	0.7934046

SOLUTION WAS A SADDLE POINT

TABLE 6.19(b): Y2 SELENIUM UPTAKE BY ROOT (Se, Te, Tl)

```
Minitab release 81.1 *** copyright - Penn State Univ. 1981
aug. 20, 1986 *** Avon Universities, Bristol
storage available  50000

 -- read 'EmanSe' c1-c8
olumn        c1            c2            c3            c4            c5
c6
ount         20            20            20            20            20
20
row
   1    -1.00000      -1.00000      -1.00000        9.5000        6.580
3.150
   2     1.00000      -1.00000      -1.00000       10.6600       46.450
2.600
   3    -1.00000       1.00000      -1.00000       11.0600        4.230
25.380
   4    -1.00000      -1.00000       1.00000        0.5000        4.620
6.100
  . . .

column        c7            c8
ount         20            20
row
   1       5.810      0.111000
   2      10.560      0.144000
   3       6.500      0.131000
   4      76.200      0.082000
  . . .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c5 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    3.81 +  25.5 x1 -  1.38 x2
    +  1.39 x3 +  20.1 x4 -  3.22 x5
    +  2.53 x6 +  1.42 x7 -  2.29 x8
    + 0.813 x 9

                                        st. dev.     t-ratio =
       column       coefficient       of coef.      coef/s.d.
       --             3.807             2.676          1.42
x1     c1            25.485             1.777         14.35
x2     c2            -1.384             1.777         -0.78
x3     c3             1.385             1.777          0.78
x4     c11           20.066             1.731         11.59
x5     c12           -3.219             2.320         -1.39
x6     c13            2.534             2.320          1.09
x7     c22            1.421             1.731          0.82
x8     c23           -2.286             2.320         -0.99
x9     c33            0.813             1.731          0.47

the st. dev. of y about regression line is
s = 6.562

with (  20-10) =  10 degrees of freedom

r-squared = 97.2 percent
r-squared = 94.7 percent, adjusted for d.f.

analysis of variance

 due to      df          ss          ms=ss/df
regression    9     14896.88          1655.21
residual     10       430.63            43.06
total        19     15327.51

further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9     14896.88
c1            1      8861.86
c2            1        26.14
c3            1        26.18
c11           1      5771.08
c12           1        82.88
c13           1        51.36
c22           1        26.07
c23           1        41.82
c33           1         9.49

            x1            y      pred. y      st.dev.
row         c1           c5        value   pred. y      residual      st.res.
  1      -1.00         6.58        -2.35      5.37          8.93        2.37r
 10      -1.68         8.01        17.63      5.11         -9.62       -2.34r

r denotes an obs. with a large st. res.

durbin-watson statistic = 2.39
 -- end
 -- stop

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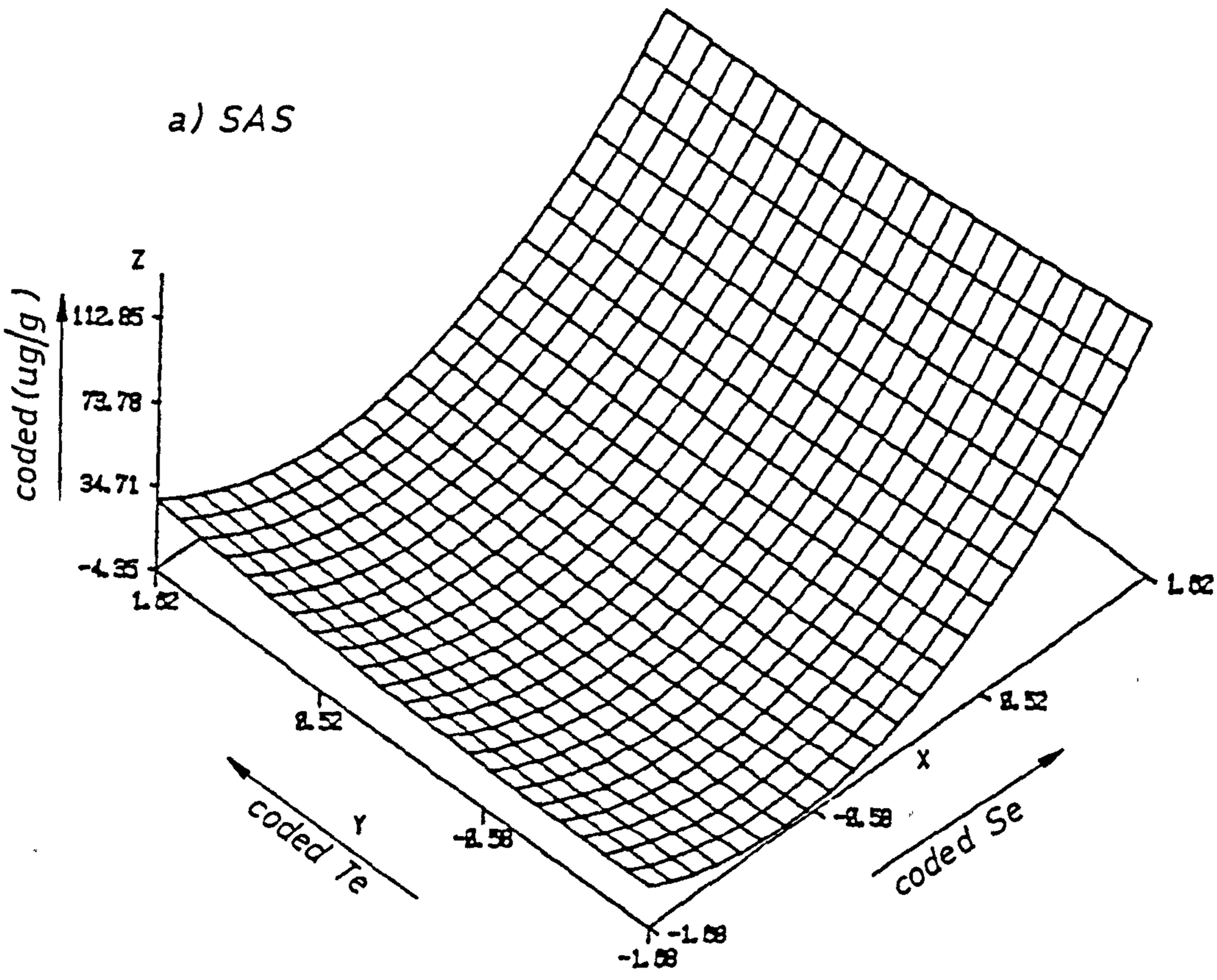
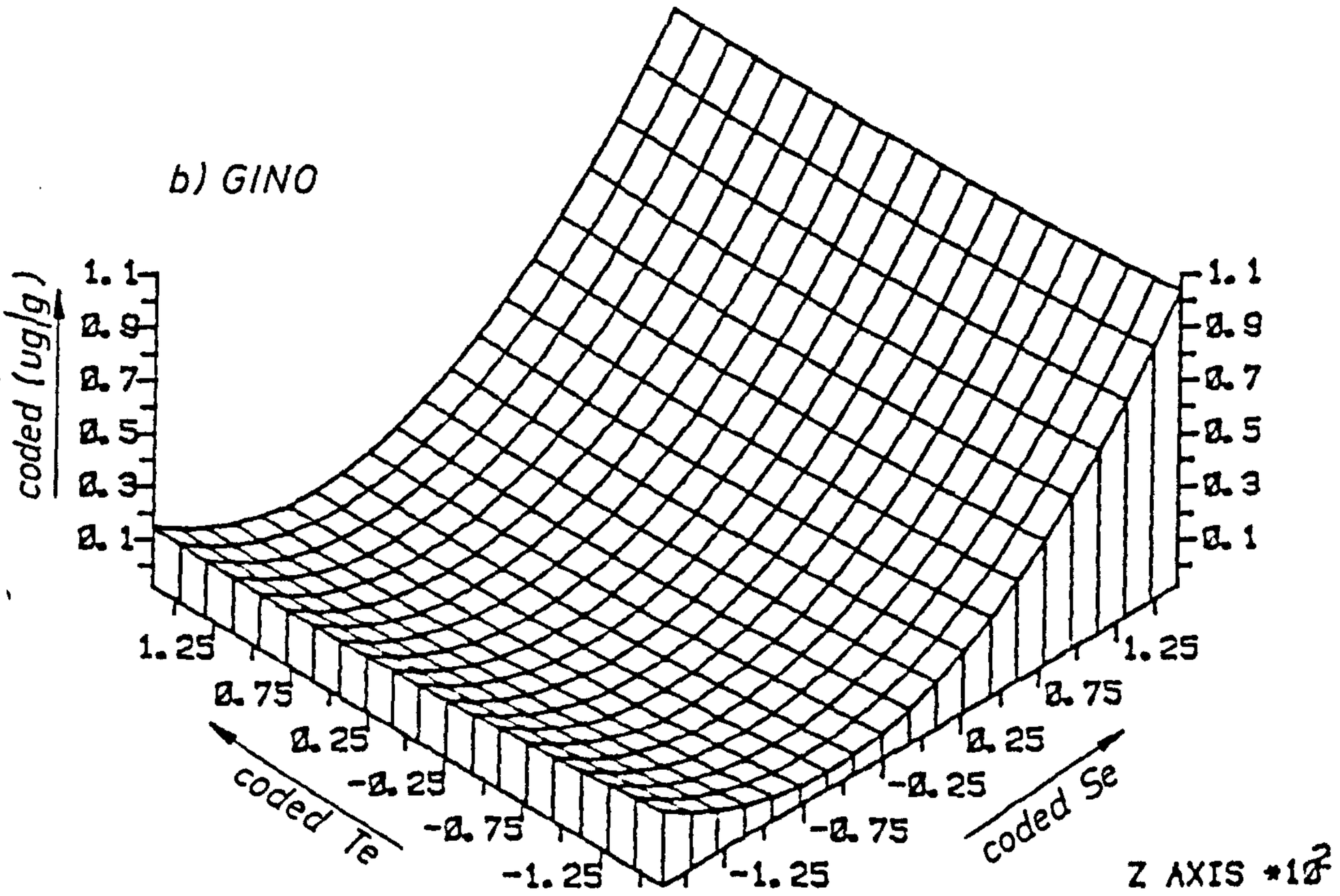
storage available   50000
```

FIG. 6.46 RESPONSE SURFACE FOR SELENIUM UPTAKE BY ROOT SEEDLINGS AT CODED T1 = 0

a) SAS



b) GINO



Se(VI) , Te(VI) , Se uptake by Root

FIG. 6.47 CONTOUR PLOT OF RESPONSE SURFACE FOR SELENIUM UPTAKE BY ROOT SEEDLING OF LOLIUM PERENNE AT CODED Tl = 0

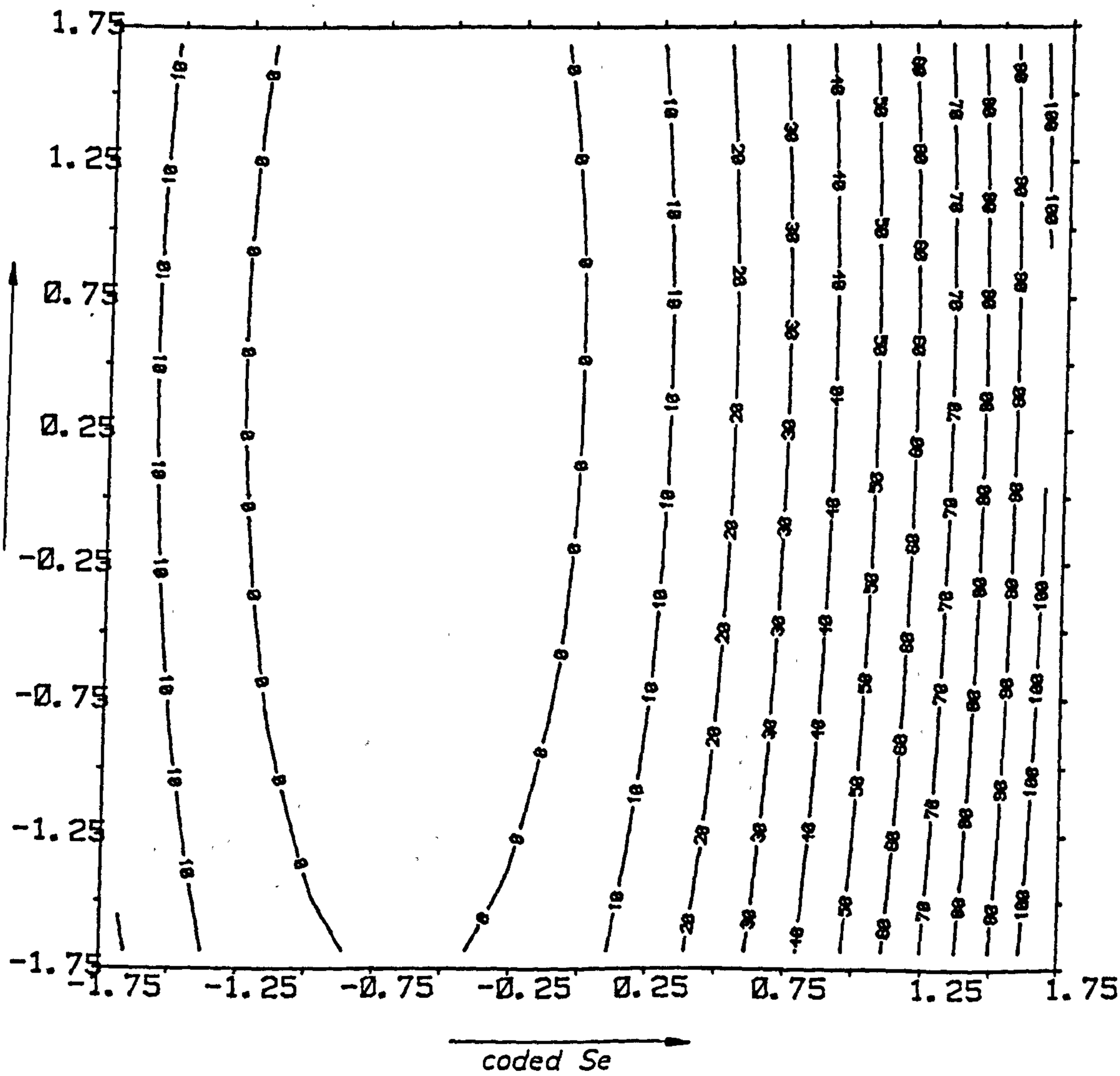
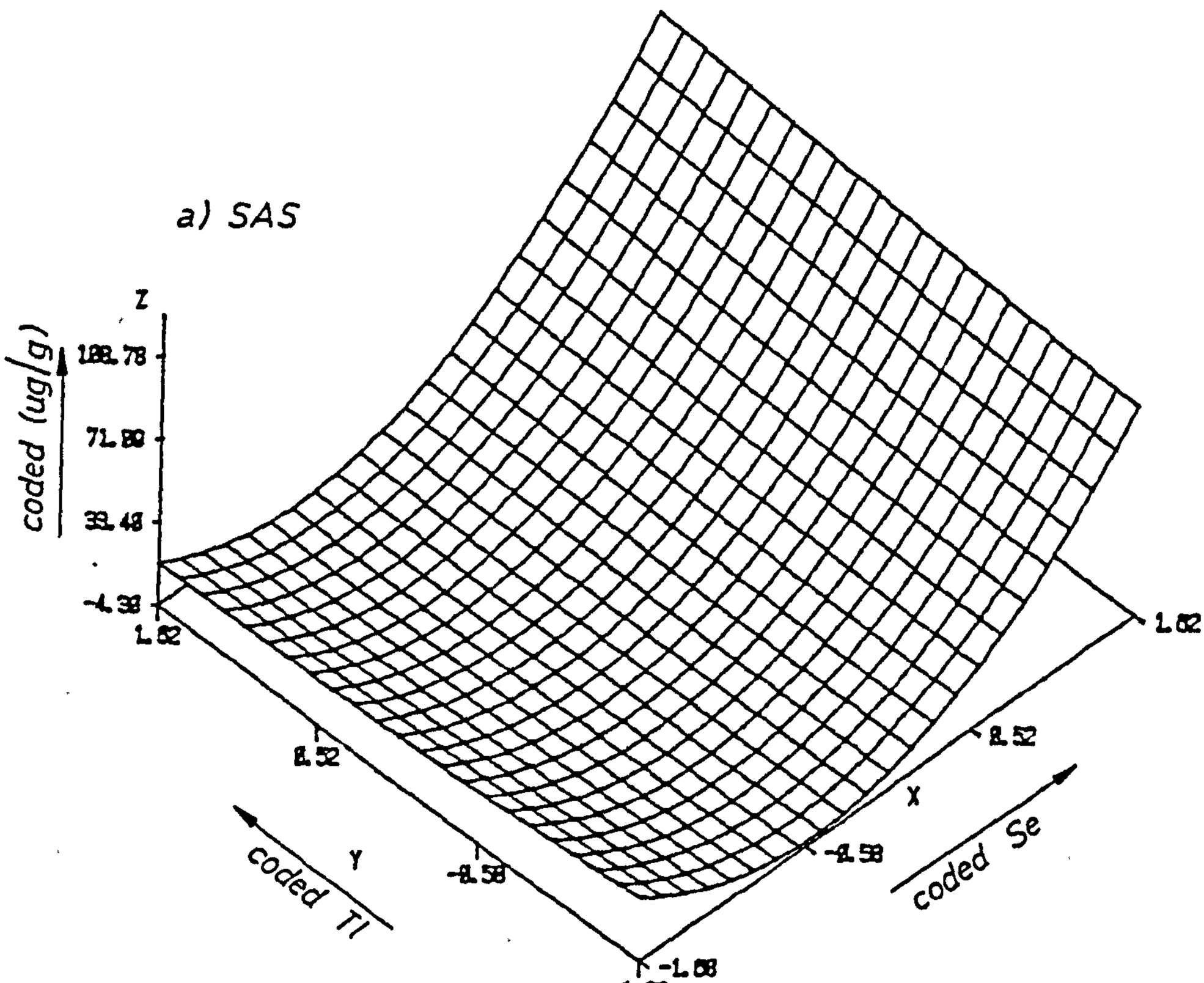
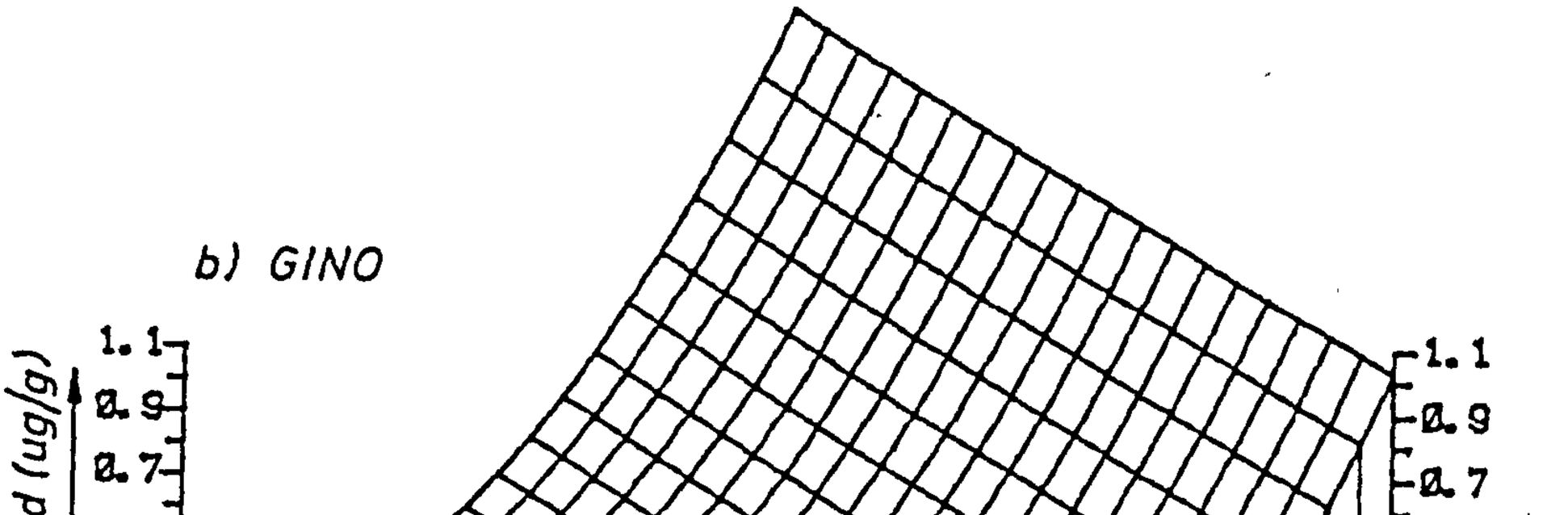


FIG. 6.48 RESPONSE SURFACE FOR SELENIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te =0

a) SAS



b) GINO



Se(VI) , Tl(I) , Se uptake by Root

FIG. 6.49 CONTOUR PLOT OF RESPONSE SURFACE FOR SELENIUM UPTAKE BY ROOT AT CODED Te = 0

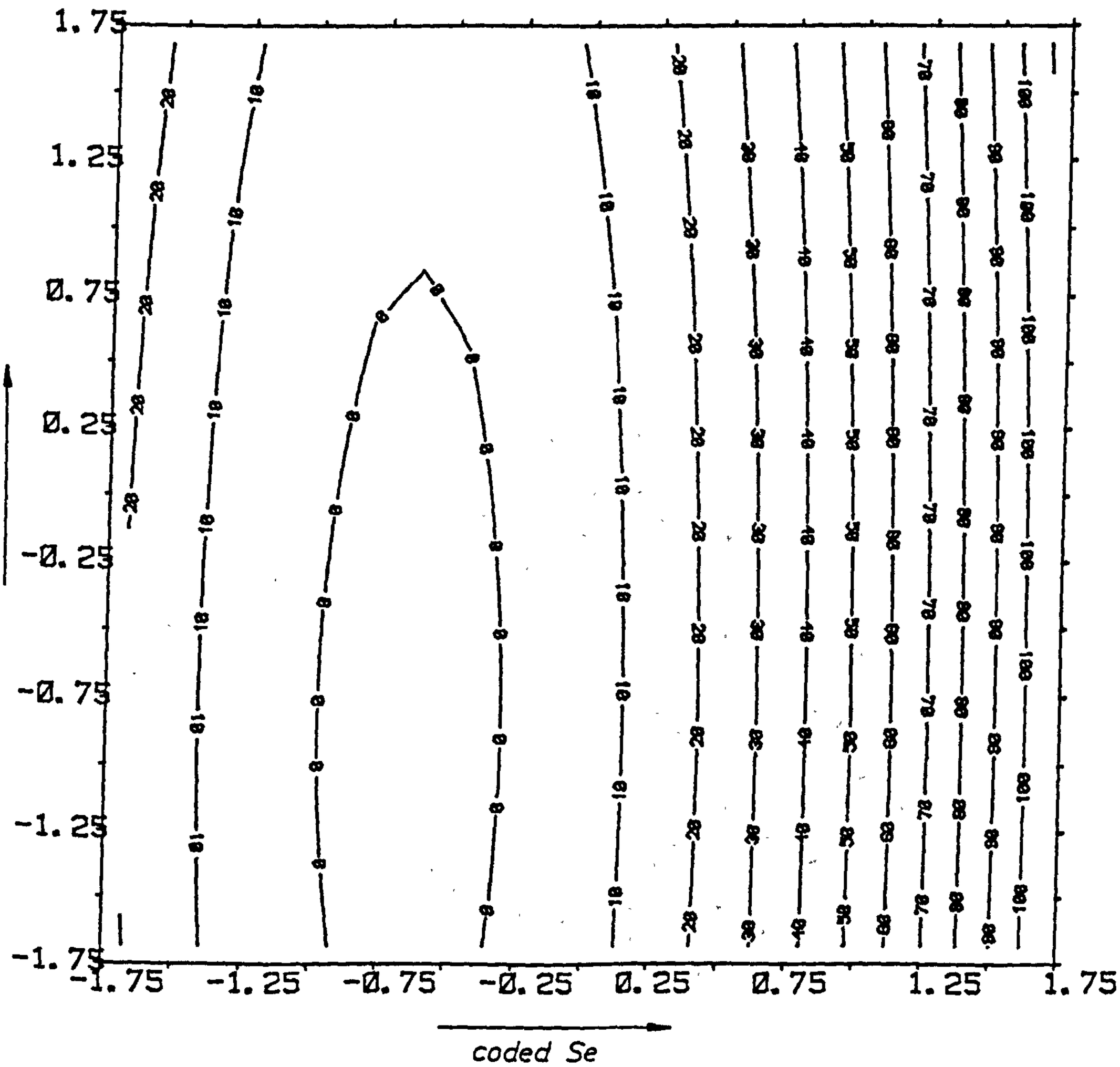
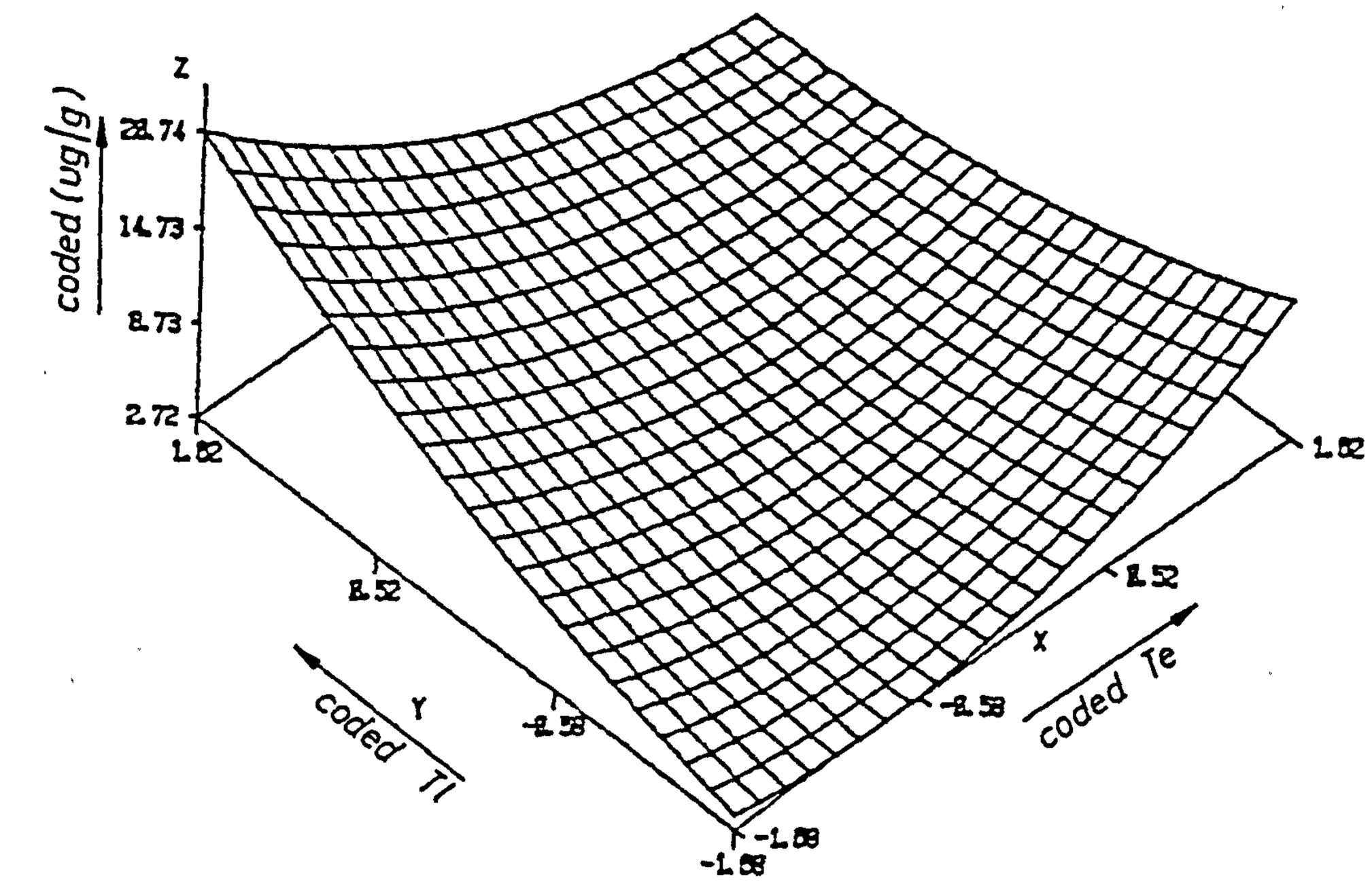
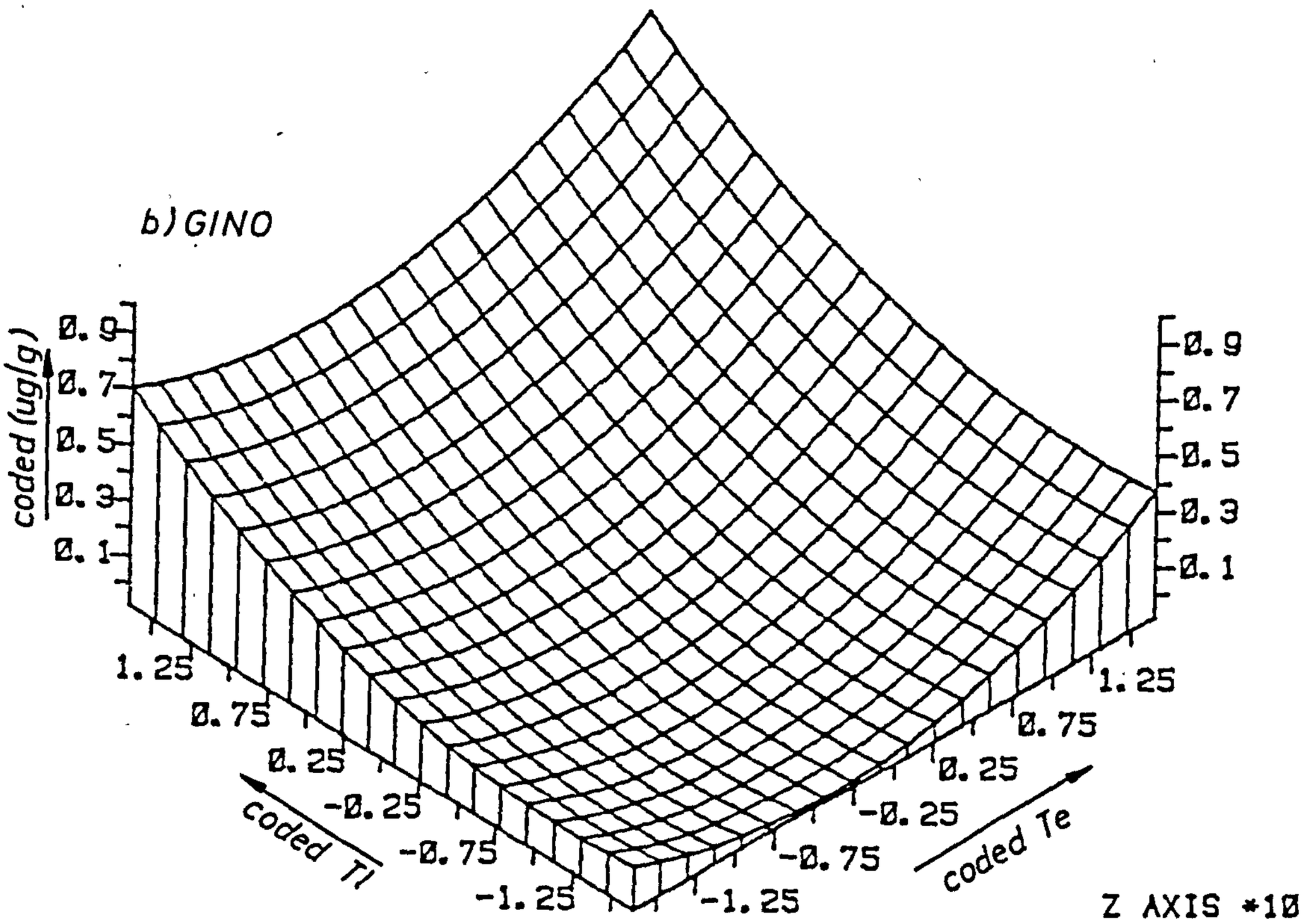


FIG. 6.50 RESPONSE SURFACE FOR SELENIUM UPTAKE BY ROOT SEEDLING AT CODED Se = 0

a) SAS

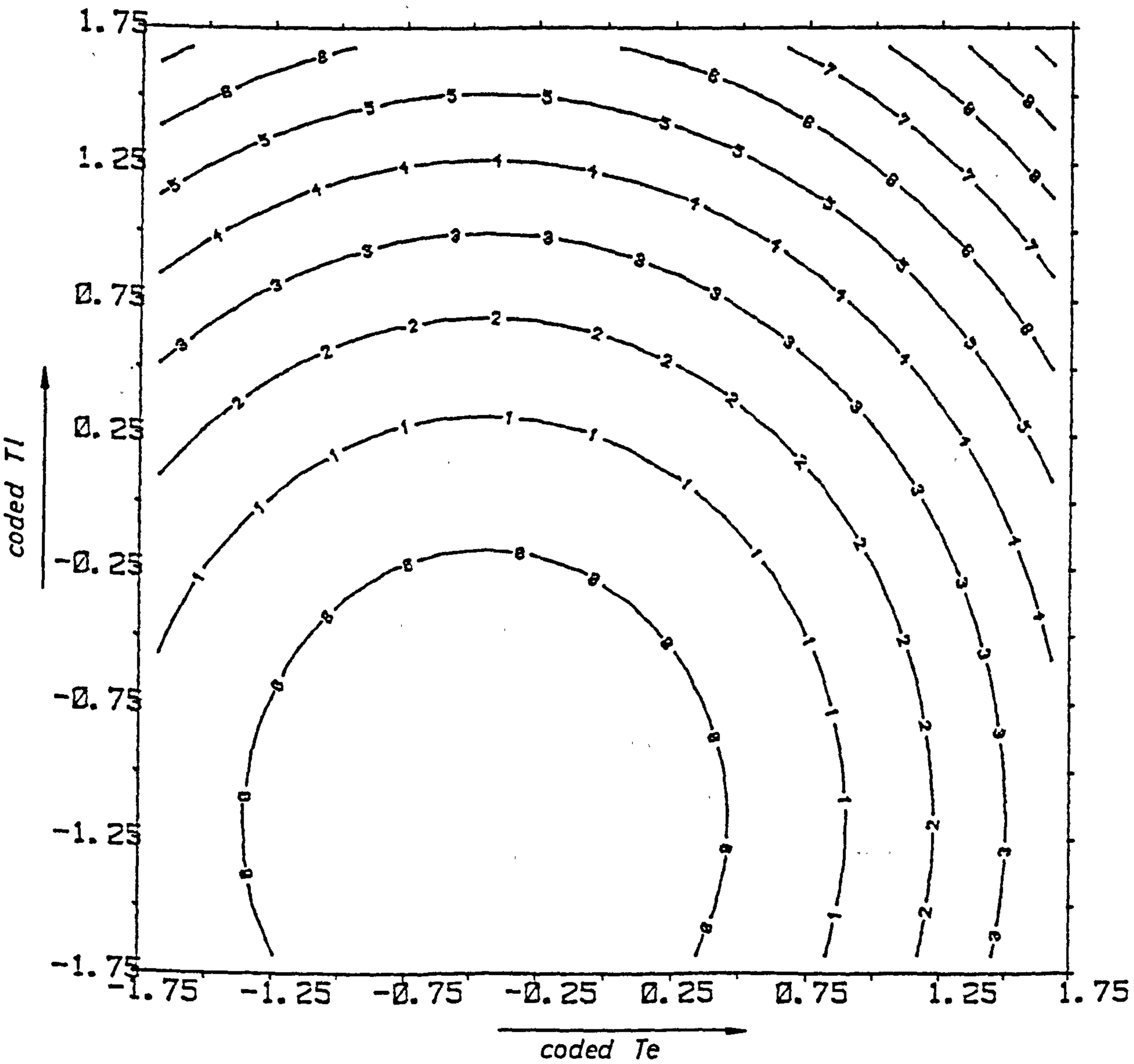


b) GINO



Te(VI), Tl(I), Se uptake by Root

FIG. 6.51 CONTOUR PLOT OF RESPONSE SURFACE FOR SELENIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT Se = 0



The Te uptake by roots is similar to that just described for the Se uptake; see Table 6.7 Response Y3, and Table 6.20(a) which describes the response parameters for the Te uptake by roots.

In this case the fit of R^2 is only 0.7885, being an 18% lesser fit (than for the Se uptake by roots), but allowing an R value of about 0.888, showing the extreme sensitivity of the R^2 value to slight changes in the fit of the model. The probability of regression values in terms of linear and quadratic terms is very high, being greater than 98% that they are factors in the model. Notice, however, that the probability of the cross-product term is very small indeed; so once again insignificant interaction terms are to be expected. The only variable of importance is the Te(VI) content of the nutrient solution, there being no effect due to the concentrations of Se(VI) or Tl(I) in the solution. An inspection of the PROB values [17] will confirm these facts; where only the linear Te(VI) and quadratic Te(VI)*Te(VI) are deemed significant. As expected they are significant at the greater than 99% level, whilst, overall, the Te(VI) content is also greater than 99% significant [18].

The Lack of Fit of the model is deemed highly significant because of the extremely small value of the Type 1 SS for the pure error values. The response surfaces confirm these findings; Figures 6.52 and 6.56 and also Figure 6.54 show the dome surface; while Figures 6.53; 6.55 and 6.57 illustrate the corresponding contour diagrams.

TABLE 6.20(a) TELLURIUM UPTAKE BY ROOT (Se,Te,Tl)

RESPONSE SURFACE FOR VARIABLE Y3

RESPONSE MEAN	17.178
ROOT MSE	22.86009
R-SQUARE	0.7885512
COEF OF VARIATION	1.330777

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	9651.335	0.3905	6.16	0.0122
QUADRATIC	3	9831.691	0.3978	6.27	0.0115
CROSSPRODUCT	3	5.579137	0.0002	0.00	0.9997
TOTAL REGRESS	9	19488.61	0.7886	4.14	0.0184

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	5223.154	1044.631	1945.067	0.0001
PURE ERROR	5	2.685333	0.5370667		
TOTAL ERROR	10	5225.839	522.5839		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	4.658563	9.322781	0.50	0.6281
X1	1	-0.389921	6.188629	-0.06	0.9510
X2	1	26.55074	6.188629	4.29	0.0016
X3	1	1.494284	6.188629	0.24	0.8141
X1*X1	1	-3.02196	6.031405	-0.50	0.6272
X2*X1	1	-0.32375	8.082264	-0.04	0.9688
X2*X2	1	24.956	6.031405	4.14	0.0020
X3*X1	1	-0.70625	8.082264	-0.09	0.9321
X3*X2	1	0.30625	8.082264	0.04	0.9705
X3*X3	1	-3.58354	6.031405	-0.59	0.5656

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	138.0925	34.52313	0.07	0.9907
X2	4	18567.21	4641.802	8.88	0.0025
X3	4	219.6858	54.92146	0.11	0.9781

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE
X1	-0.0583073
X2	-0.533504
X3	0.1914414

PREDICTED VALUE AT OPTIMUM -2.2695

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
24.95778	-0.00585387	0.9999681	0.005437284
-2.85322	0.9008834	0.00763365	-0.433994
-3.75407	0.4340216	-0.00235781	0.9008994

SOLUTION WAS A SADDLE POINT

TABLE 6.20(b) : Y3 TELLURIUM UPTAKE BY ROOT (Se,Te,Tl)

```
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aug. 20, 1986 *** Avon Universities, Bristol
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 -- read 'EmanTe' c1-c8
column        c1          c2          c3          c4          c5
c6
count         20          20          20          20          20
20
row
  1     -1.00000    -1.00000    -1.00000      9.5000      6.580
3.150
  2      1.00000    -1.00000    -1.00000     10.6600     46.450
2.600
  3     -1.00000     1.00000    -1.00000     11.0600      4.230
25.380
  4     -1.00000    -1.00000     1.00000      0.5000      4.620
6.100
  . . .

column        c7          c8
count         20          20
row
  1        5.810    0.111000
  2       10.560    0.144000
  3        6.500    0.131000
  4       76.200    0.082000
  . . .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c6 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    4.66 - 0.390 x1 +  26.6 x2
     +  1.49 x3 -  3.02 x4 - 0.324 x5
     - 0.706 x6 +  25.0 x7 + 0.306 x8
     -  3.58 x 9

                                        st. dev.    t-ratio =
       column      coefficient        of coef.    coef/s.d.
       --             4.659             9.323         0.50
x1     c1            -0.390             6.189        -0.06
x2     c2            26.551             6.189         4.29
x3     c3             1.494             6.189         0.24
x4     c11           -3.022             6.031        -0.50
x5     c12           -0.324             8.082        -0.04
x6     c13           -0.706             8.082        -0.09
x7     c22           24.956             6.031         4.14
x8     c23            0.306             8.082         0.04
x9     c33           -3.584             6.031        -0.59

the st. dev. of y about regression line is
s = 22.86

with (  20-10) =  10 degrees of freedom

r-squared = 78.9 percent
r-squared = 59.8 percent, adjusted for d.f.

analysis of variance

 due to      df          ss       ms=ss/df
regression    9       19488.6         2165.4
residual     10        5225.8          522.6
total        19       24714.4

further analysis of variance
ss explained by each variable when entered in the order given

 due to      df          ss
regression    9       19488.6
c1            1           2.1
c2            1        9618.8
c3            1          30.5
c11           1         356.2
c12           1           0.8
c13           1           4.0
c22           1        9291.1
c23           1           0.8
c33           1         184.5

              x1           y    pred. y    st.dev.
row           c1          c6     value  pred. y   residual     st.res.
 11         0.00      164.84    119.70    17.81      45.14       3.15r

r denotes an obs. with a large st. res.

durbin-watson statistic = 2.50
 -- end
 -- stop

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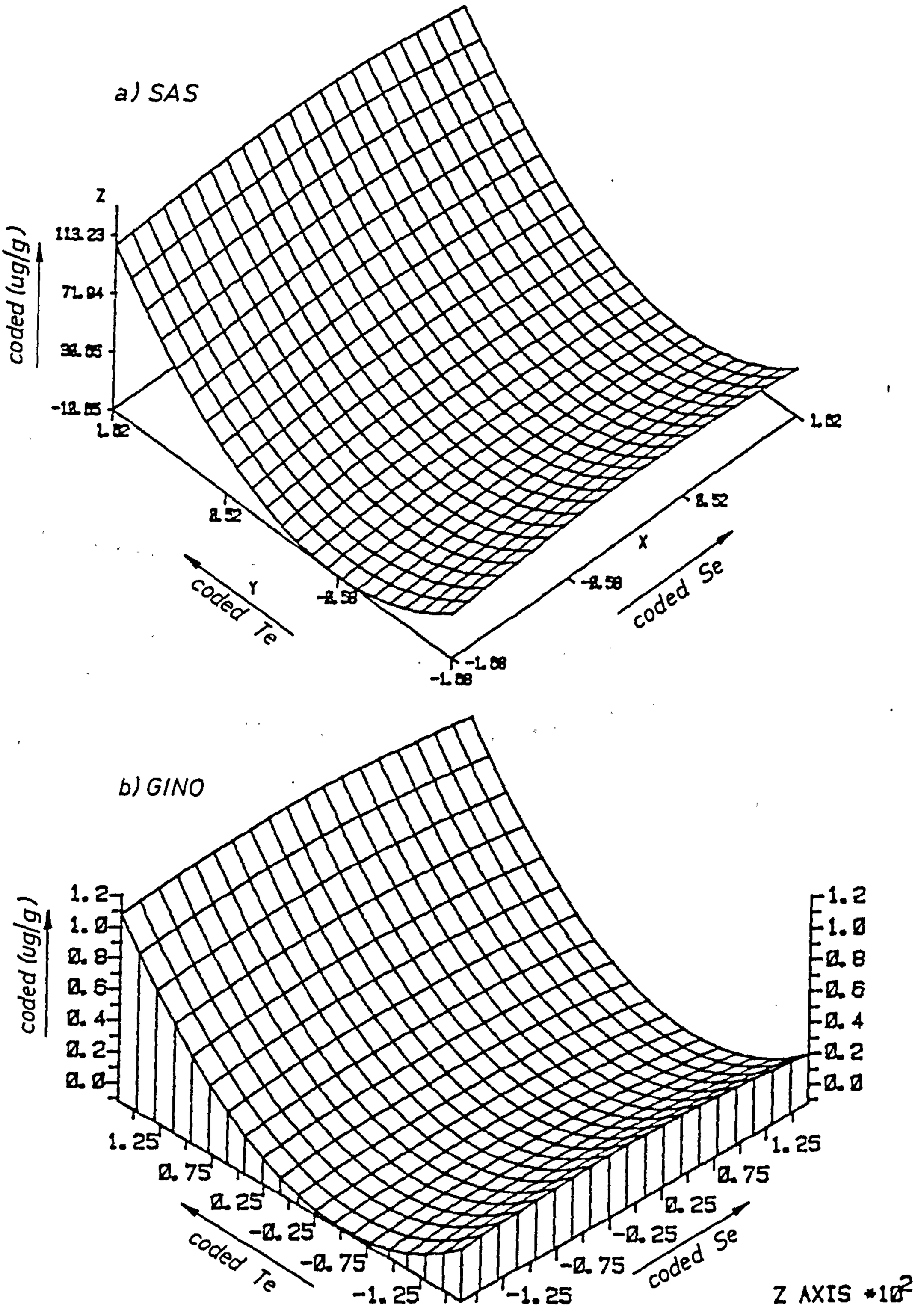
FIG. 6.52 RESPONSE SURFACE FOR TELLURIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0



Se(VI), Te(VI), Te uptake by Root

FIG. 6.53 CONTOUR PLOT OF RESPONSE SURFACE FOR TELLURIUM UPTAKE BY ROOT SEEDLING OF LOLIUM PERENNE AT CODED Tl = 0

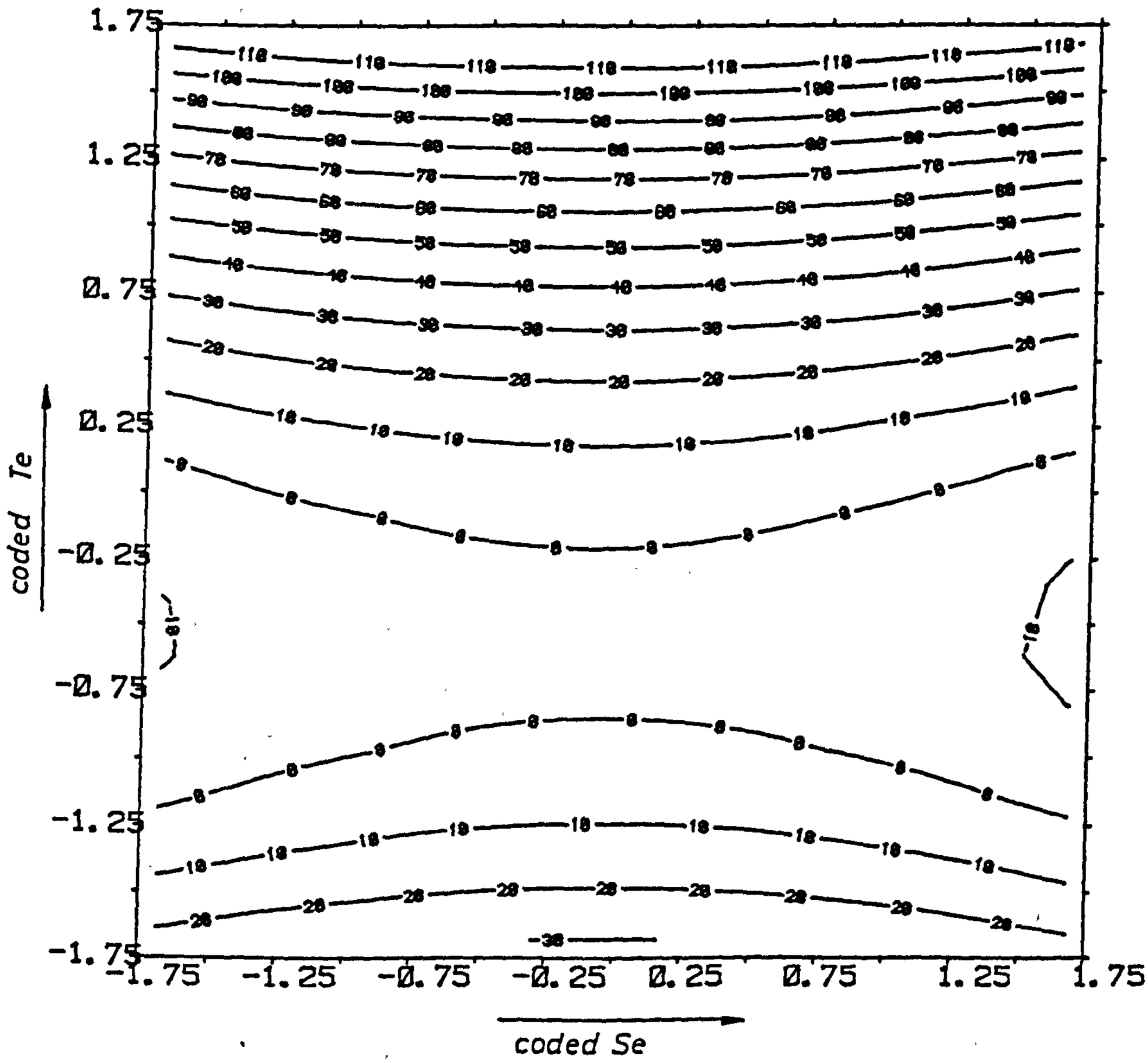
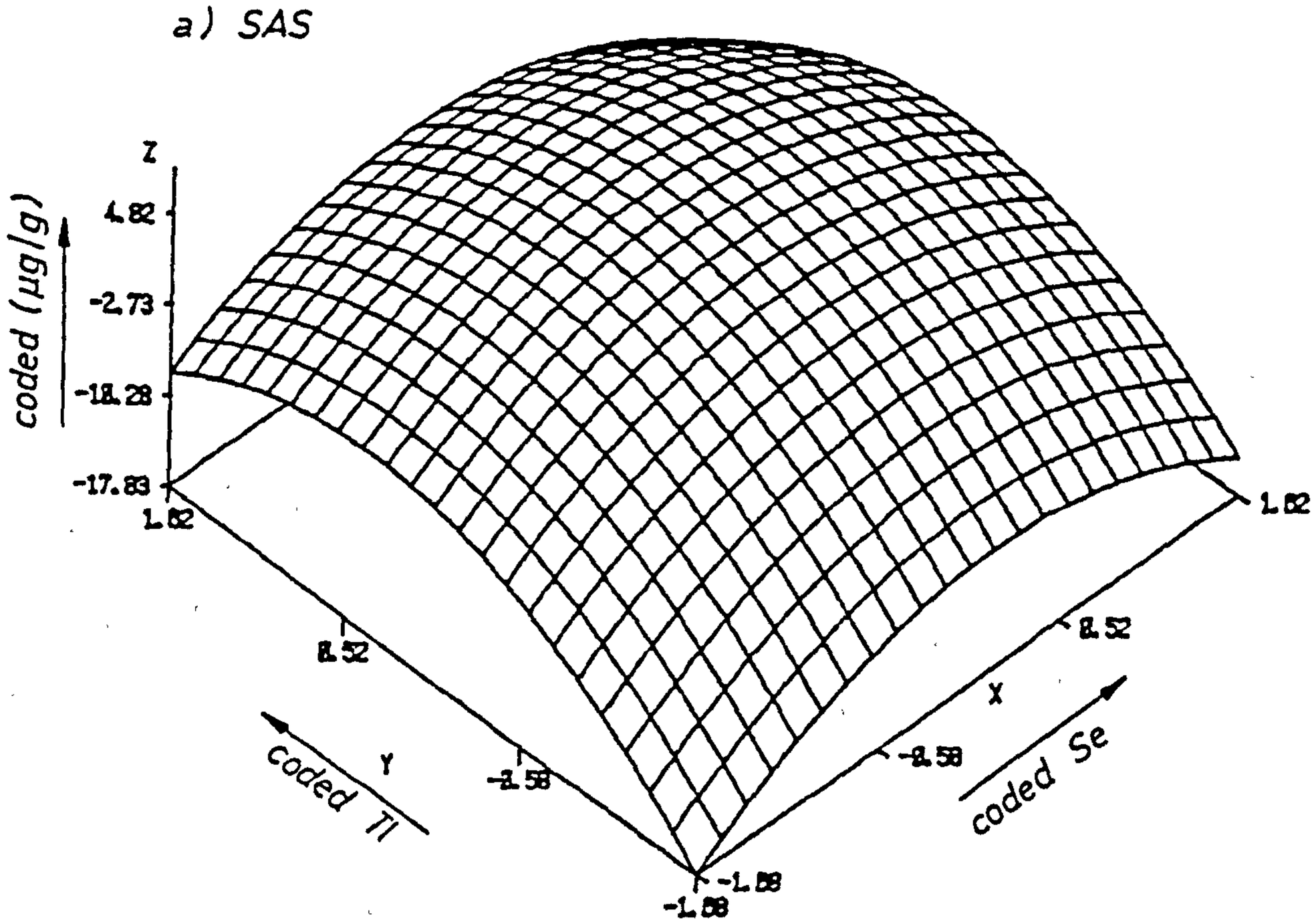
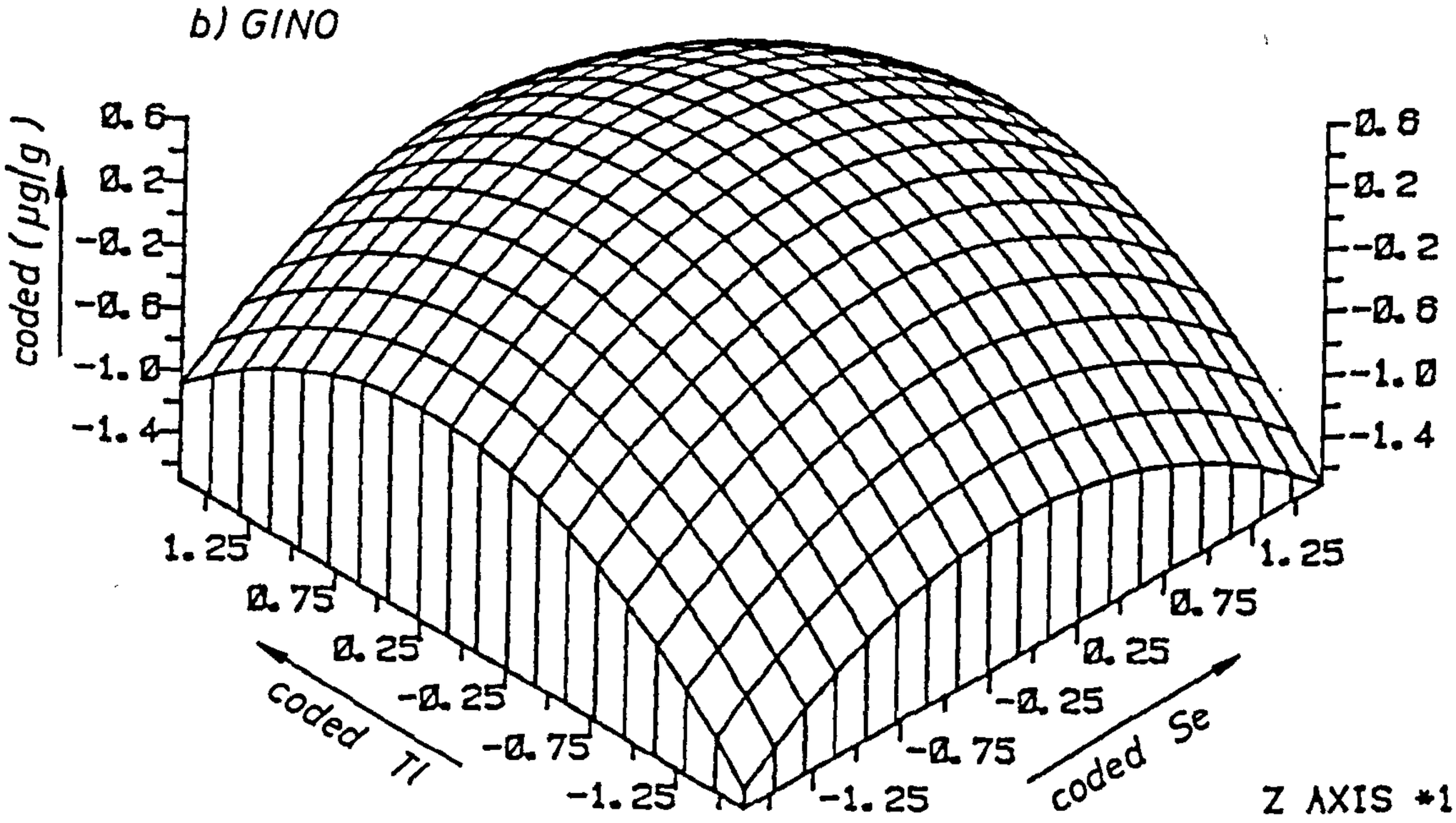


FIG. 6.54 RESPONSE SURFACE FOR TELLURIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te =0

a) SAS



b) GINO



Se(VI), Tl(I), Te uptake by Root

FIG. 6.55 CONTOUR PLOT OF RESPONSE SURFACE FOR TELLURIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

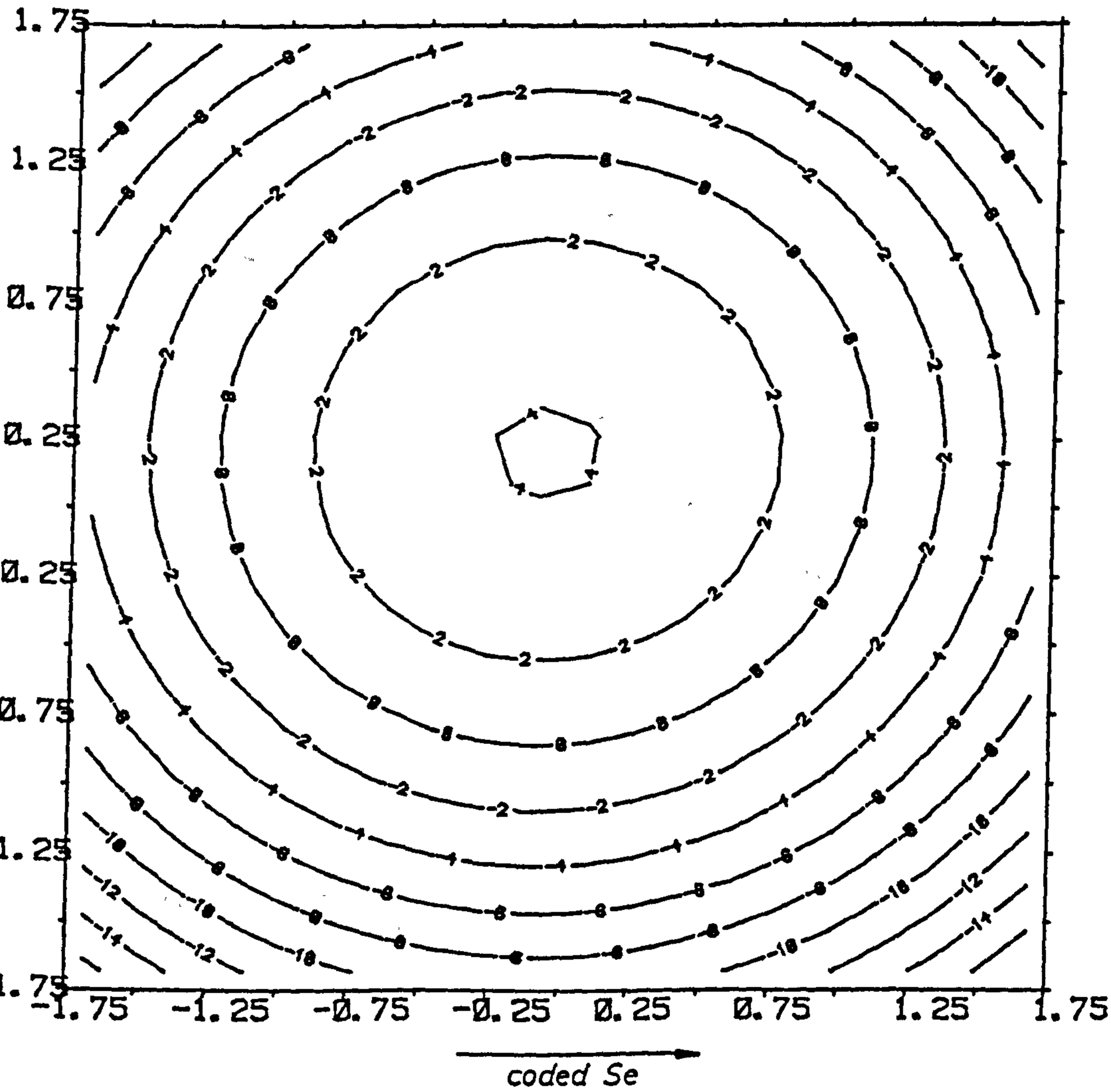
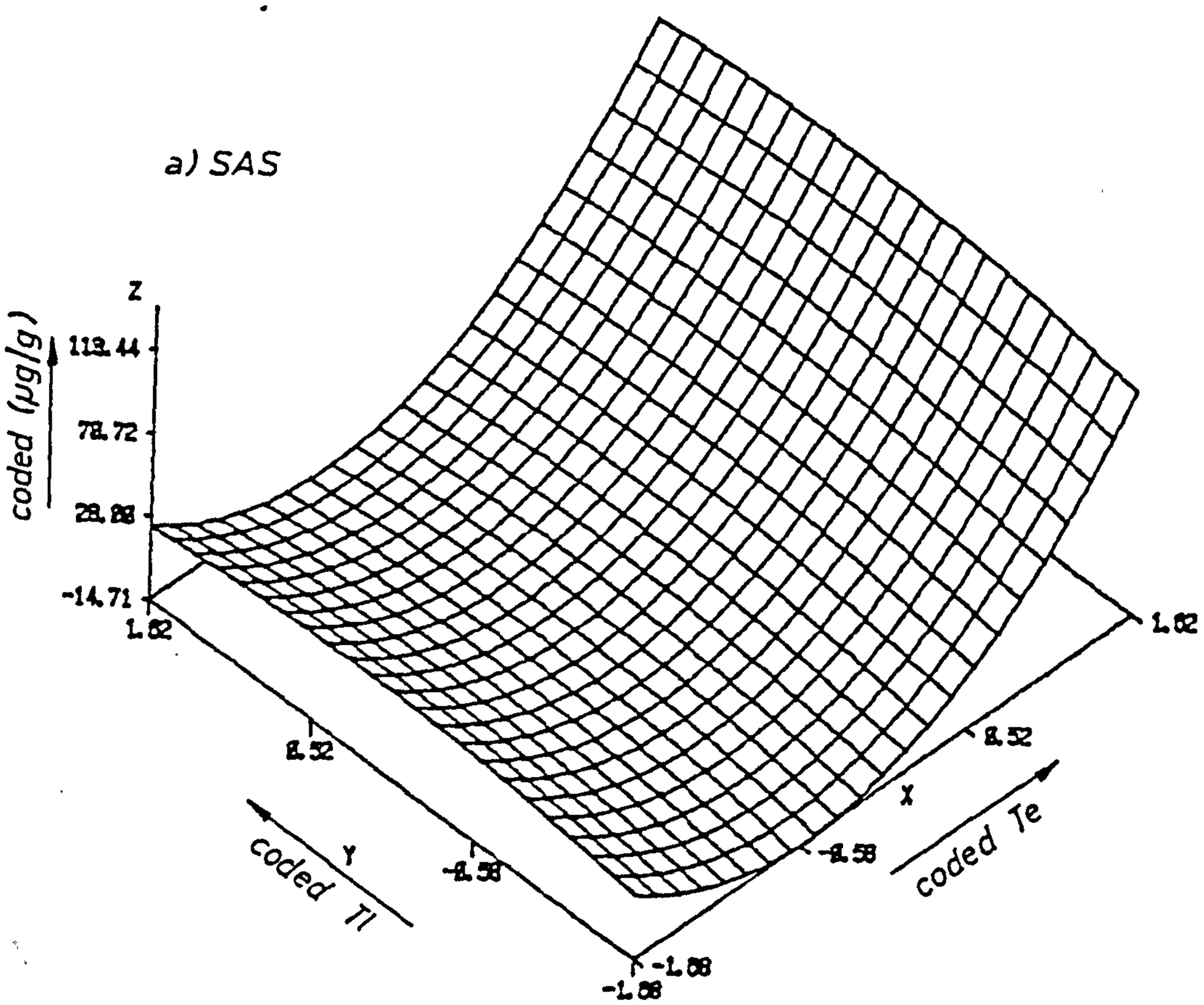
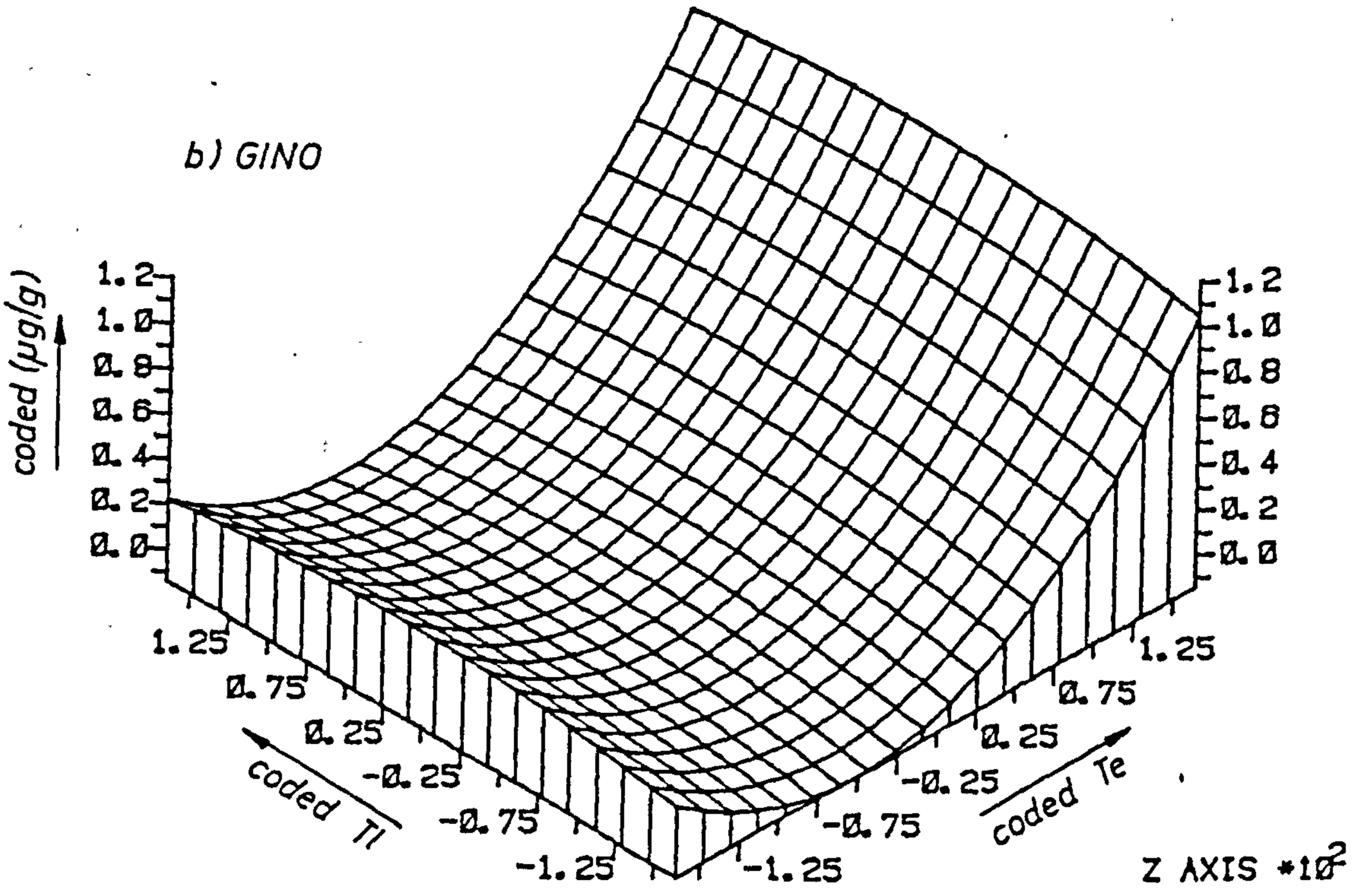


FIG.6.56 RESPONSE SURFACE FOR TELLURIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

a) SAS

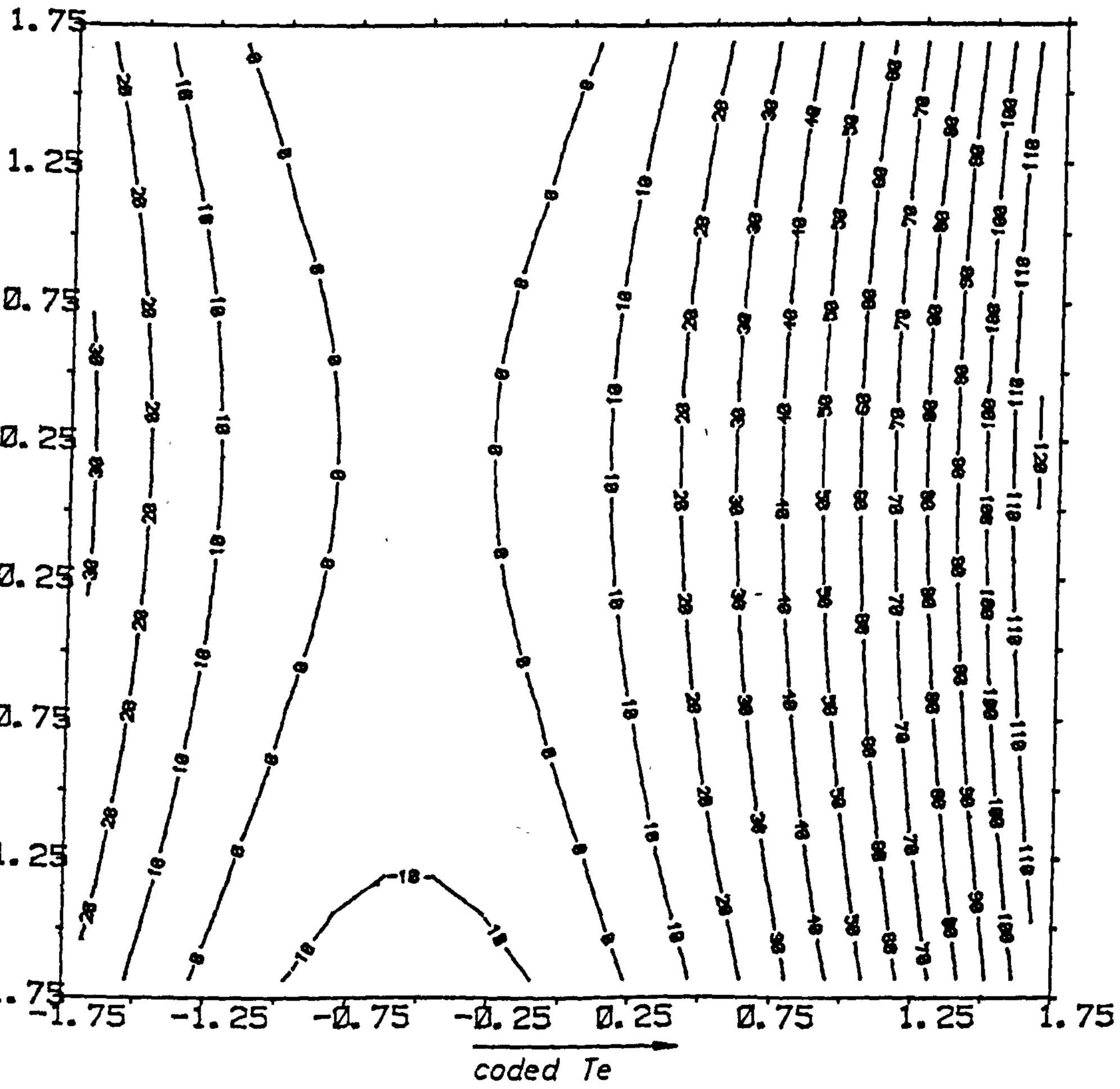


b) GINO



Te(VI), Tl(I), Te uptake by Root

FIG. 6.57 CONTOUR PLOT OF RESPONSE SURFACE FOR TELLURIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0



In an analogous manner to the selenium (VI) or tellurium (VI) uptake, the thallium (I) uptake from nutrient solution into the root system appears to be only dependent on the Tl(I) concentration in the nutrient solution. Table 6.21(a) describes the response variable statistics for this uptake.

Note that the fit of R^2 is 0.8417, being lower than the Se(VI) figure but better than the Te(VI) value. Again very similar values for the probabilities of each of the factors being involved in the process of uptake are noted, except that Tl(I) is the dominant factor, whilst the other two are non-significant. Thus the probability of there being no effect by the Se(VI) and Te(VI) on Tl(I) uptake is greater than 0.91 in each case. However, for the Tl(I) concentration in solution the same figure is 0.0007, i.e. there is less than a 1 in 10^5 chance that the Tl(I) concentration is not involved. Again the lack of interaction terms is not completely unexpected because of the lack of competition of Se(VI) or Te(VI) with Tl(I) as an interferent in the K^+-transport system.

Examination of the response surfaces in Figures 6.58 and 6.60 will confirm these findings, as soon as Tl(I) concentration in solution is above a coded value of -0.5, then the concentration of Tl(I) in the tissue rises almost exponentially. Clearly the Se(VI) or Te(VI) concentrations have almost no effect on the situation.

However, the lack of fit of the model to the data is exemplified by Figures 6.62 and 6.62, where although the toxicities of Se(VI) and Te(VI) and their effect on Tl(I) uptake are shown, clearly they are much exaggerated, especially since a negative concentration cannot be obtained.

TABLE 6.21(a) THALLIUM UPTAKE BY ROOT (Se,Te,Tl)

RESPONSE SURFACE FOR VARIABLE Y4

RESPONSE MEAN	48.0895
ROOT MSE	48.54602
R-SQUARE	0.8417321
COEF OF VARIATION	1.009493

REGRESSION	DF	TYPE I SS	R-SQUARE	F-RATIO	PROB
LINEAR	3	69707.79	0.4681	9.86	0.0025
QUADRATIC	3	55523.13	0.3729	7.85	0.0055
CROSSPRODUCT	3	108.6829	0.0007	0.02	0.9972
TOTAL REGRESS	9	125339.6	0.8417	5.91	0.0052

RESIDUAL	DF	SS	MEAN SQUARE	F-RATIO	PROB
LACK OF FIT	5	23540.99	4708.198	899.509	0.0001
PURE ERROR	5	26.17093	5.234187		
TOTAL ERROR	10	23567.16	2356.716		

PARAMETER	DF	ESTIMATE	STD DEV	T-RATIO	PROB
INTERCEPT	1	23.93764	19.79799	1.21	0.2544
X1	1	-1.00495	13.14226	-0.08	0.9406
X2	1	0.3268938	13.14226	0.02	0.9806
X3	1	71.46767	13.14226	5.44	0.0003
X1*X1	1	-10.204	12.80838	-0.80	0.4441
X2*X1	1	-3.62875	17.16361	-0.21	0.8368
X2*X2	1	-12.1545	12.80838	-0.95	0.3650
X3*X1	1	-0.16875	17.16361	-0.01	0.9923
X3*X2	1	0.62375	17.16361	0.04	0.9717
X3*X3	1	57.75939	12.80838	4.51	0.0011

FACTOR	DF	SS	MEAN SQUARE	F-RATIO	PROB
X1	4	1615.118	403.7795	0.17	0.9481
X2	4	2232.153	558.0381	0.24	0.9112
X3	4	117621.1	29405.28	12.48	0.0007

SOLUTION FOR OPTIMUM RESPONSE

FACTOR	CRITICAL VALUE
X1	-0.044886
X2	0.004271045
X3	-0.618756

PREDICTED VALUE AT OPTIMUM 1.850379

EIGENVALUES	EIGENVECTORS		
	X1	X2	X3
57.76091	-0.00136146	0.004496029	0.999989
-9.12023	0.8584176	-0.51294	0.003474931
-13.2399	0.5129497	0.8584129	-0.00316112

SOLUTION WAS A SADDLE POINT

TABLE 6.21(b): Y4 THALLIUM UPTAKE BY ROOT (Se,Te,Tl)

```
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 -- read 'EmanTl' c1-c8
column        c1          c2          c3          c4          c5
c6
count         20          20          20          20          20
20
row
   1    -1.00000    -1.00000    -1.00000      9.5000      6.580
3.150
   2     1.00000    -1.00000    -1.00000     10.6600     46.450
2.600
   3    -1.00000     1.00000    -1.00000     11.0600      4.230
25.380
   4    -1.00000    -1.00000     1.00000      0.5000      4.620
6.100
   .  .  .

column        c7          c8
count         20          20
row
   1       5.810    0.111000
   2      10.560    0.144000
   3       6.500    0.131000
   4      76.200    0.082000
   .  .  .

 -- let c11=c1*c1
 -- let c22=c2*c2
 -- let c33=c3*c3
 -- let c12=c1*c2
 -- let c13=c1*c3
 -- let c23=c2*c3
 -- regress c7 9 c1-c3,c11-c13,c22,c23,c33

the regression equation is
y =    23.9 -  1.00 x1 + 0.327 x2
    +  71.5 x3 -  10.2 x4 -  3.63 x5
    - 0.169 x6 -  12.2 x7 + 0.624 x8
    +  57.8 x 9

                                        st. dev.    t-ratio =
      column      coefficient         of coef.    coef/s.d.
      --             23.94              19.80         1.21
x1    c1             -1.00              13.14        -0.08
x2    c2              0.33              13.14         0.02
x3    c3             71.47              13.14         5.44
x4    c11           -10.20              12.81        -0.80
x5    c12            -3.63              17.16        -0.21
x6    c13            -0.17              17.16        -0.01
x7    c22           -12.15              12.81        -0.95
x8    c23             0.62              17.16         0.04
x9    c33            57.76              12.81         4.51

the st. dev. of y about regression line is
s = 48.55

with (  20-10) =  10 degrees of freedom

r-squared = 84.2 percent
r-squared = 69.9 percent, adjusted for d.f.

analysis of variance

 due to     df          ss       ms=ss/df
regression   9      125340          13927
residual    10       23567           2357
total       19      148907

further analysis of variance
ss explained by each variable when entered in the order given

 due to     df          ss
regression   9      125340
c1           1          14
c2           1           1
c3           1       69693
c11          1        2985
c12          1         105
c13          1           0
c22          1        4613
c23          1           3
c33          1       47925

          x1           y    pred. y    st.dev.
row       c1          c7     value   pred. y   residual     st.res.
 13     0.00       401.4     307.0      37.8       94.3       3.10r

r denotes an obs. with a large st. res.

durbin-watson statistic = 2.17
 -- end
 -- stop

*** Minitab *** Statistics Dept * Penn State Univ. * release 81.1 *
storage available   50000
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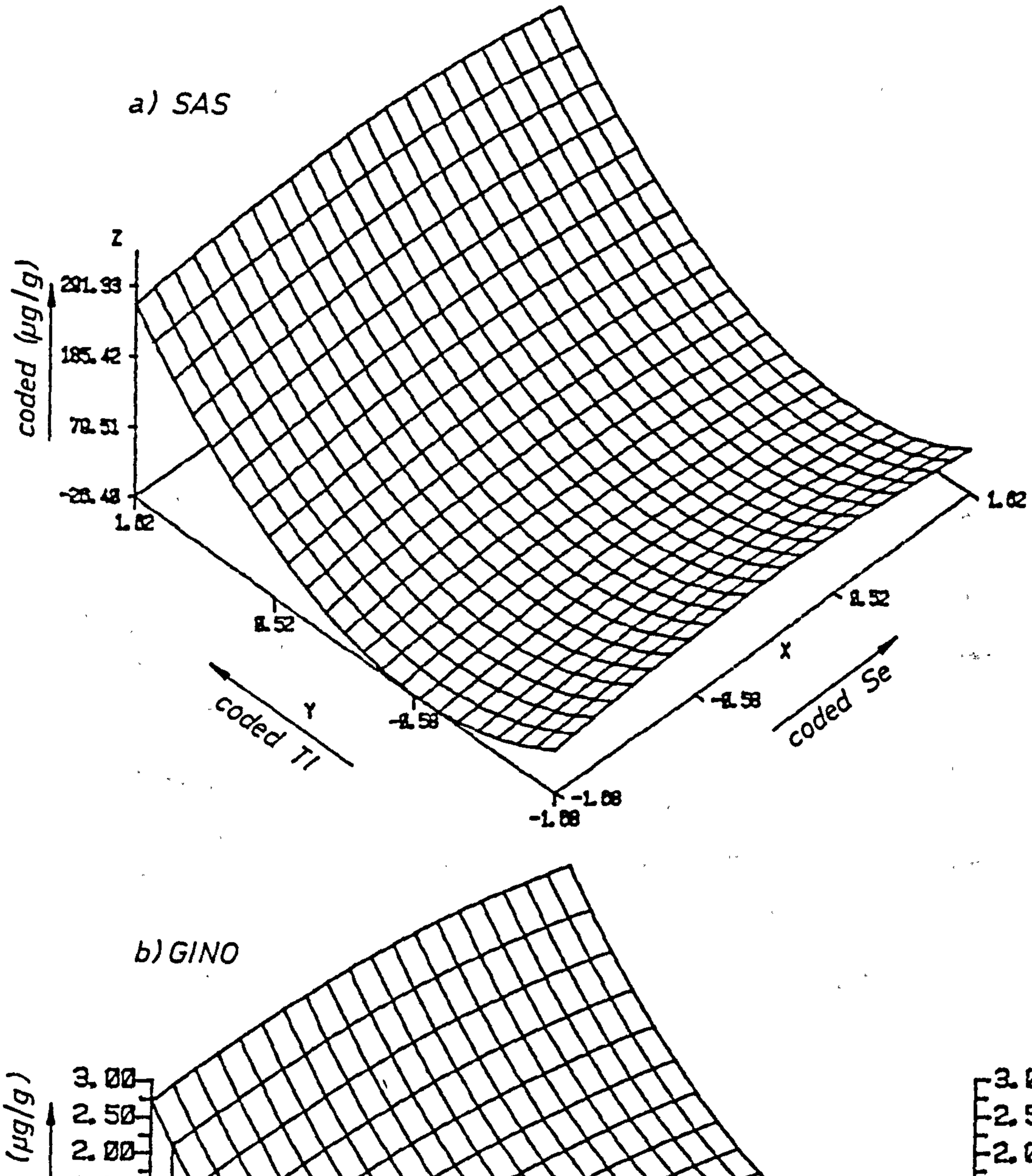
FIG. 6.58 RESPONSE SURFACE FOR THALLIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0



Se(VI). Tl(I). Tl uptake by Root

FIG. 6.59 CONTOUR PLOT FOR THALLIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Te = 0

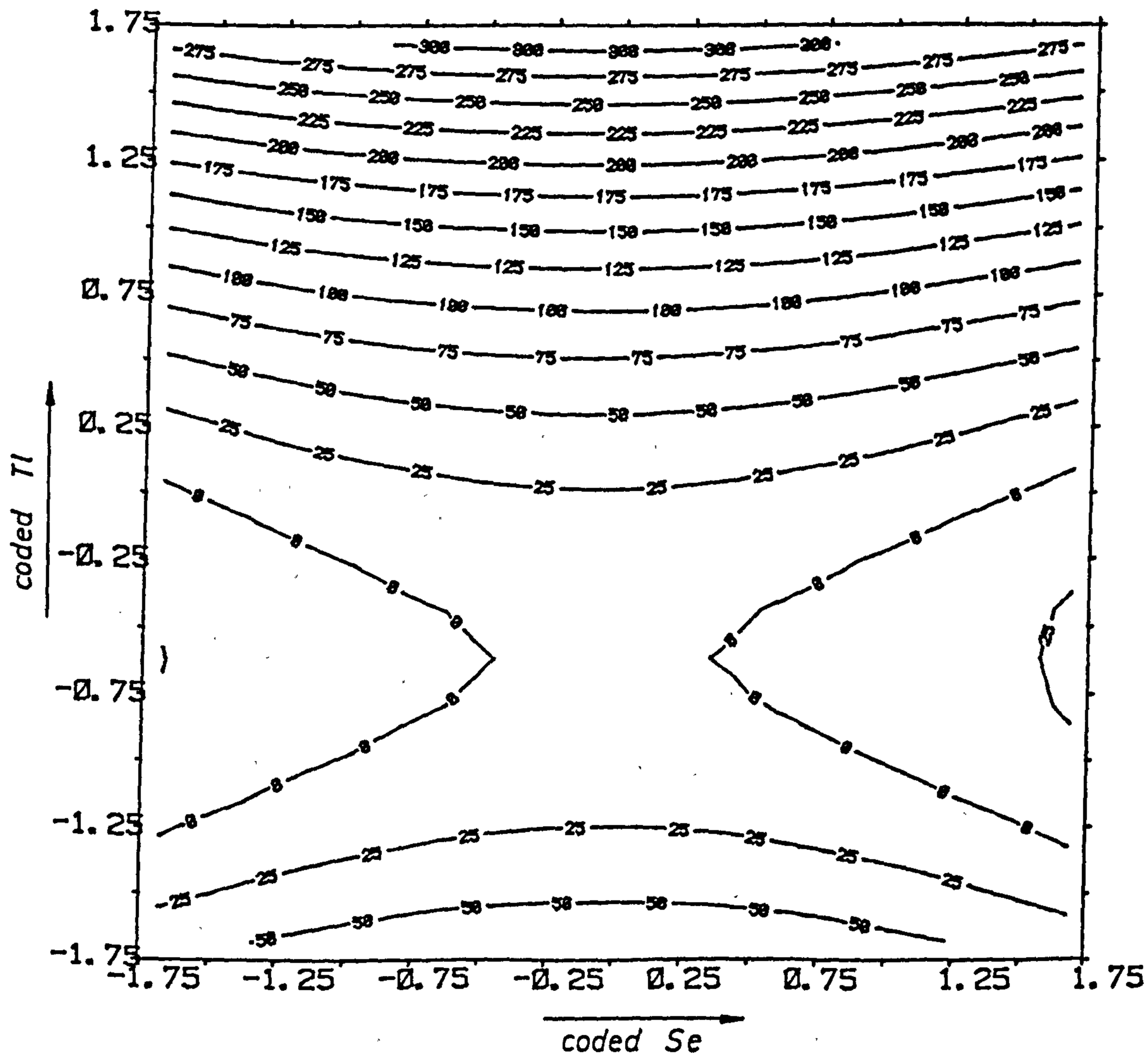
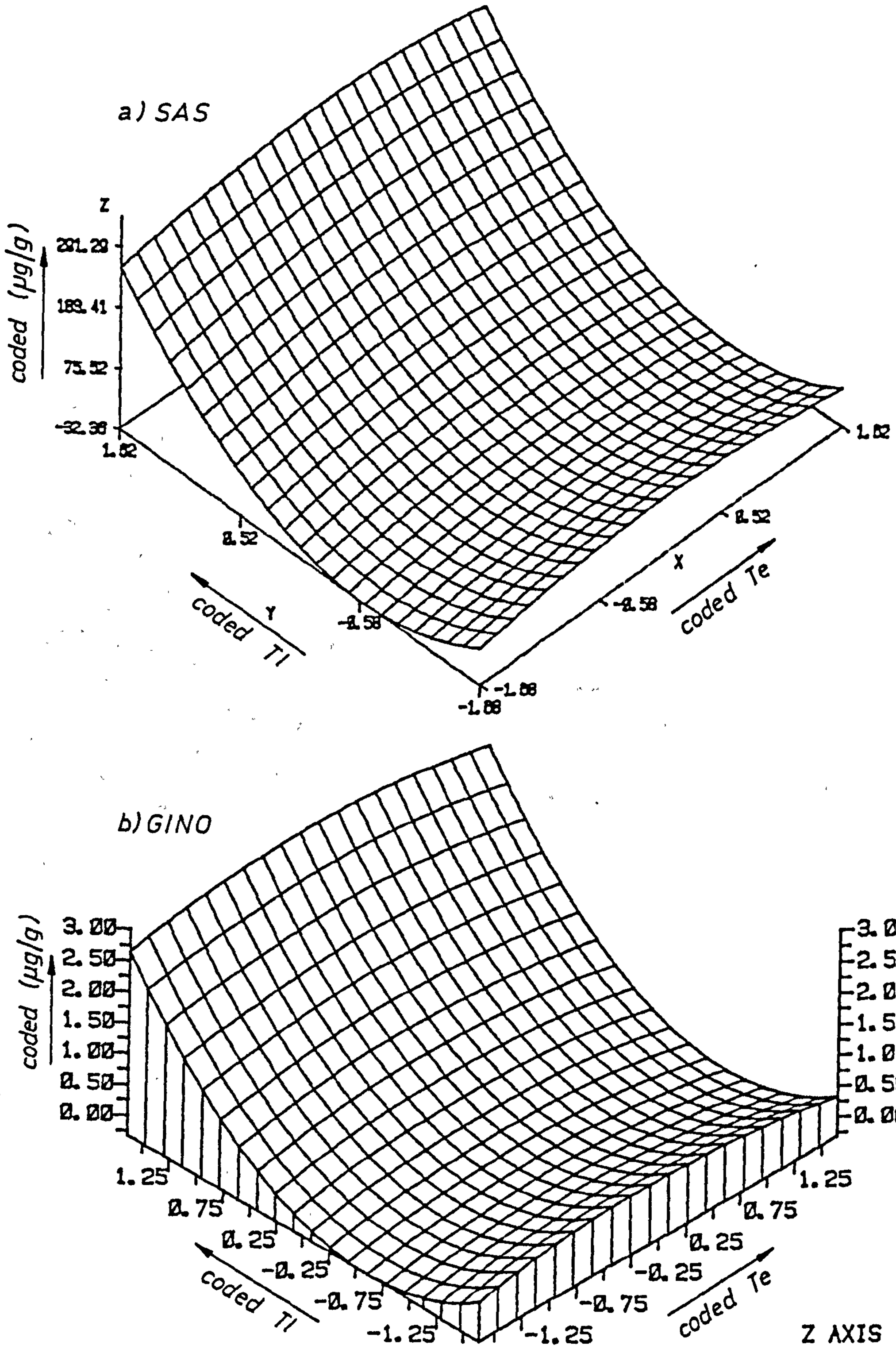


FIG. 6.60 RESPONSE SURFACE FOR THALLIUM UPTAKE BY ROOT SEEDLING OF LOLIUM PERENNE AT CODED Se = 0



Te(VI), Tl(I) , Tl uptake by Root

FIG.6.61 CONTOUR PLOT OF RESPONSE SURFACE FOR THALLIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED Se = 0

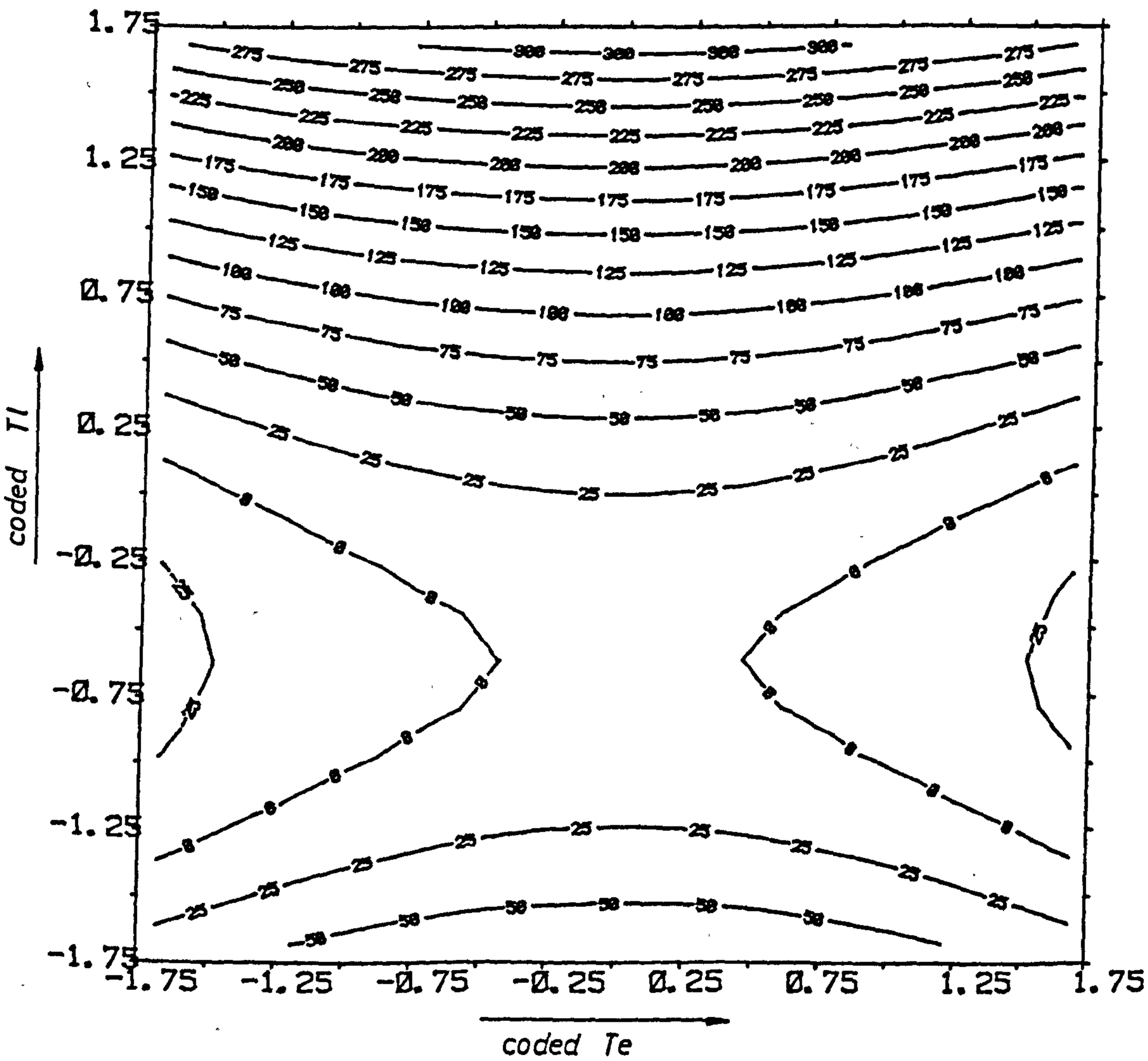
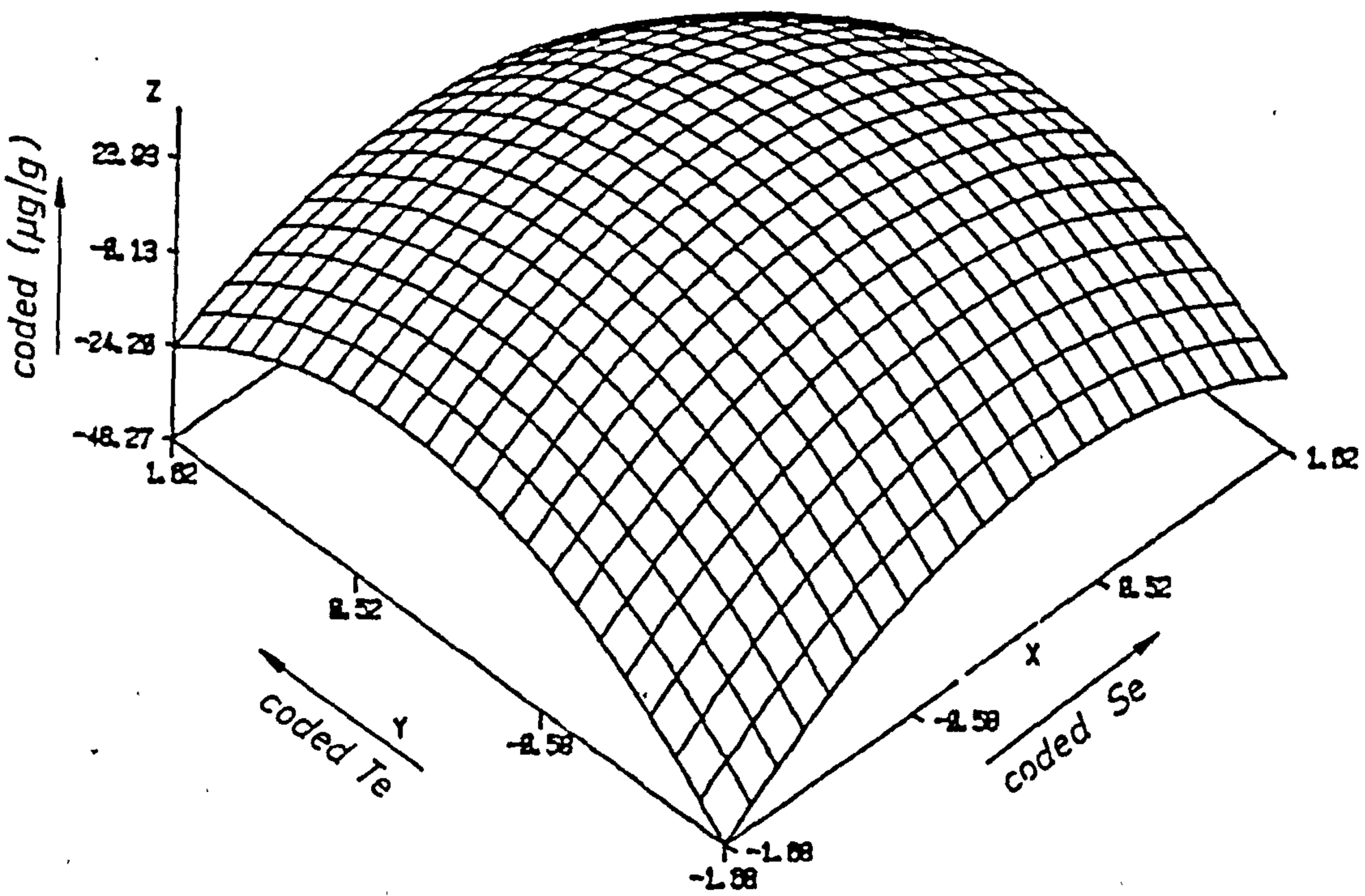
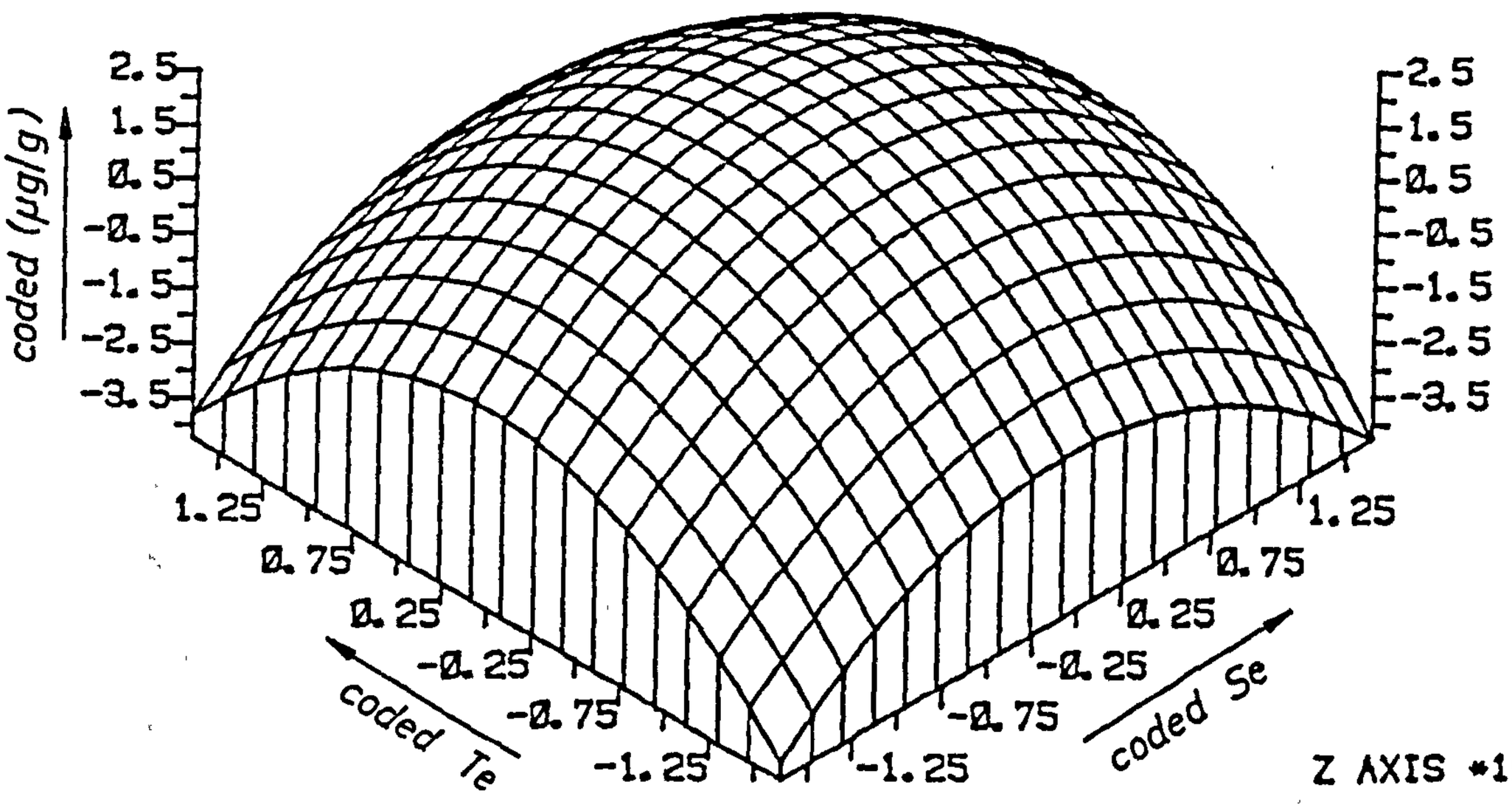


FIG. 6.62 RESPONSE SURFACE FOR THALLIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0

a) SAS

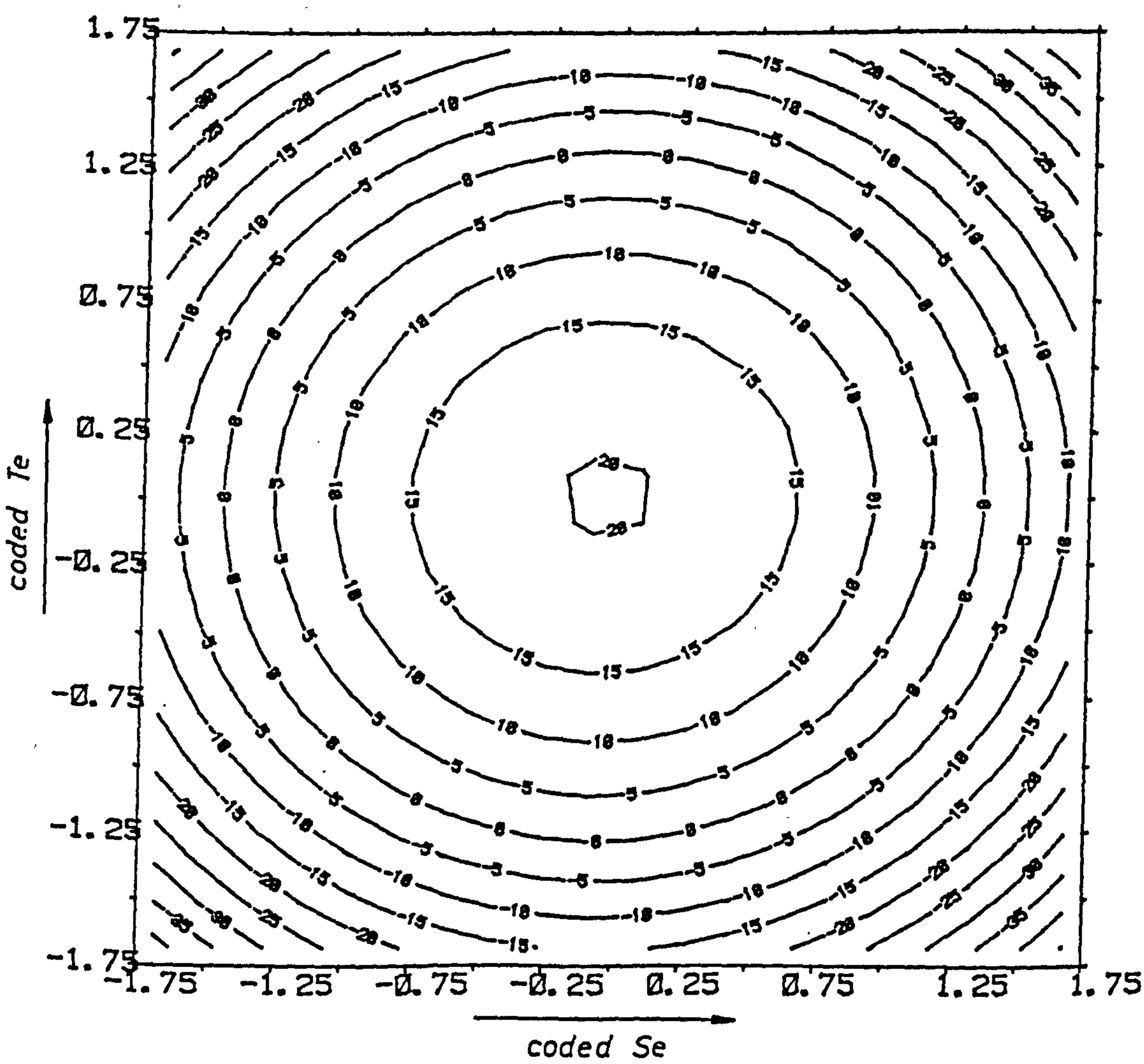


b) GINO



Se(VI), Te(VI), Tl uptake by Root

FIG. 6.63 CONTOUR PLOT OF RESPONSE SURFACE FOR THALLIUM UPTAKE BY ROOT SEEDLINGS OF LOLIUM PERENNE AT CODED TI = 0

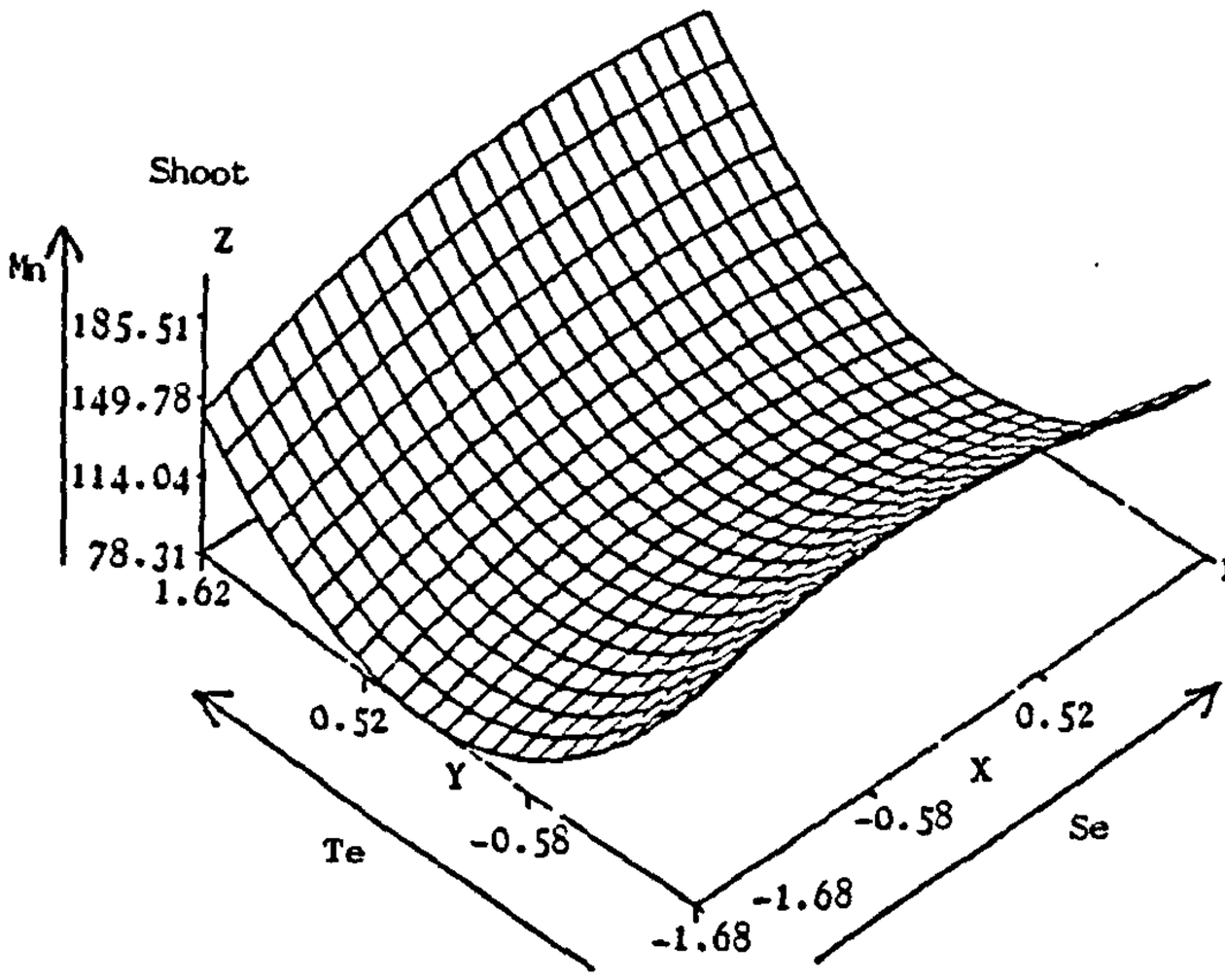


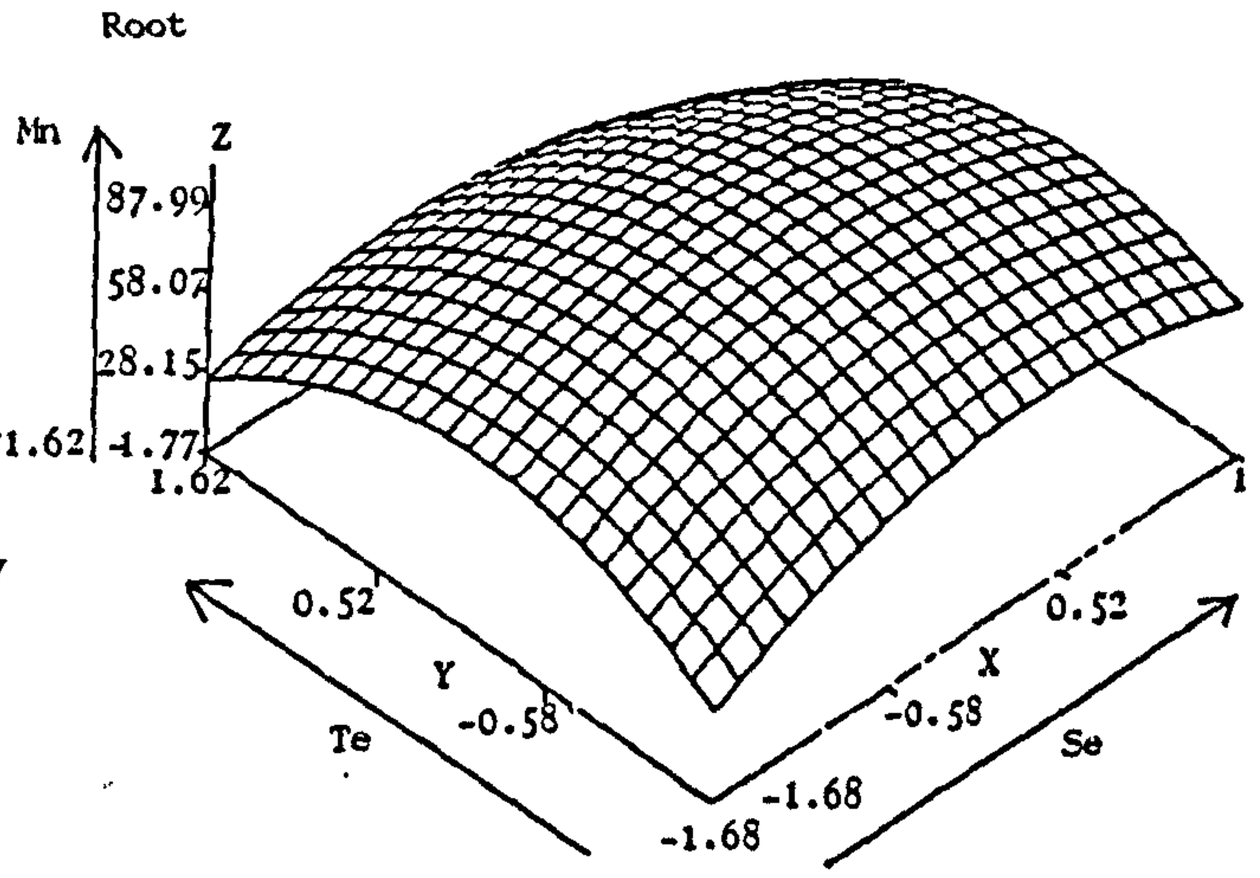
Turning to the essential metals, particularly Cu, Fe, Mn and Zn, their level in the plant may be affected by interaction (competitive or otherwise) by the level of the added metals on the nutrient solution. The essential metals were present at normal concentrations in all the nutrient solutions involved. For example, the uptake of manganese as a function of the concentration of added elements, for both roots and shoots of Lolium perenne seedlings, is shown in Figure 6.64.

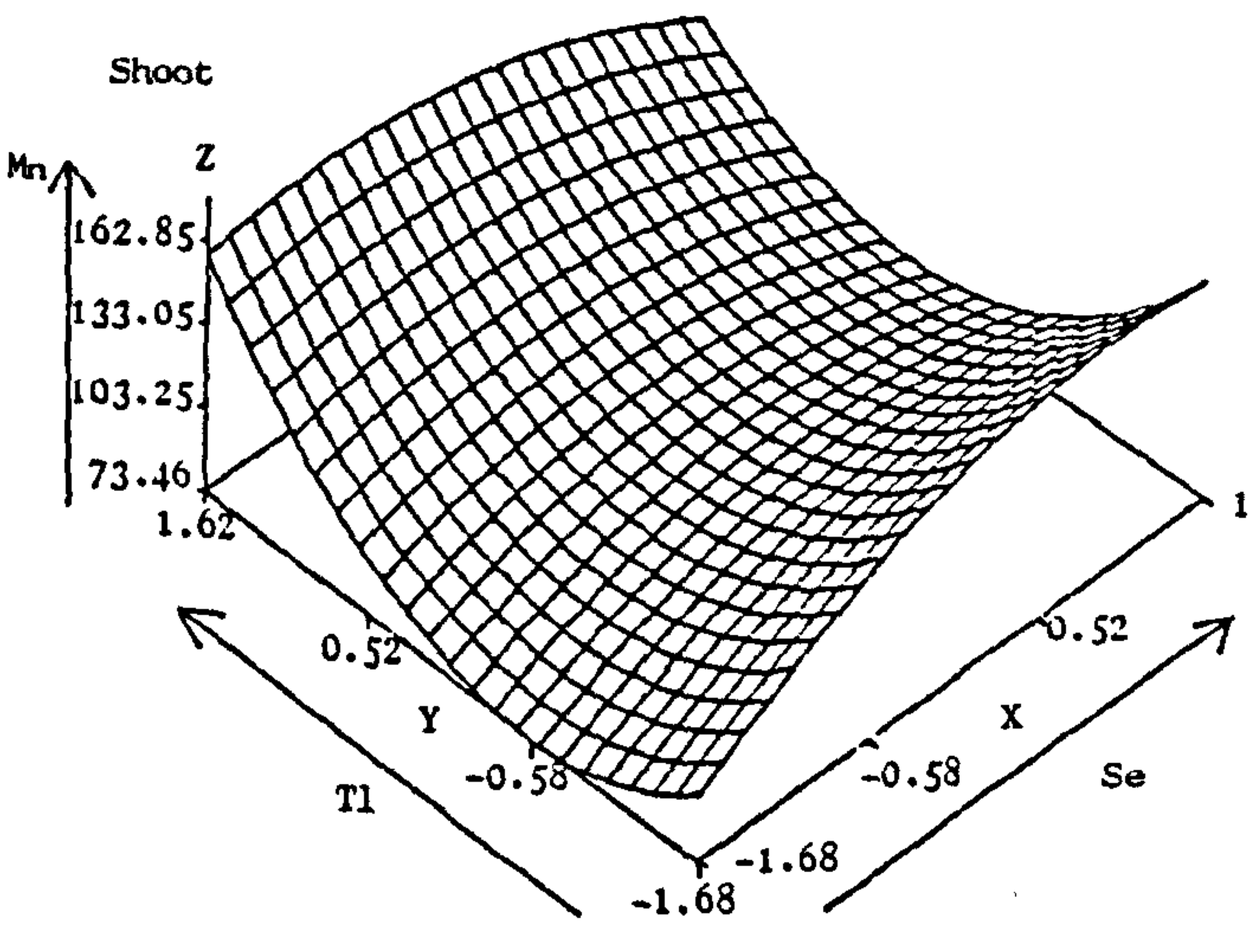
For the roots, regretfully no value is really significant (see R^2 values, Table 6.11), although all three surfaces are of the maximum. (dome-shaped) type. Interestingly, the levels of manganese are lower in the roots than in the shoots. For all surfaces, the Se(VI) level does not greatly affect the level of Mn(II) present. However, for the twin toxic elements of Te(VI) and Tl(I), note the very clear effect with the toxicity of both added elements being shown, resulting in very low levels of Mn(II) in the roots. The explanation is supported from the statistics presented in Table 6.11, where only the Te(VI)*Te(VI) and Tl(I)*Tl(I) terms approach significance.

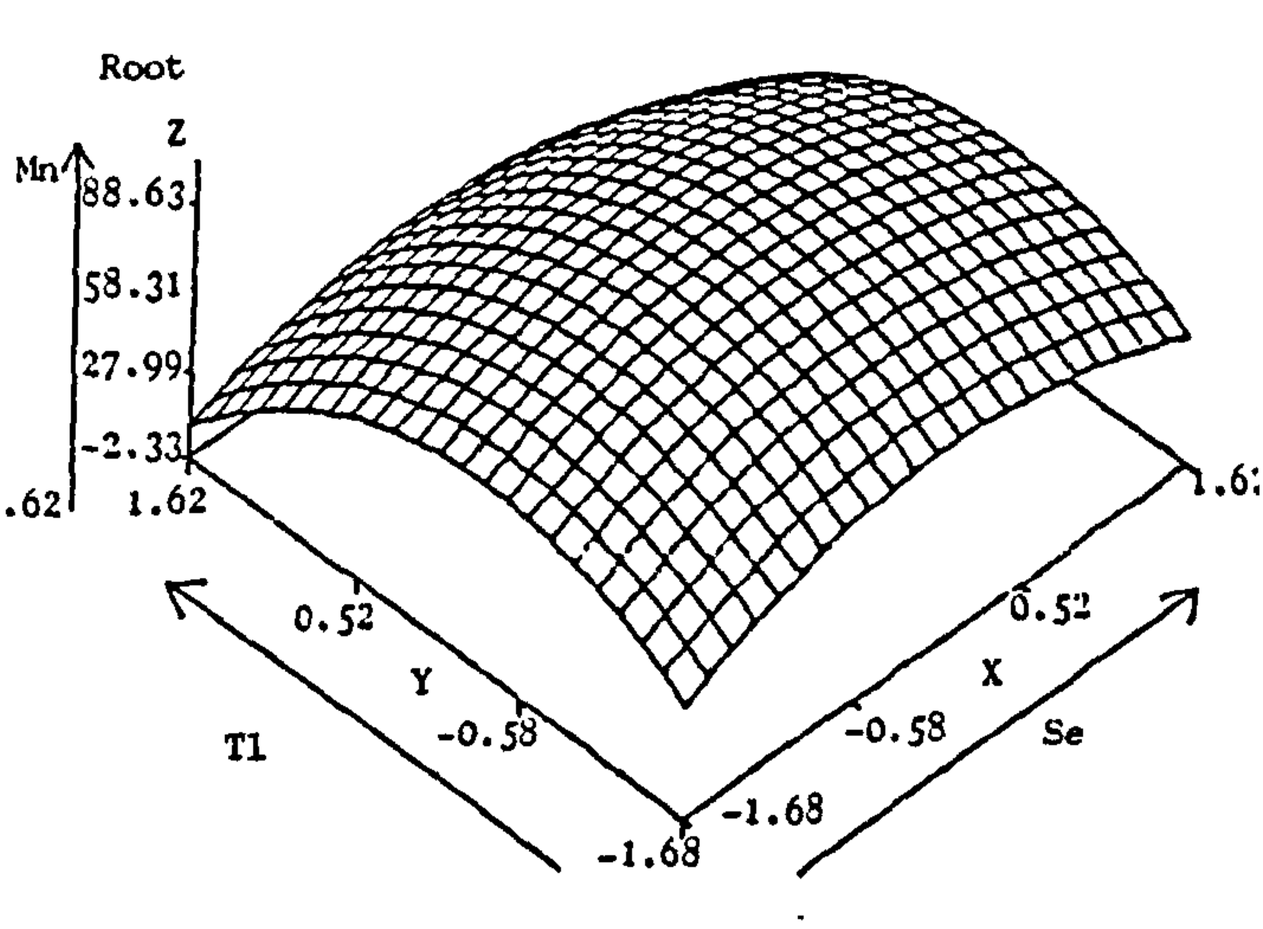
On examination of the shoot data, all the surfaces are similar in shape, being a saddle or minimum system. Here, the levels of Mn are much higher than in the roots. In all surfaces, the Se(VI) affects the shoot levels of Mn to a greater extent than in the roots. Again the effect of two toxic elements produces a real minimum, with Mn levels increasing markedly at high toxic element values. The only values to attain or approach significance are the Te(VI)*Te(VI) and Tl(I)*Tl(I) values (see Table 6.10).

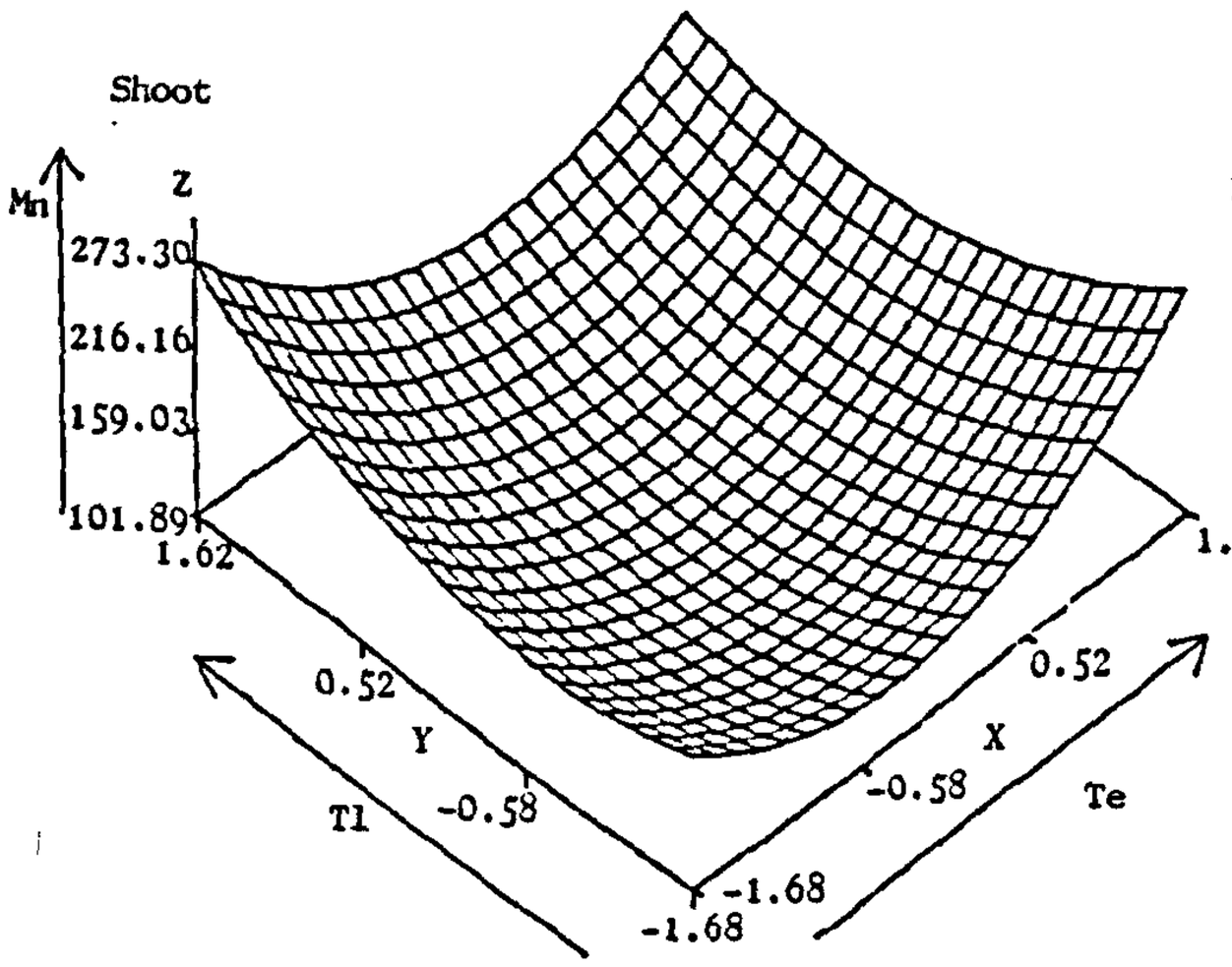
Figure 6:64. Response surface for Manganese taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment II (Se, Te, Tl) with third element at coded level = 0

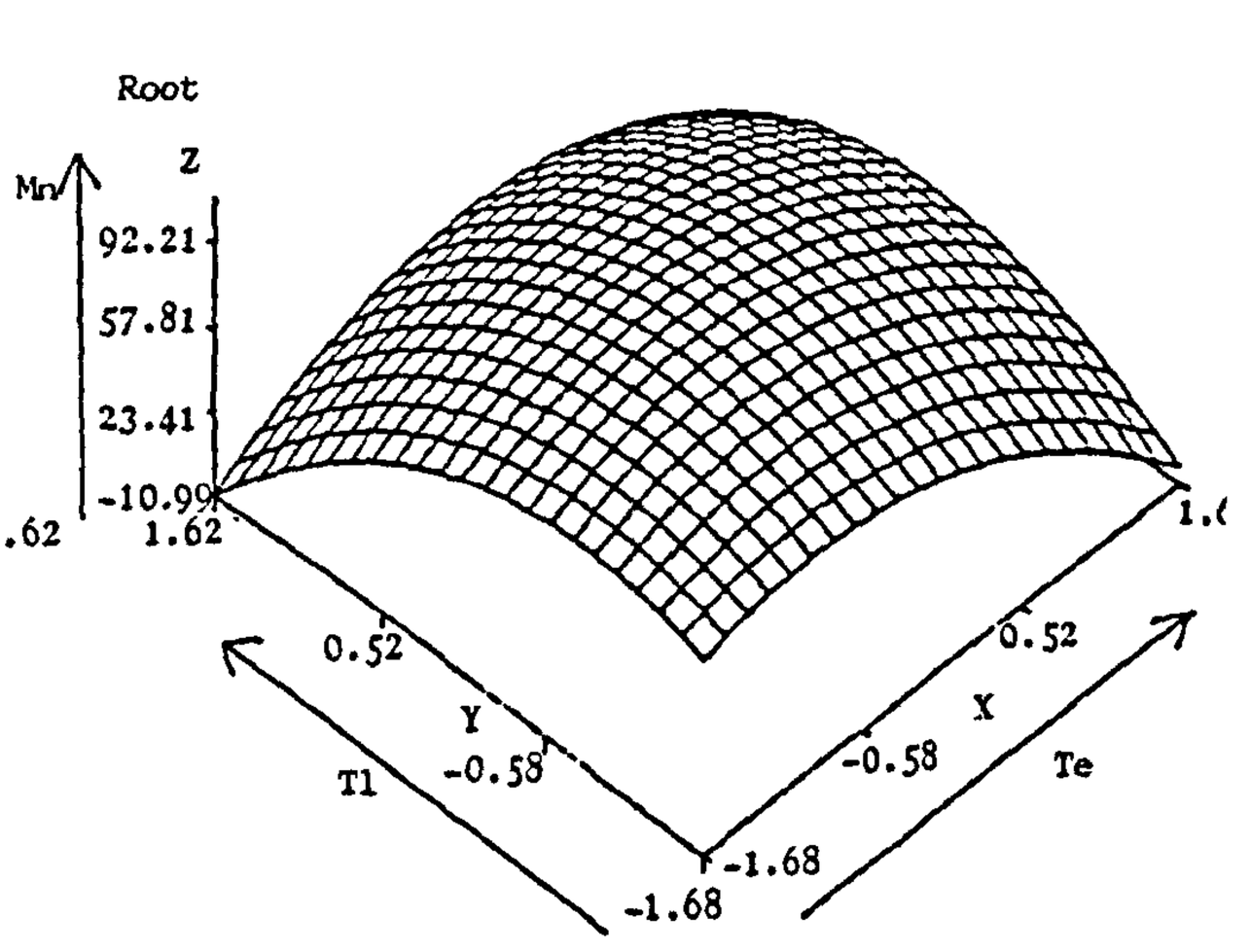










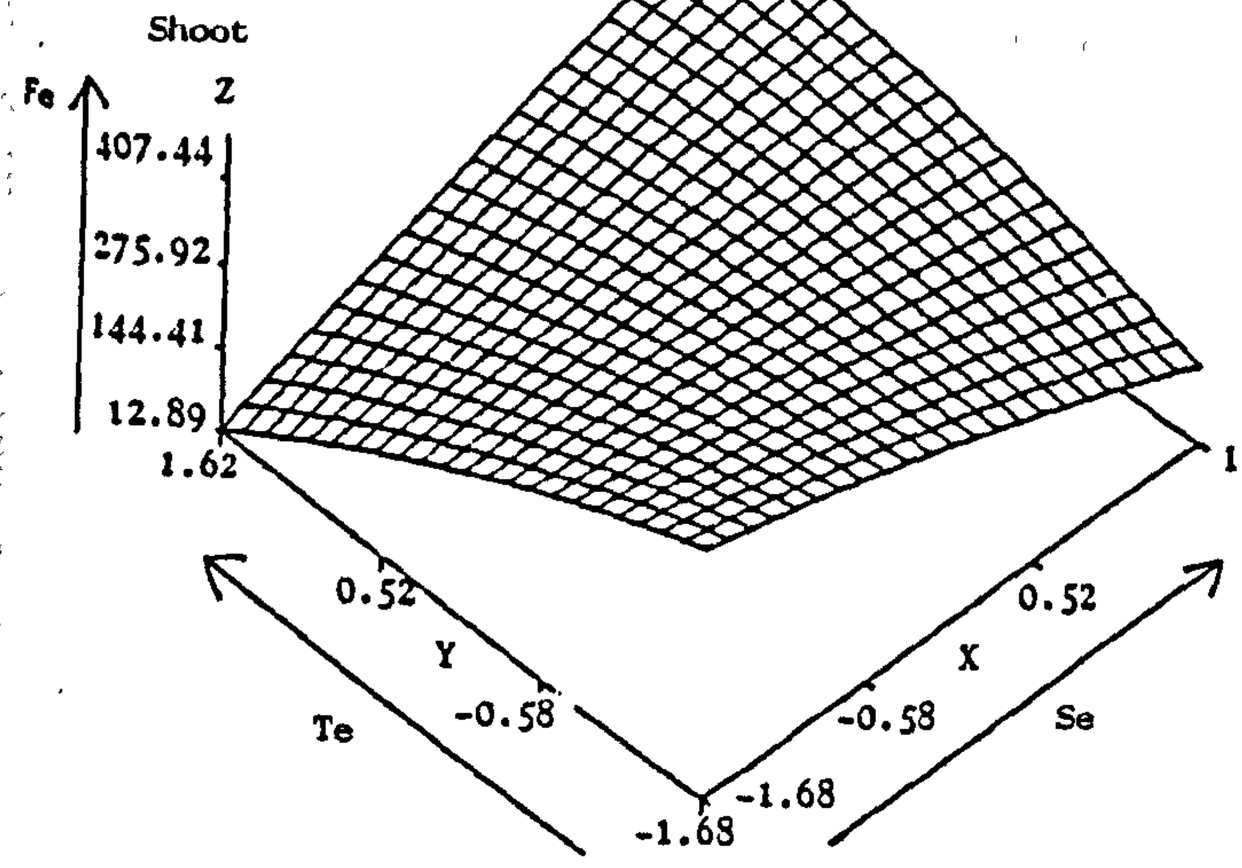


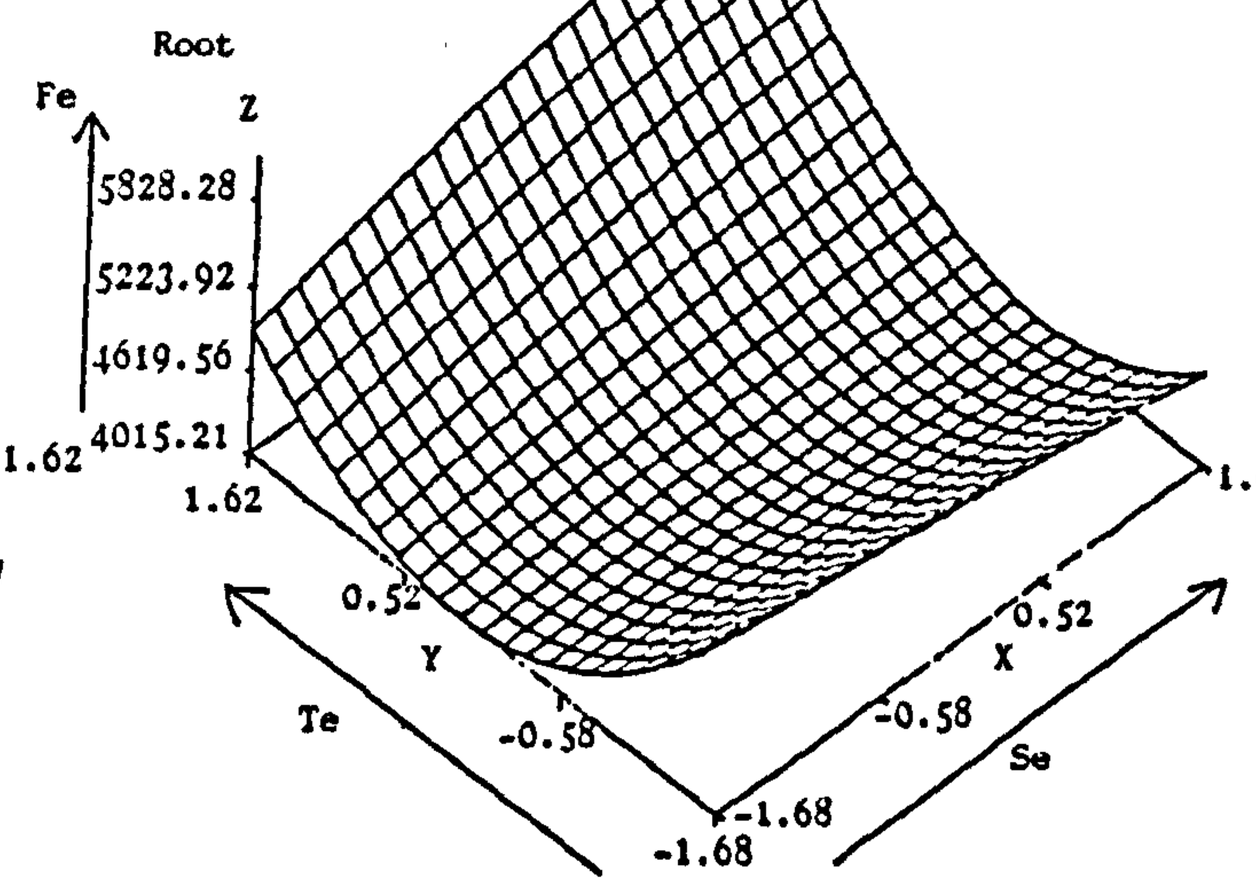
The uptake of iron as a function of the added elements, for both roots and shoots of Lolium perenne seedlings, are listed in Tables 6:6 and 6:7 as response Y6.

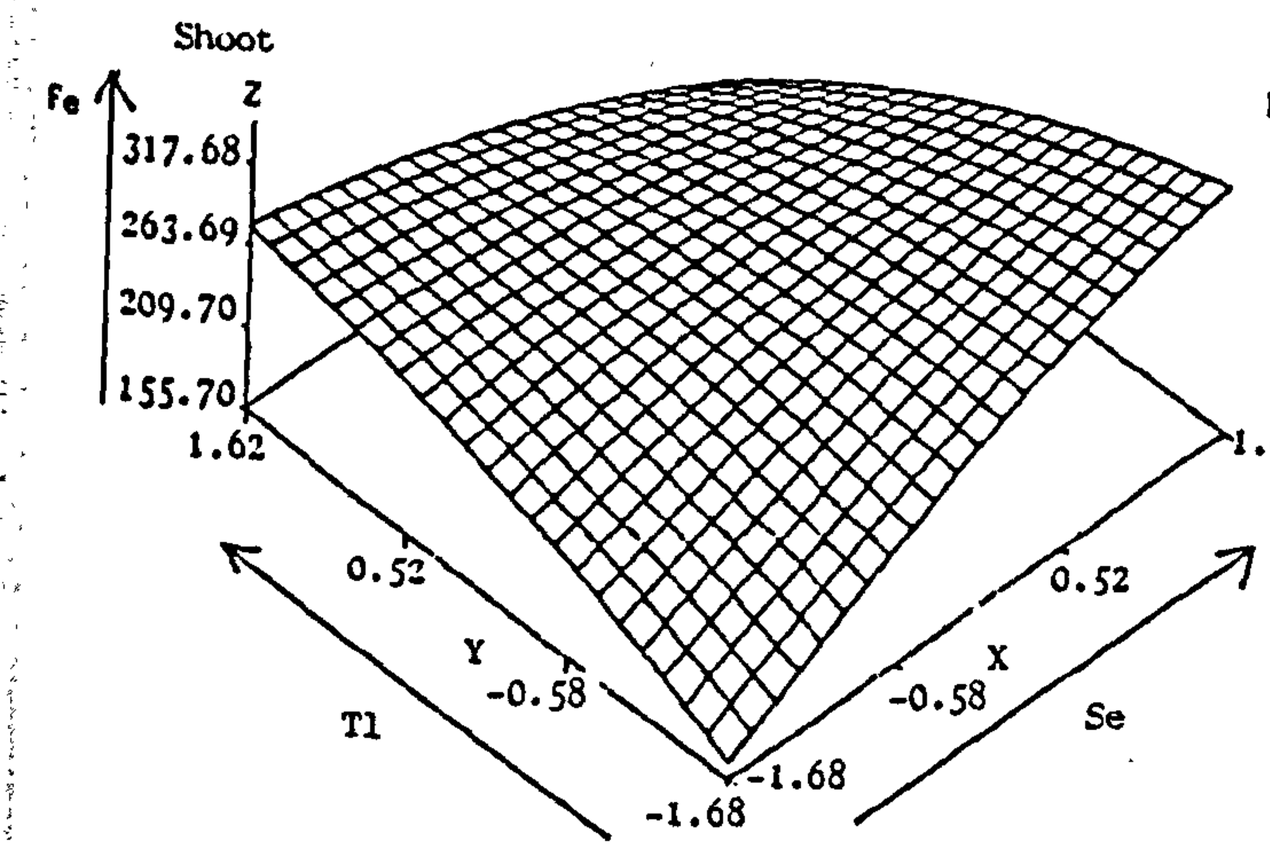
For the roots the R^2 value of 0.7968 is a better fit than for the manganese data. However, the pure error variation arising from the coded (0,0,0) population accounts for almost 25% of the total error, so a good fit cannot be expected. Interestingly, the levels of iron in the roots are very much higher than in the shoots, in contrast to the manganese values (see Tables 6.6 and 6.7). Generally for the roots no parameter was significant except for X3 (Tl(I)) as a linear effect and the Te(VI)*Te(VI) factor as a quadratic. The statistical parameters are given in Table 6.11. For the roots, the effect of Se(VI) on Fe levels is minimal, while the effect of Te(VI) is not much greater. However, the level of Tl(I) in nutrient solution clearly has a major effect on the Fe levels, which appear to increase as the Tl(I) level rises - a clear indication of the toxicity of Tl(I) to the root system, with the plant possibly trying to overcome the problem by taking in more iron.

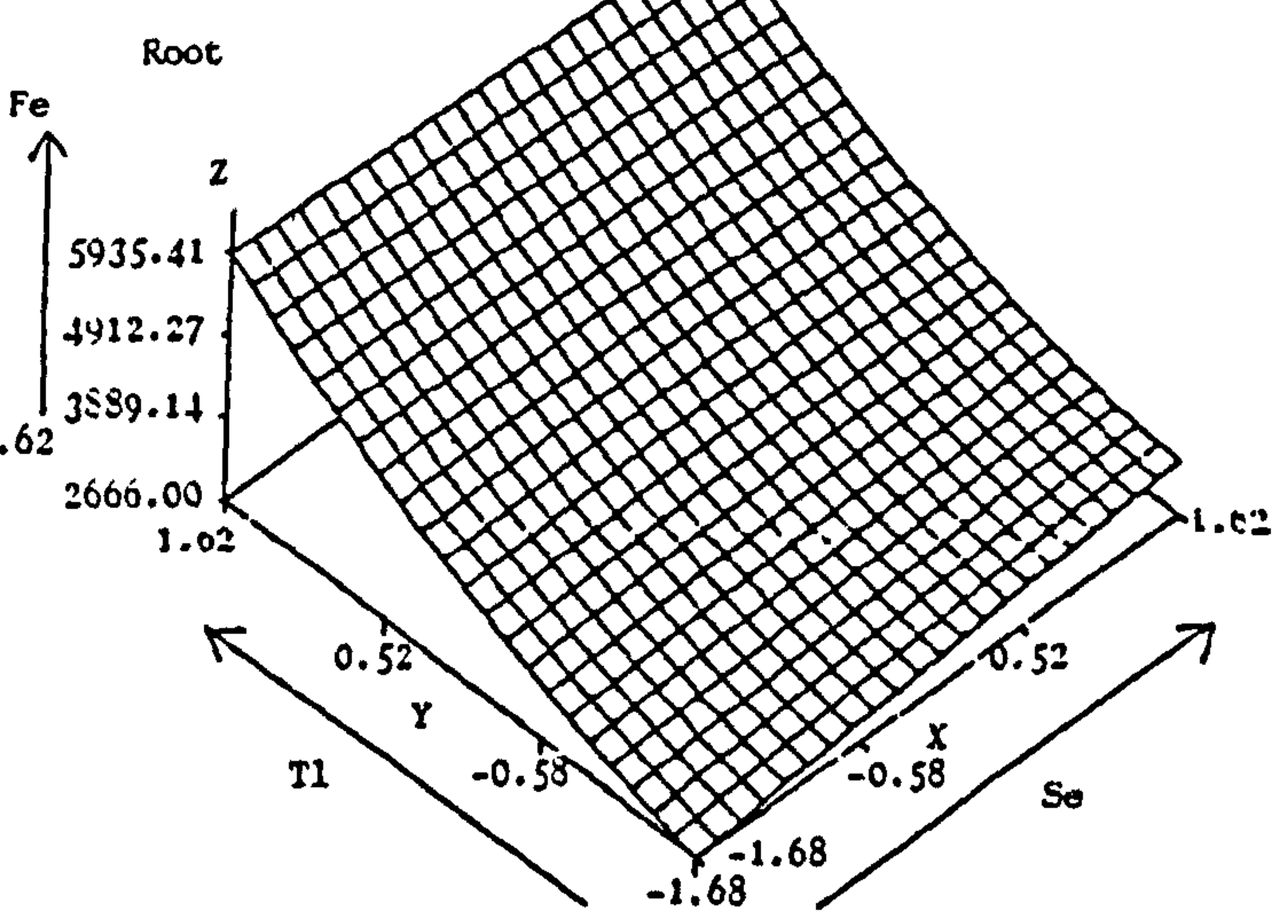
Regretfully, examination of the shoot data shows that via the R^2 value, the model proposed is an extremely poor fit for the data. Again, the pure error figure is high but the lack of fit figure is almost significant, certainly so that the 90% probability level. However, the levels of iron in the shoots are much lower than in the roots. When Tl(I) concentration is increased, so does the iron concentration in the shoots, but the lack of fit can be seen in the opposing trends following increase in Se nutrient concentration; in once case the Fe level decreases, whilst in the other case it increases. A similar effect can be noticed for the iron response to Te(VI) levels.

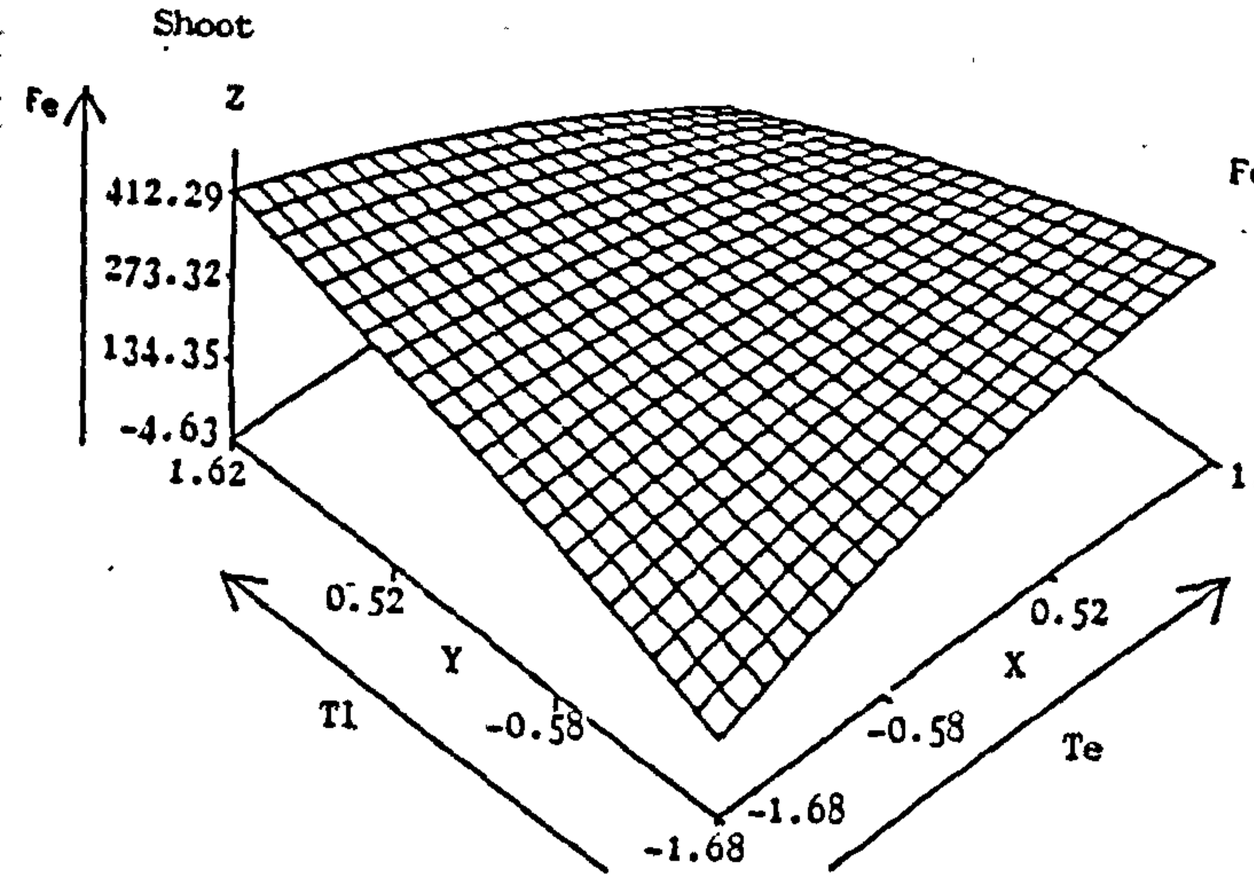
Figure 6:65. Response Surface for Iron taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment II (Se, Te, Tl) with third element at coded level = 0

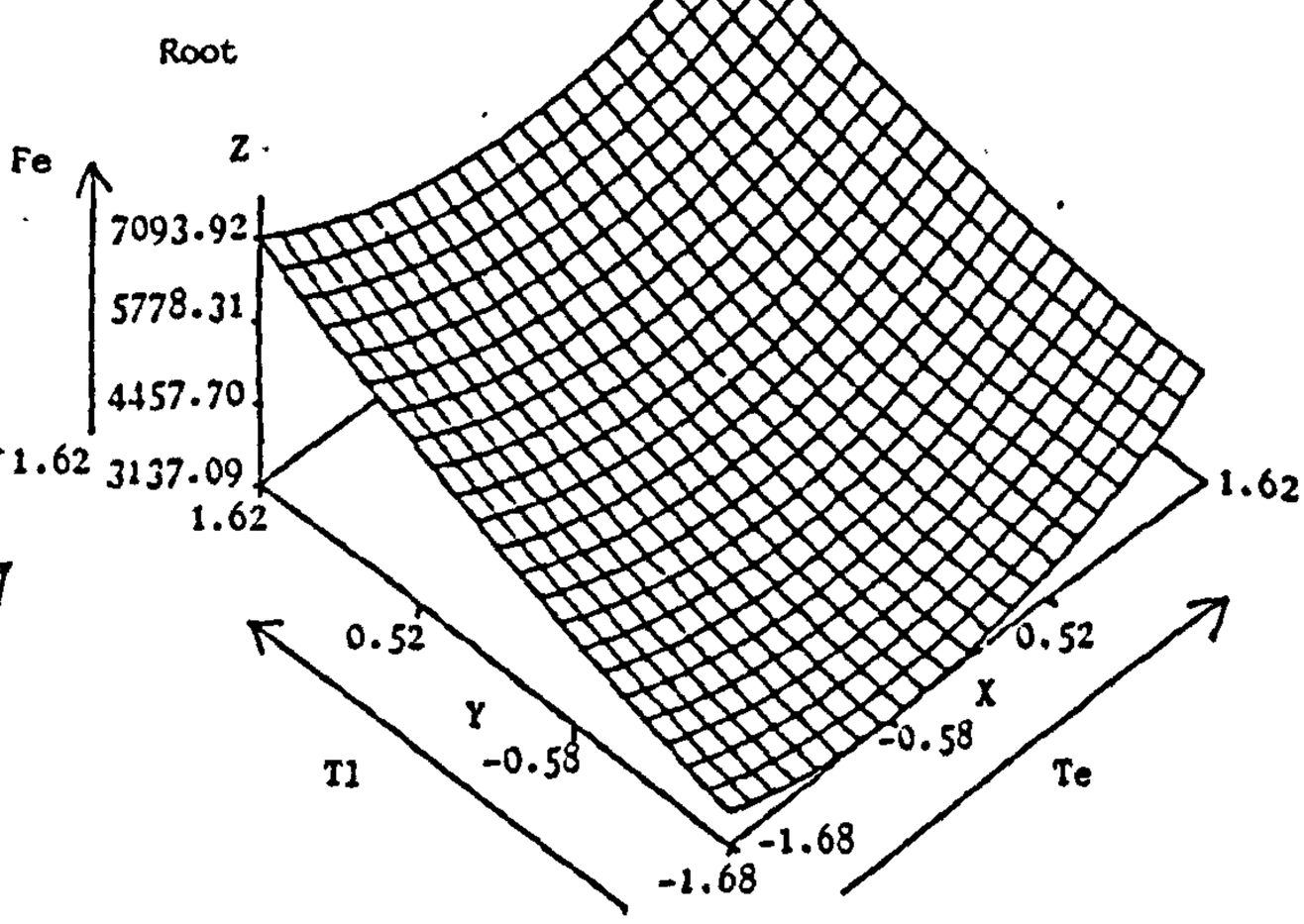










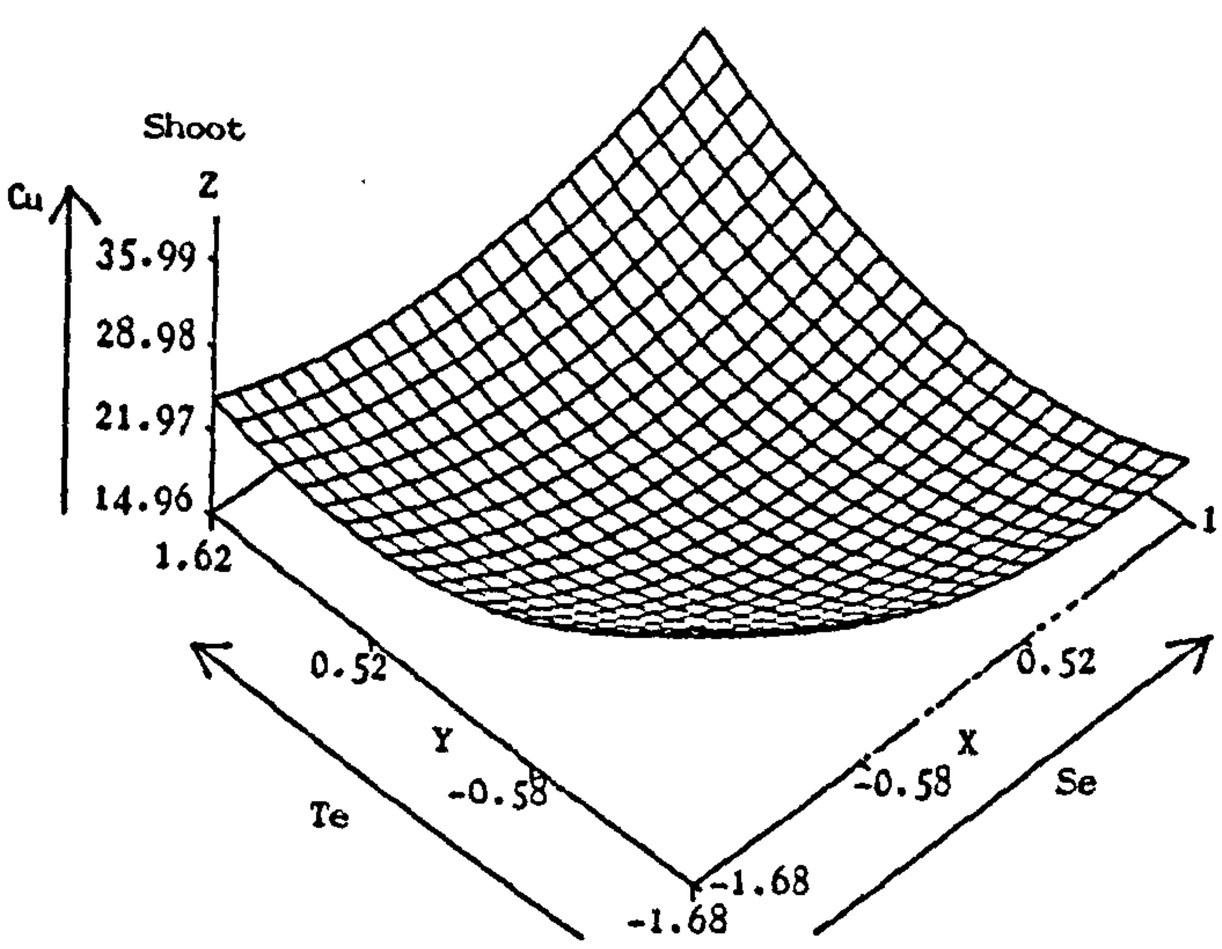


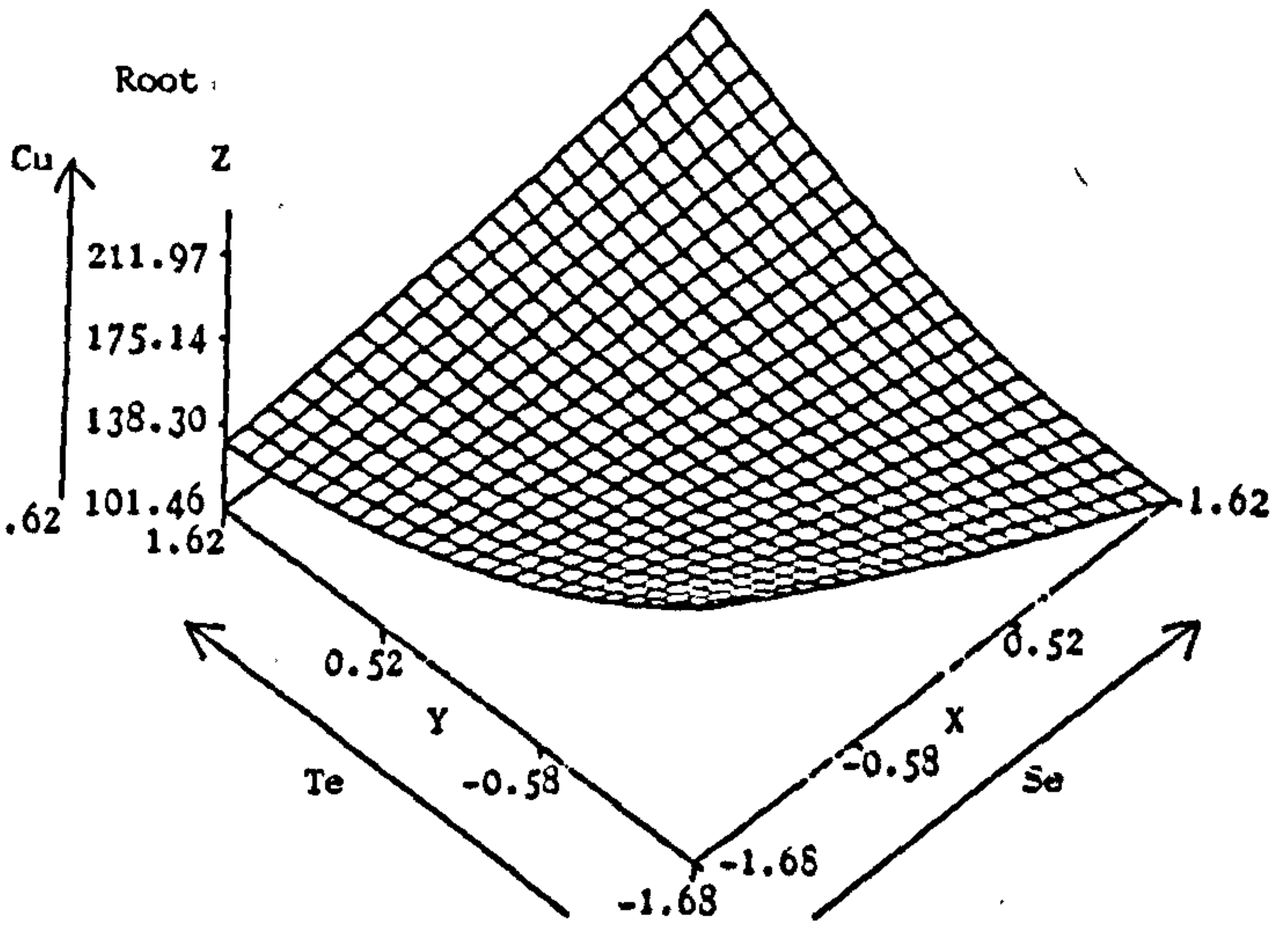
Clearly, little credence can be placed in these surfaces (see Figure 6.65) or the parameters for the model. Obviously, the general second order quadratic equation is not a good approximation for the iron response and a more detailed set of experiments, probably over a limited range of toxicant concentrations, is necessary.

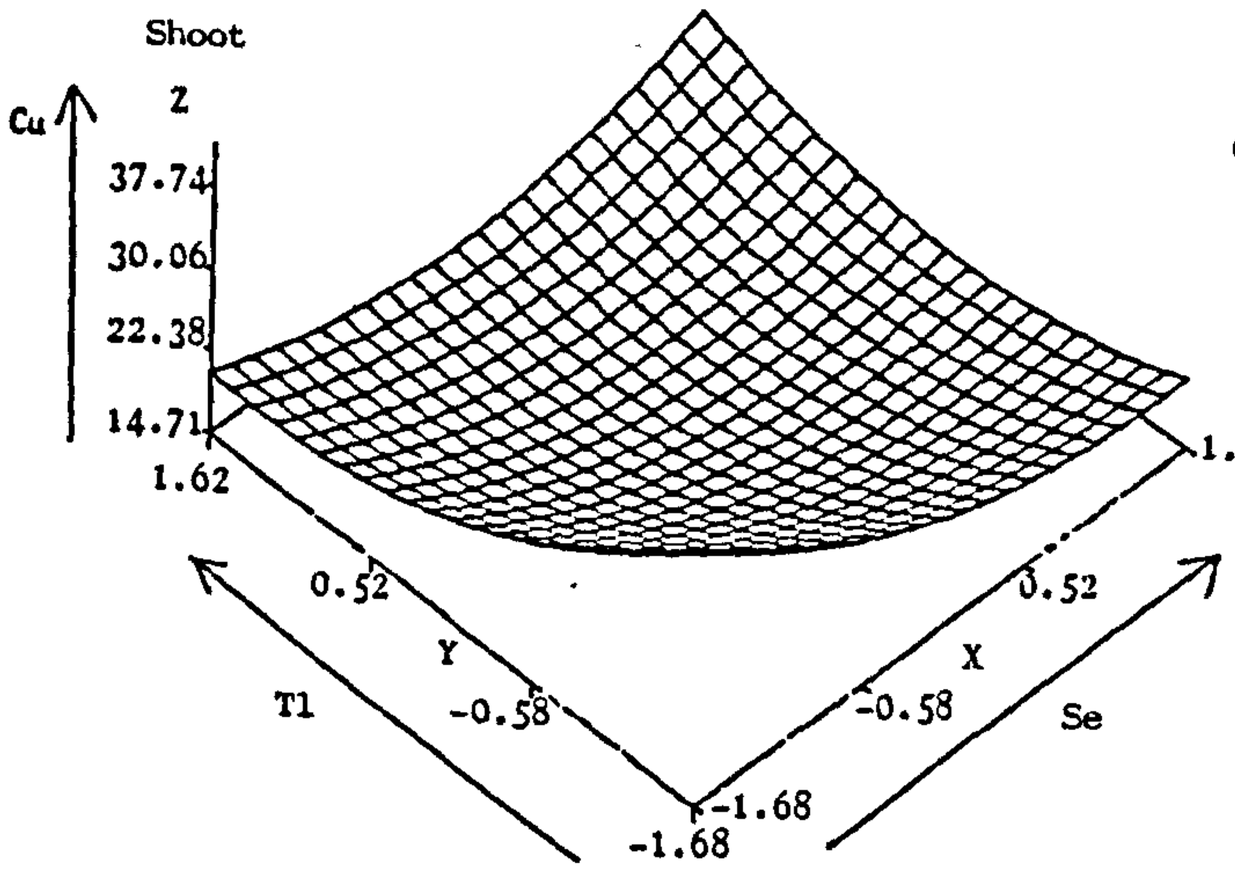
The uptake of copper by Lolium perenne in the presence of the spiked toxic elements for both roots and shoots is listed in Tables 6.6 and 6.7 as response Y7.

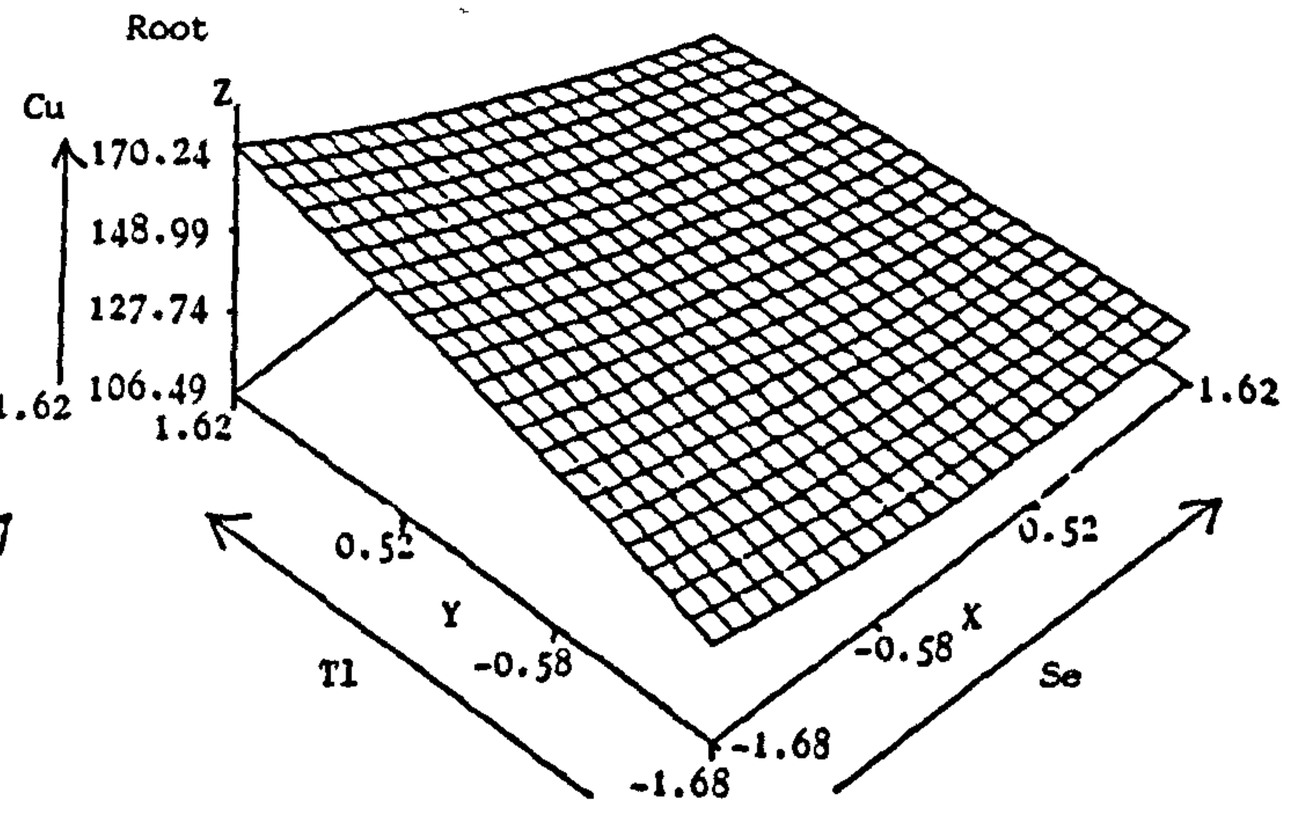
For the roots case, the fit of R^2 is unexpectedly low, being only 0.365; this fact further being reinforced in that the lack of fit of model to the data is significant (greater than 95%) being almost highly significant (99%). The pure error only accounts for less than 10% of the total error, so making the lack of fit parameter an important guide. With this level of error in the experimental data fitting the proposed model, it is not surprising that none of the parameters for roots are significant, i.e. there appears to be no support for the hypothesis that the level of Cu in the roots is affected by the presence of Se(VI), Te(VI) or Tl(I) even when they are present over a very large concentration range. Notice that the suggested levels of Cu do not change markedly, no more than a factor of 2, even with a 10^4 change in concentration of the spiked elements. Thus it is not unexpected that the response surfaces (as shown in Figure 6.66) are almost planar. All that can be said is that the levels of Cu in the roots appear not to be affected to any significant extent by the levels of these particular toxicants in the nutrient solution.

Figure 6:66. Response Surface for Copper taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment II (Se, Te, Tl) with third element at coded level = 0









However, for the shoots the fit is somewhat better, the R^2 value in this case being 0.65, but not anywhere near a significant fit. In fact, the lack-of-fit of the model to the data is almost significant (at the 95% level). The levels of Cu found in the shoots is lower than in the roots and appear to change little with increase in toxicant concentration. Although the Cu levels are low, because of the adequate sensitivity of the atomic absorption method for Cu, plus the non-variability in Cu levels in the coded centre point, the pure error accounts for only 20% of the total error. Clearly, Cu is taken into the roots and thence to the shoots by a mechanism which is not affected by competition from a very wide range of concentrations of the three added elements. Perhaps the Cu being an element which forms very strong chelates with organic materials, e.g. amino acids, is taken into the plant in such a complexed form. Clearly, our particular elements, two in the form of anions, the other element [Tl(I)] does not form strong complexes with, in particular, nitrogen donors, and so will not affect the uptake of Cu by the rooting system.

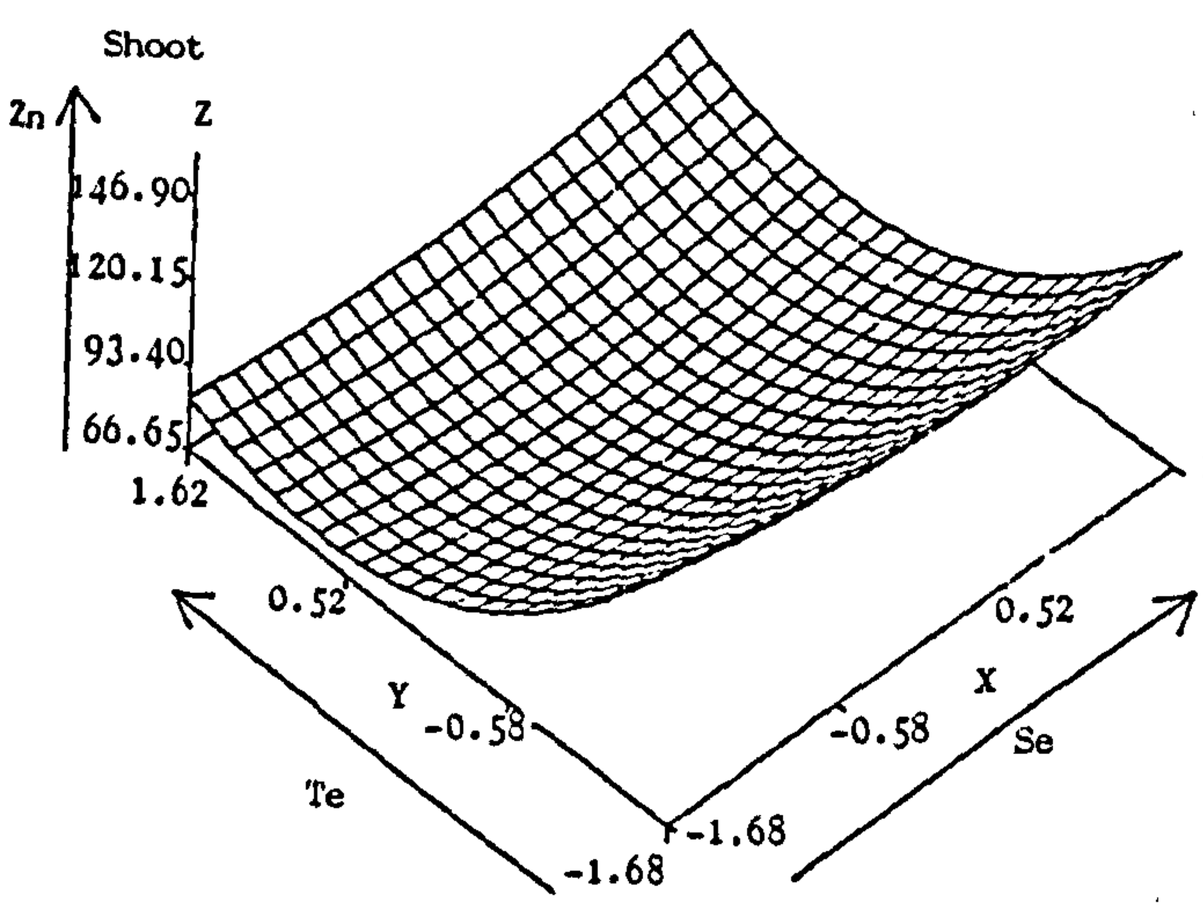
The last example of the possible effect of the added toxicants on the level of essential metal is that of the uptake of zinc by both roots and shoots.

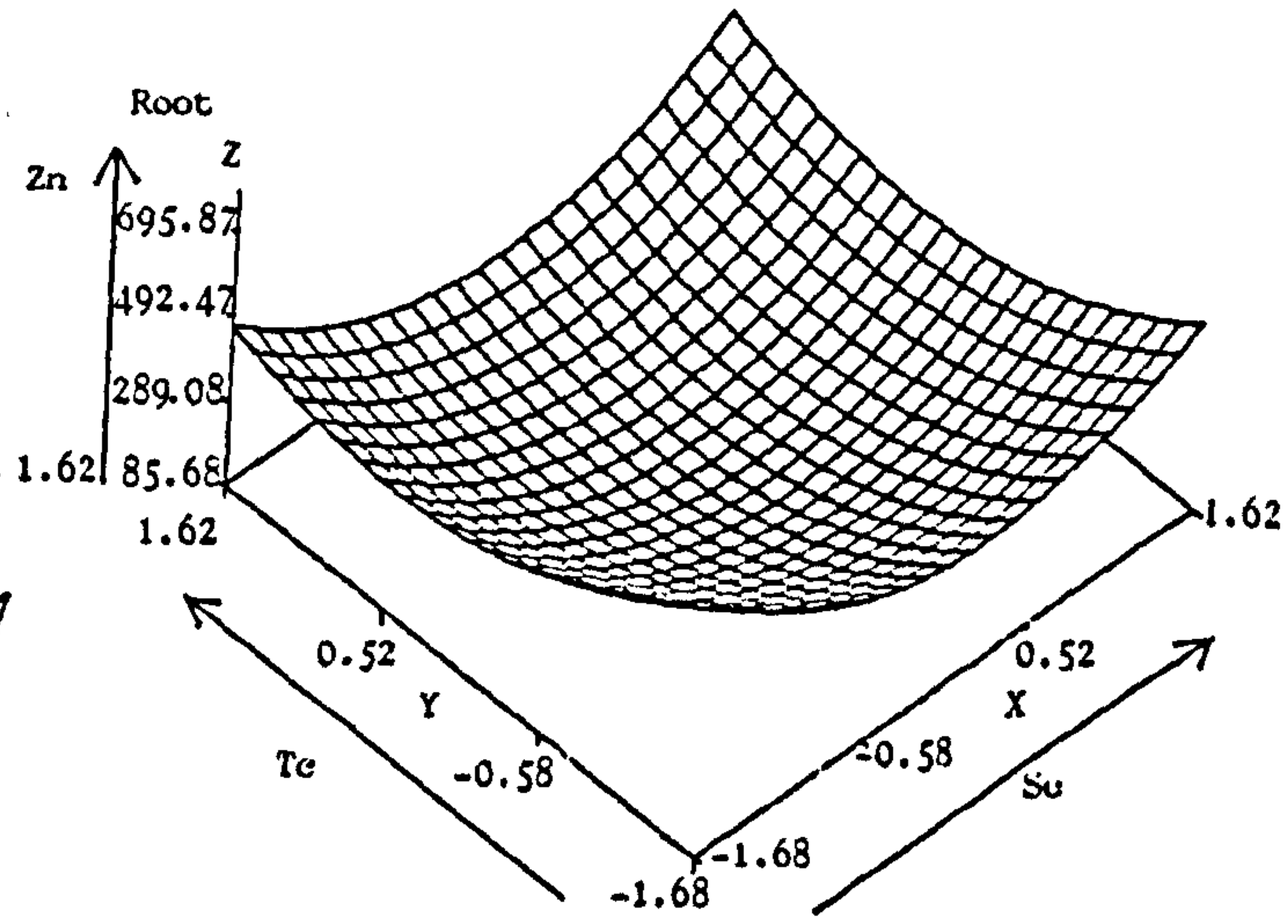
For the roots, the R^2 value is only 0.52, a rather low value and not surprisingly, due to a combination of the high levels of zinc found in the roots, the high sensitivity and reproducibility of the AAS method for zinc, the lack-of-fit term is very significant indeed. Here the pure error term accounts for only 0.5% of the total error, a very low figure. Not surprisingly in view of the low R^2 value, none of the experimental variables turn out to be of significance on the uptake of zinc into the roots. If it is a real effect, the cross-product term Se(VI)*Tl(I) is the only one approaching significance and is the first cross-product term to do so in the entire experiment. The levels of zinc in the roots are much higher than those present in the shoot system. Of the three added elements Se(VI) and Te(VI) appear to make little effect on the uptake of zinc, while increasing Tl(I) concentrations appear to cause an increase in the zinc concentration, in a rather similar manner to that observed for Fe and to a much lesser extent by Cu.

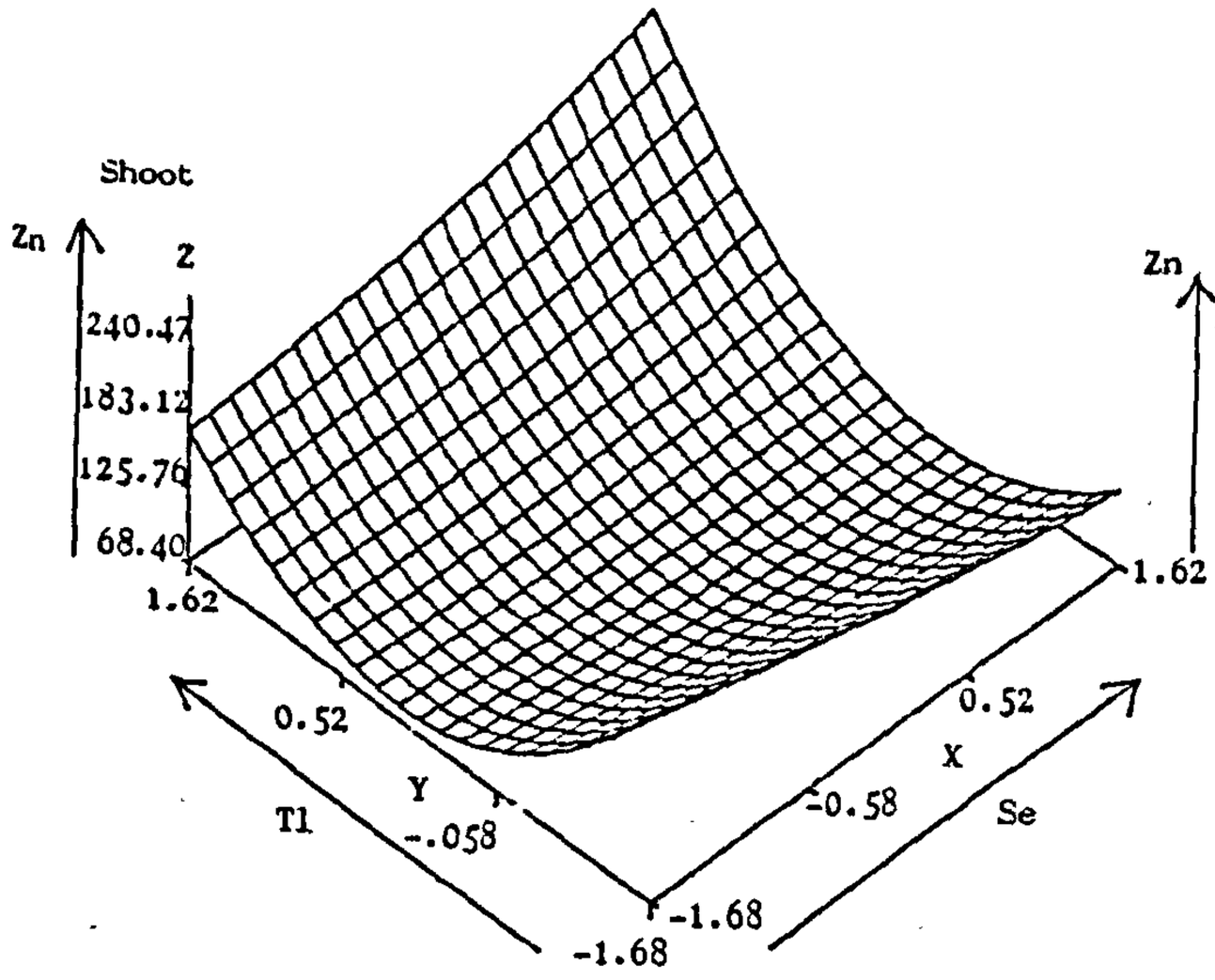
Although a similar situation appears to hold for the level of zinc in the shoots, in that the R^2 value is 0.57 (rather than 0.52 - roots) and that the lack-of-fit term is deemed highly significant, a marked difference does occur. Here, the Tl(I) concentration, in spite of the lack of fit, appears to have some effect on the level of zinc in the shoots. Thus the Tl(I)*Tl(I) term is significant at practically the 99% level and certainly overall there is a 90% probability that the level of Tl(I) is a significant factor in determining the level of zinc in shoots. Notice that the Se(VI) and Te(VI) concentrations are unimportant in their effect on the zinc levels. Possibly, therefore,

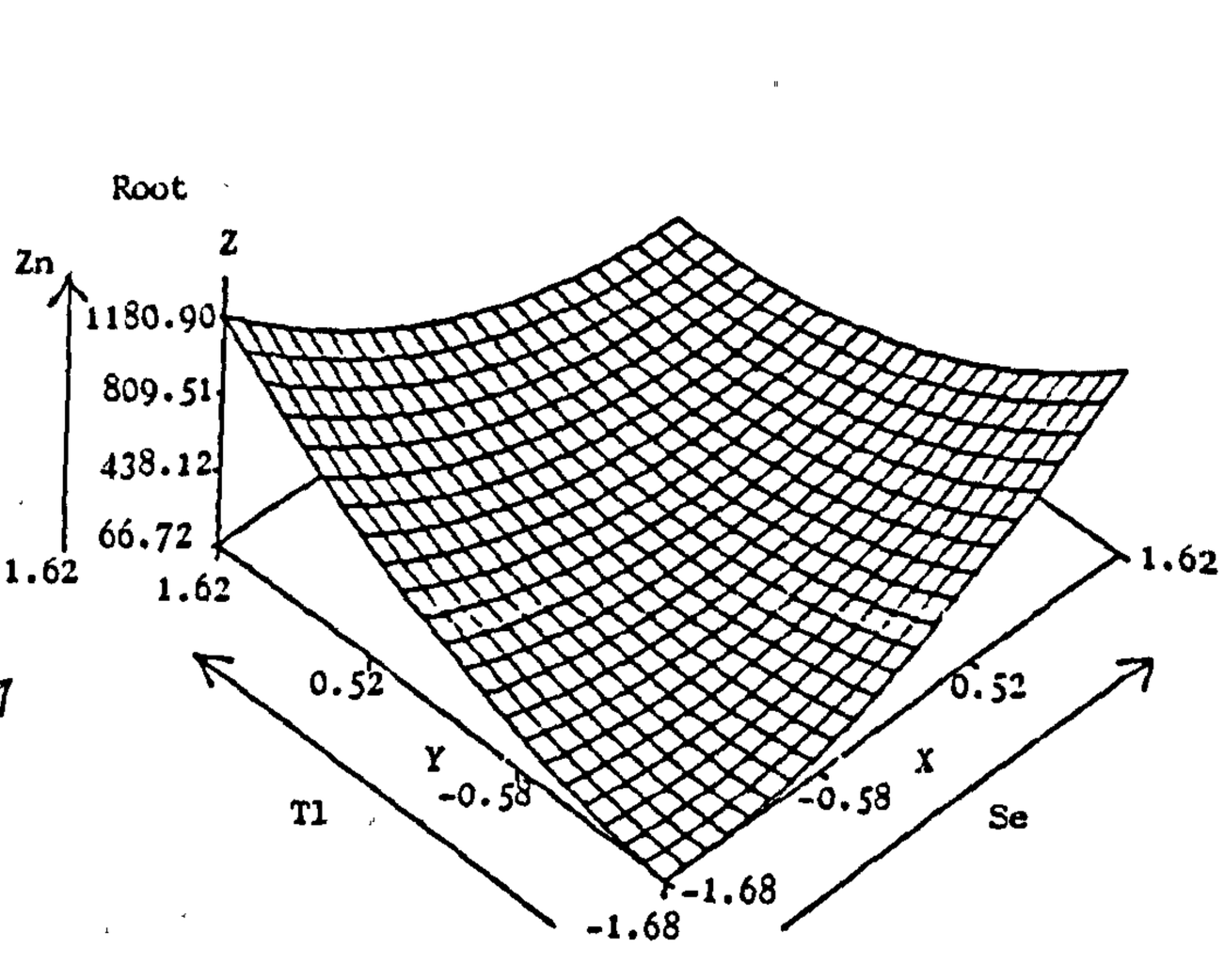
the Tl(I) ion at higher concentrations interferes with the mechanism causing control of zinc level in shoots and hence as the Tl(I) value continues to rise, so does the Zn level (see Figure 6.67).

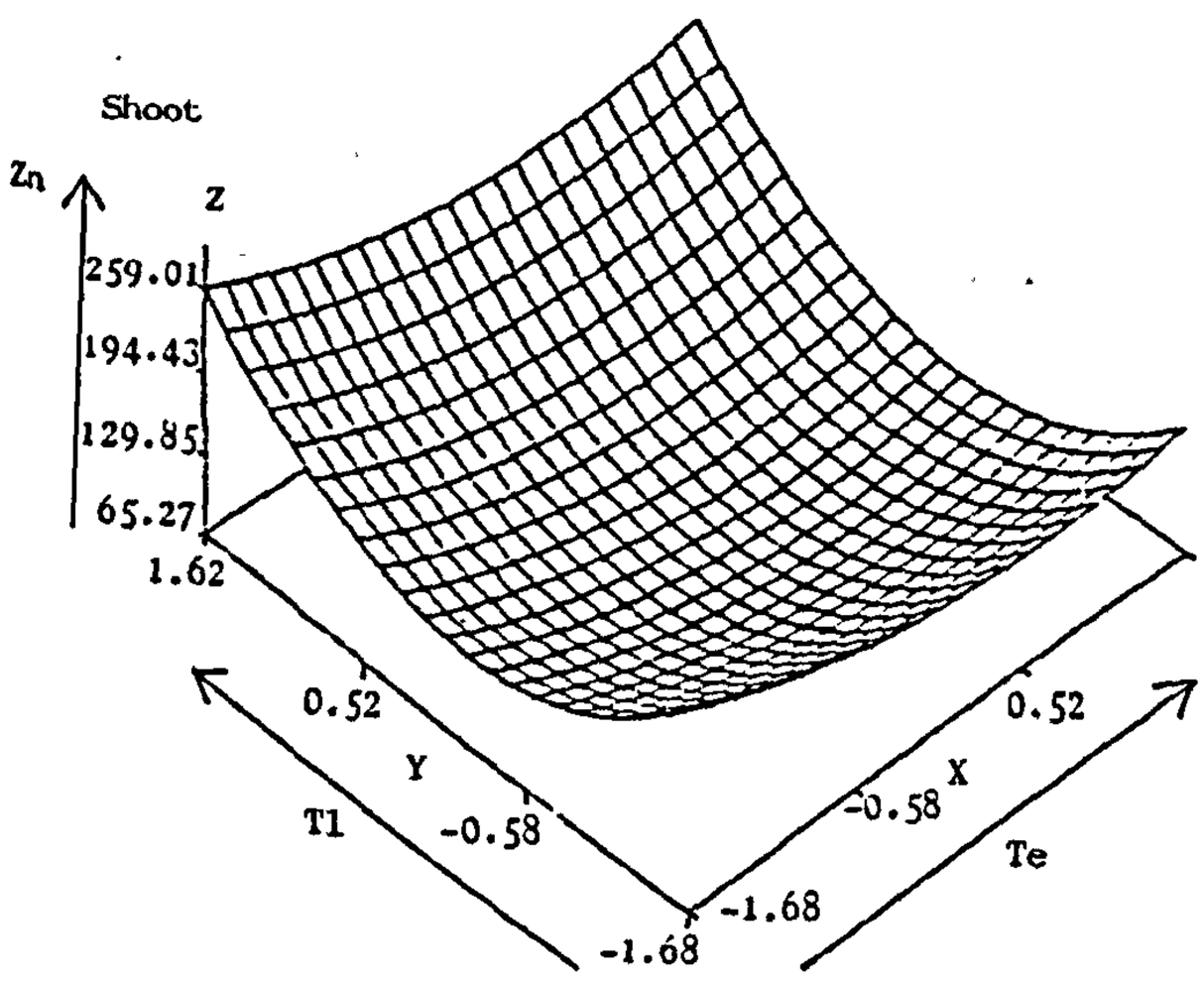
Figure 6:67. Response Surface for Zinc taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment II (Se, Te, Tl) with third element at coded level = 0

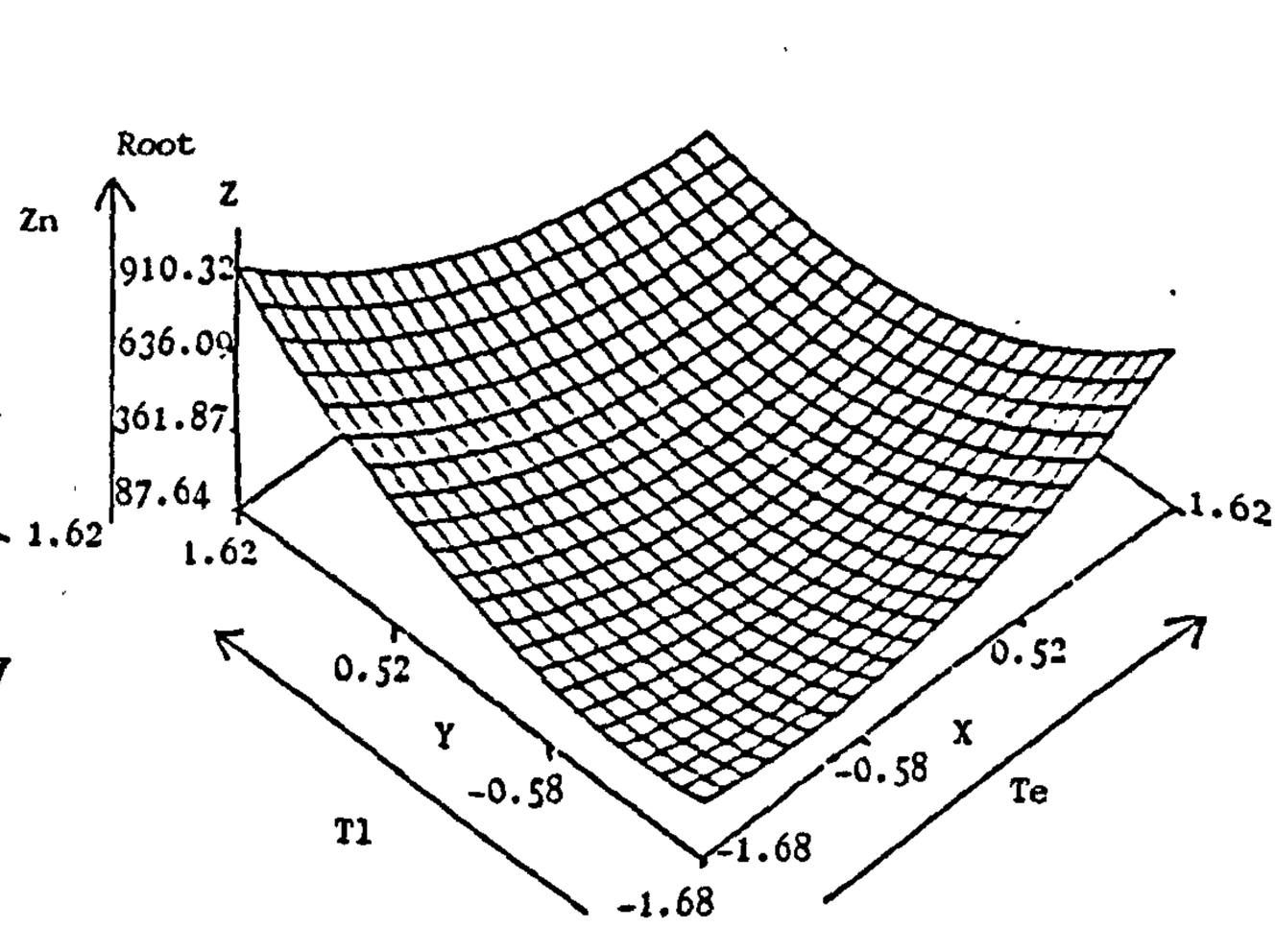












ii) Interactive Effects of Se(VI), Cd(II) and Hg(II) on Plants

In Experiment 1, the elements fed to the Lolium perenne seedlings were cadmium (as cadmium acetate) plus mercury (II) (as mercuric acetate) in conjunction with Se(VI) as sodium selenate. The plants were grown for a period of three weeks. Then the plant material was divided into roots and shoots on harvesting, and a set of data similar to that described for Experiment 2 (Se(VI), Te(VI), Tl(I)) was obtained. The response surfaces were generated using REREG and G3D programs in the SAS package and also by using the MINITAB and GINO programs, again similar to Experiment 2. Generally all surfaces in Experiment 2 generated by SAS and GINO were very similar, so in discussing Experiment 1 (Se, Cd, Hg), in order to avoid needless repetition (and save space), only the SAS surfaces will be shown or discussed.

For the shoots harvested from Experiment 1 [Se(VI), Cd, Hg], Table 6.4 lists the values of the response variables obtained for some nine parameters, two gross forms of biological importance (shoot length and biomass), and seven forms concerned with the elemental analyses for the various metals or selenium content of the plant material. Table 6.4 lists these variables in terms of the three experimental variables, X1, X2 and X3 which represent the concentrations of Se(VI), Cd and Hg in the nutrient solution. Table 6.8 lists in a very condensed form the statistical parameters and values from trying to fit the general second order equation to the various response data collected.

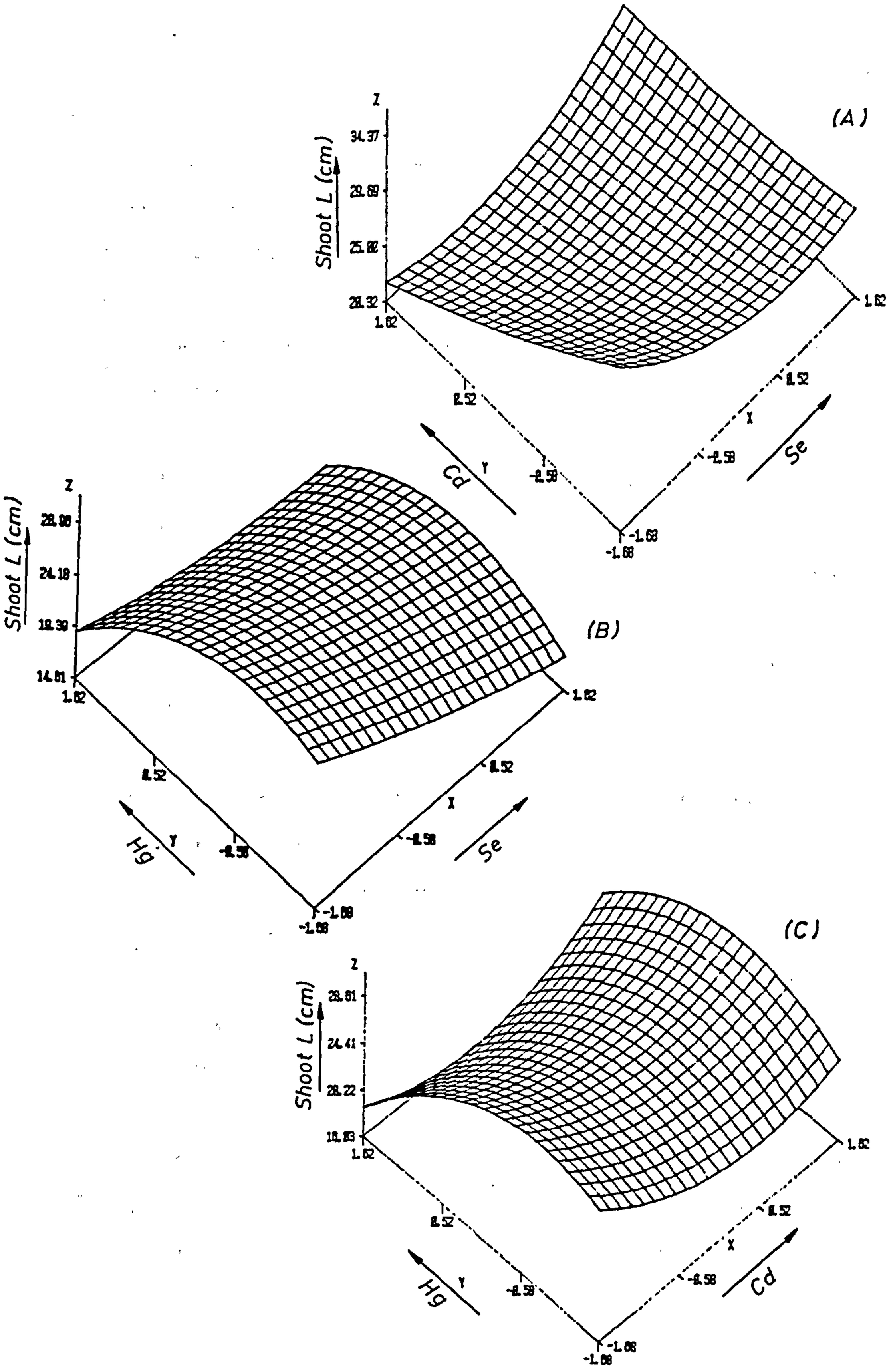
Taking the first response measured, shoot length, the R^2 value is 0.67, so our second order equation is only a reasonable descriptor for the process.

With the very significant probability terms [10] it is obvious that only some of the linear terms are important but not the quadratic or the cross-product terms. The pure error term accounts for only 8% of the total error, thus rendering the lack-of-fit term highly significant.

Examination of the PROB values [17] seems to confirm that Cd and Hg have a significant effect on shoot length, whilst Se(VI) does not. Notice that this effect is also carried on into only the X3*X3 (Hg*Hg) term which is significant, while the other quadratic and the cross-product terms are non-significant. The greater toxicity of Hg is thus confirmed, but with Cd and Hg have a much greater effect on growth. Notice how the shoot length decreases for the increasing concentrations of these two toxicants.

The response surfaces confirm these findings; in Figure 6.68 where in each case increasing Se(VI) concentration has only a rather small effect on growth.

FIG. 6.68 RESPONSE SURFACES FOR SHOOT LENGTH OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT 1 (Se, Cd, Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



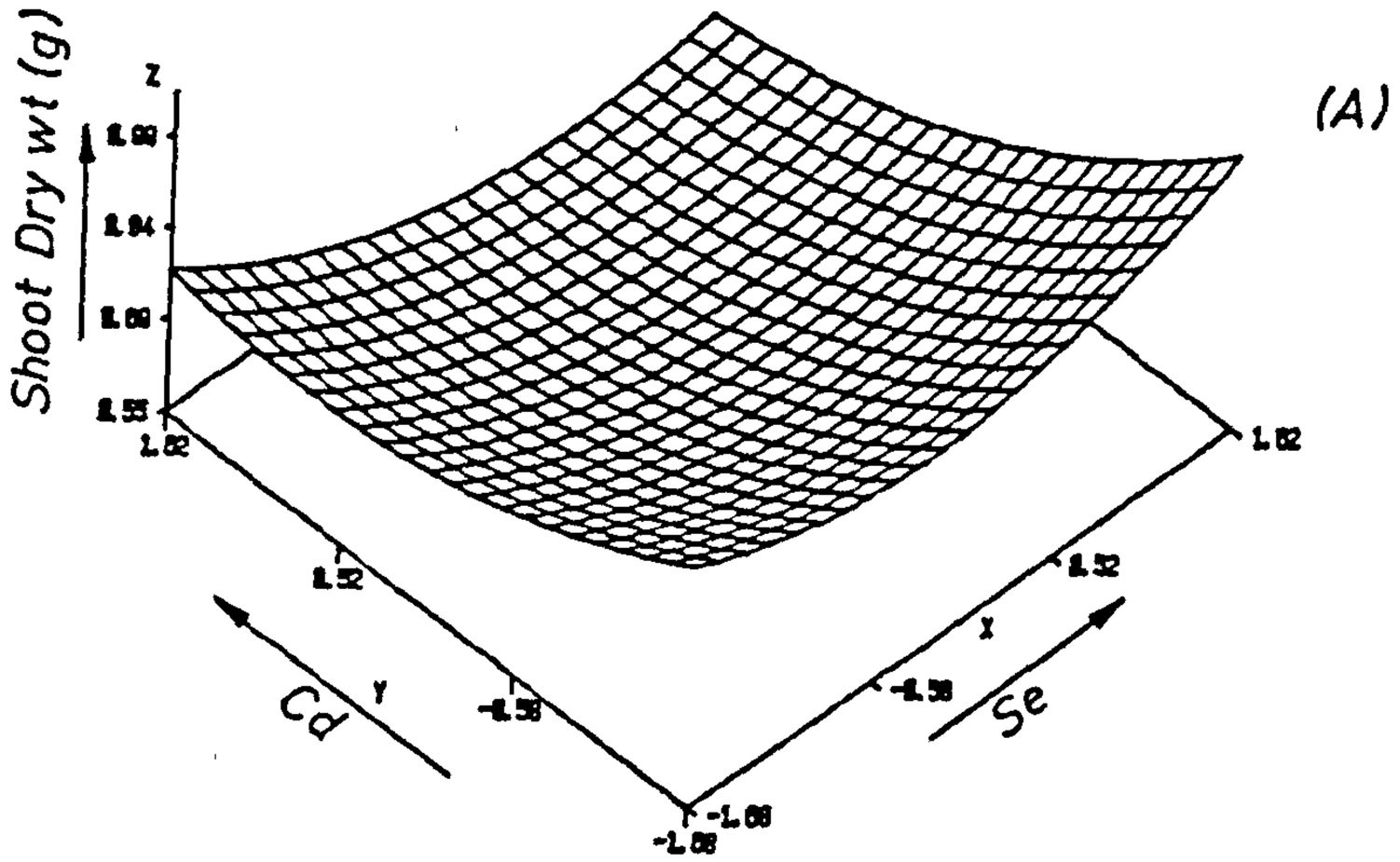
Turning to the other biological parameter, the dry weight of shoots as response variable (Y9, Table 6.4), with the regression statistics listed in Table 6.8; regretfully, in this case, the fit of R^2 value is 0.272, the lowest fit value for all the shoots. The pure error accounts for only 5% of the total error, so the lack-of-fit is highly significant. Thus the model equation is not a good fit at all in this situation.

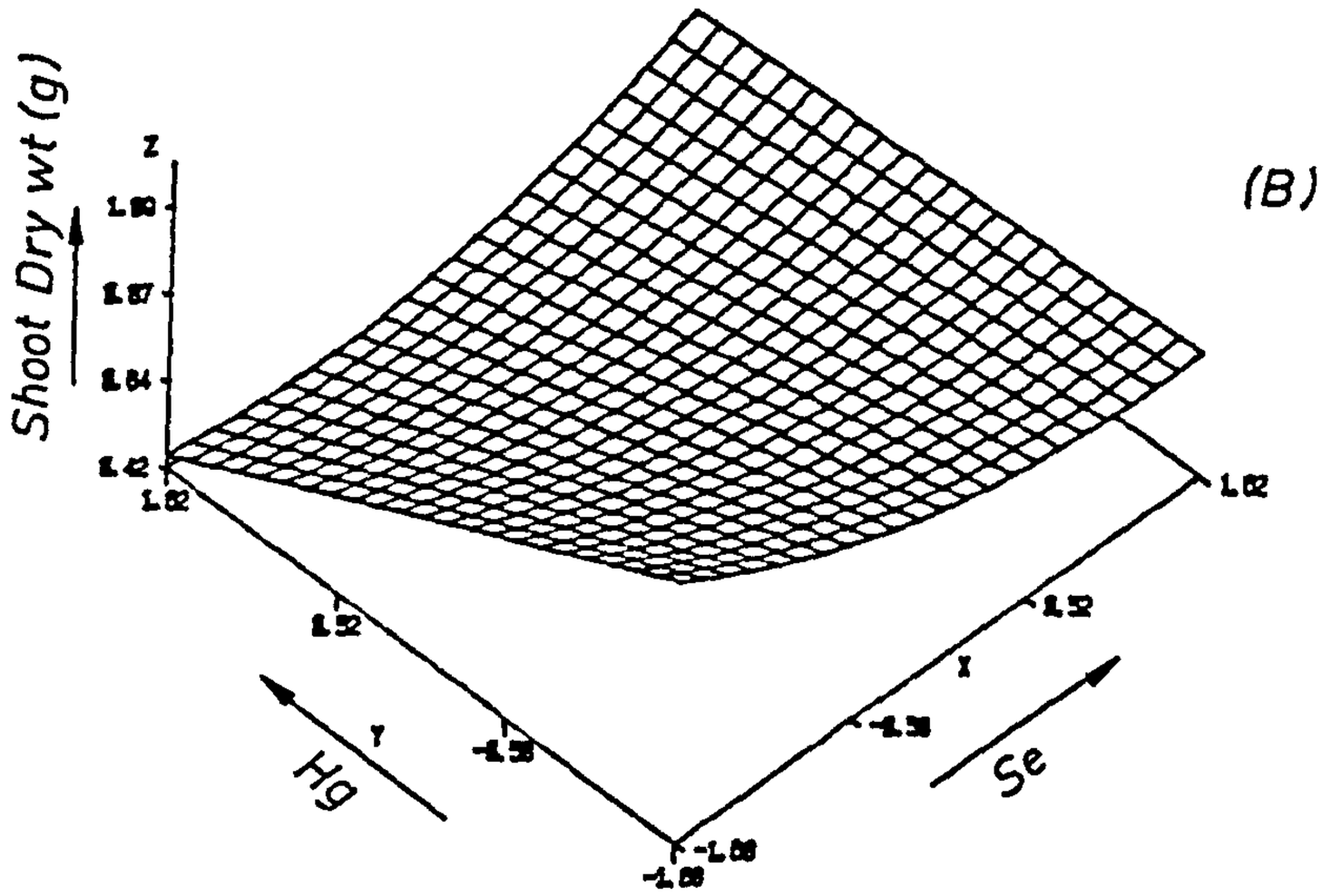
The two R^2 values of 0.67 and 0.272 for this particular experiment should be compared with the values obtained for Experiment 2, discussed earlier. In the latter case the values were 0.91 and 0.75 respectively. Thus in Experiment 1 there is some feature which causes a very significant lack-of-fit of the general second order equation to the data. The major difference between the two experiments is that in Experiment 1 two rather toxic elements and a supposedly beneficial metalloid were used, whilst in Experiment 2, one highly toxic metal with two metalloids, one beneficial, the other more toxic than selenium but not as toxic as either cadmium or mercury. Perhaps when the plants are highly stressed their normal growth patterns are broken down and more attention to this aspect of the work must be made. One cannot talk about synergistic effects because the interaction terms are insignificant, but a similar experiment, perhaps over a slightly amended range of added toxicant concentrations, should be carried out. In Experiment 2, the highly toxic nature of Tl(I) tended to dominate over the other added elements and so only one set of terms (those containing X3) in the second order equation were relevant.

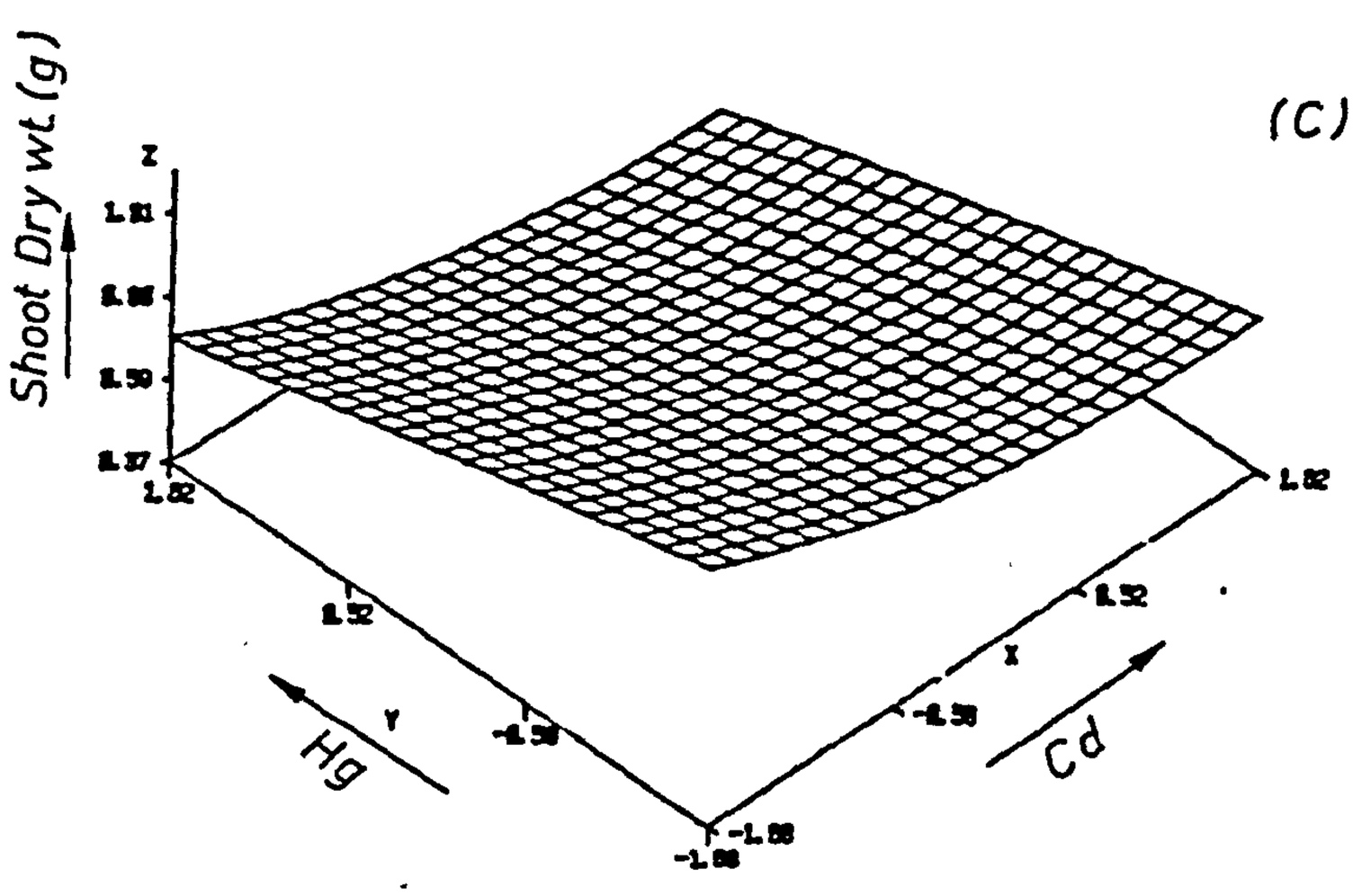
As expected, no value is at all close to significance, although from Table 6.4 the dry weight of the shoots appears to decrease with increasing concentration of the added toxic elements, especially with

Cd and Hg, but not so markedly for increasing Se(VI). The response surfaces in Figure 6.69 seem to confirm these findings. Clearly an alternative form of equation is necessary to fit the data, probably one involving exponentials, but then this particular experimental design is not satisfactory.

FIG. 6.69 RESPONSE SURFACES FOR SHOOT DRY WEIGHT OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT 1 (Se,Cd,Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0







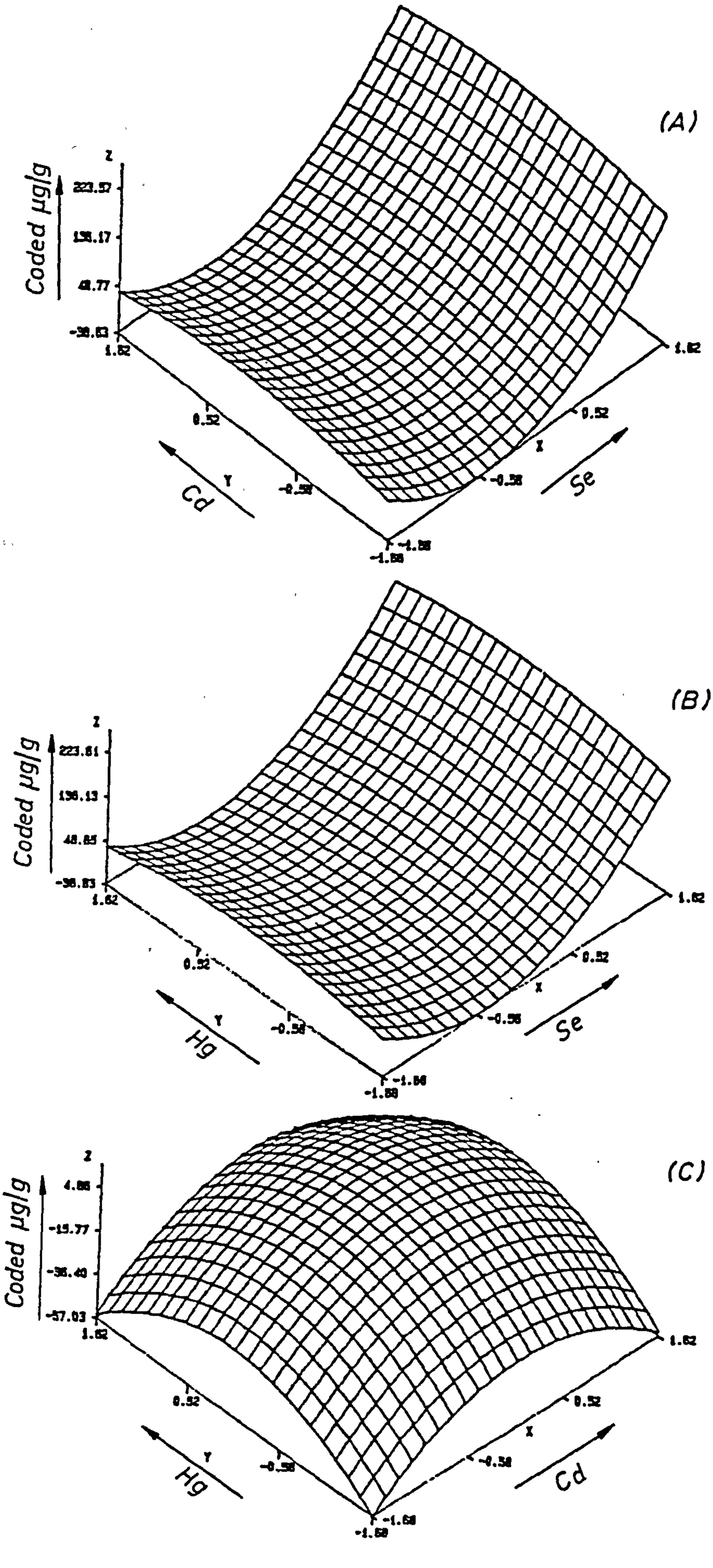
Similarly to Experiment 2 (Se, Te, Tl), a number of elemental analyses were undertaken on the plant tissue, especially important being the uptake of the "spiked" elements (in this case Se(VI), Cd and Hg(II)) from the nutrient solution into the plant. Considering selenium first, with the uptake by shoot as a response variables (response Y2, Table 6.4), while Table 6.8 represents the data treatment values. In this case, the R^2 value is only 0.692 (i.e. an R value of about 0.832 showing the extreme sensitivity of the R^2 value to slight changes in the fit of the model). The probability of the regression values [10] in terms of linear and quadratic terms is also highly significant.

Examination of the PROB values [17] seems to confirm that only Se of the linear terms is significant and similarly in the quadratic factors only Se(VI)*Se(VI) is significant. In the cross-product terms, no value is significant. Thus there appears to be no influence of the other two metals on the uptake of Se(VI). In fact, many values have a probability of close to 1 that they are not involved, e.g. the Cd term has a probability of 0.9619 of causing no effect on the uptake of Se(VI).

Obviously, no such interaction is hinted at in the PROB [18] where only the Se concentration of the nutrient solution is significant, whilst the other two variables (X2 and X3) are not significant to any degree at all.

Clearly all the response surfaces in Figure 6.70 are almost planar with respect to change in either the Cd or Hg(II) concentration, i.e. no change across the Cd or Hg(II) axes. In contrast, the Se content increases dramatically as the Se concentration in the nutrient solution is increased, especially above a coded value of -0.25.

FIG.6.70 RESPONSE SURFACES FOR SELENIUM UPTAKE BY SHOOTS OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se,Cd,Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



The Cd uptake by shoots is similar to that just described for the Se uptake (see Table 6.4, Response Y3 and Table 6.8).

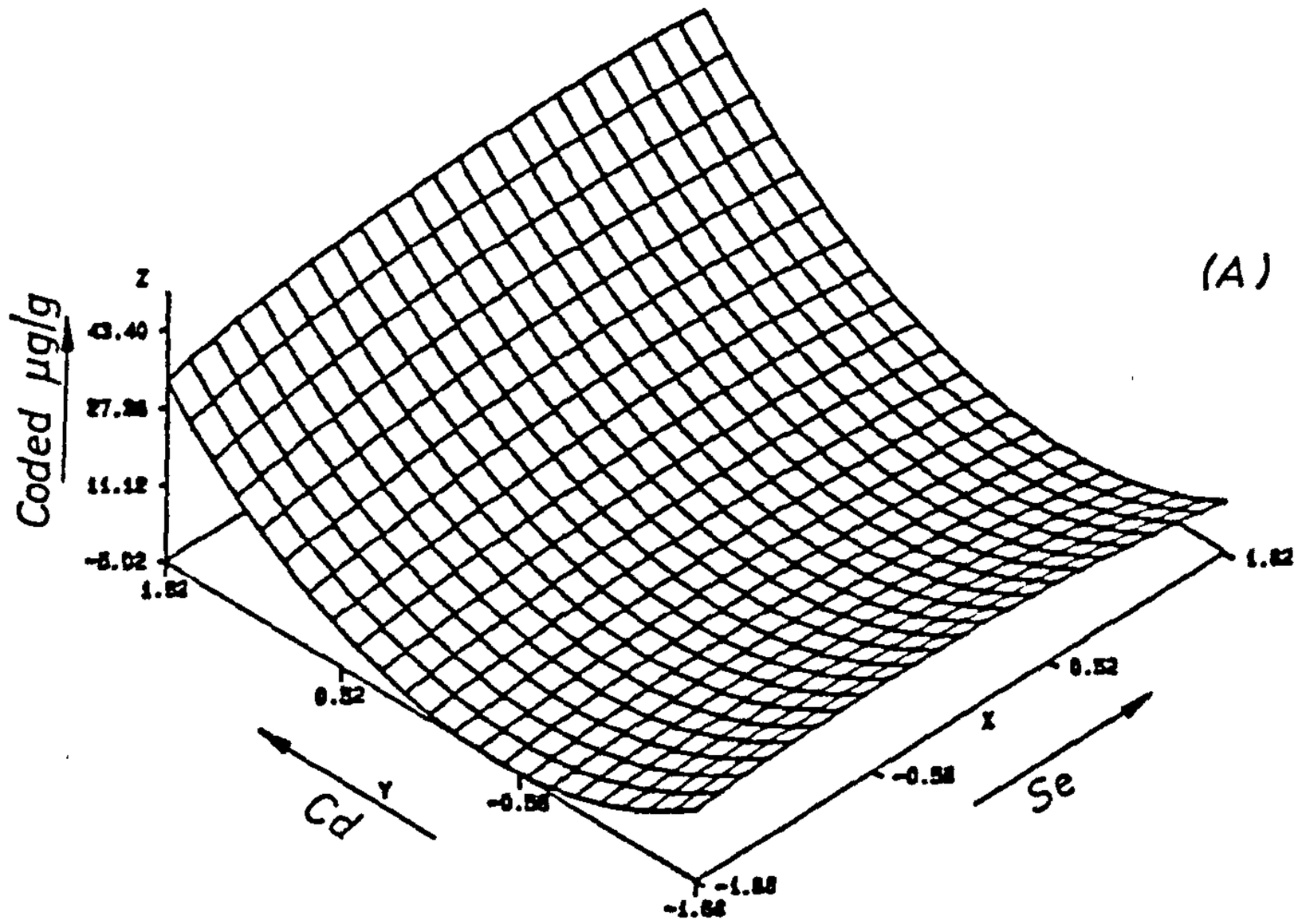
In this case the fit of R^2 is 0.7496, being the best value among nine variables for the shoots.

Examination of the PROB values [10] and [17] show similar Se uptake by shoots, i.e. linear X2 (Cd) is significant and quadratic Cd*Cd is also significant, but the remainder of the other values have no significance.

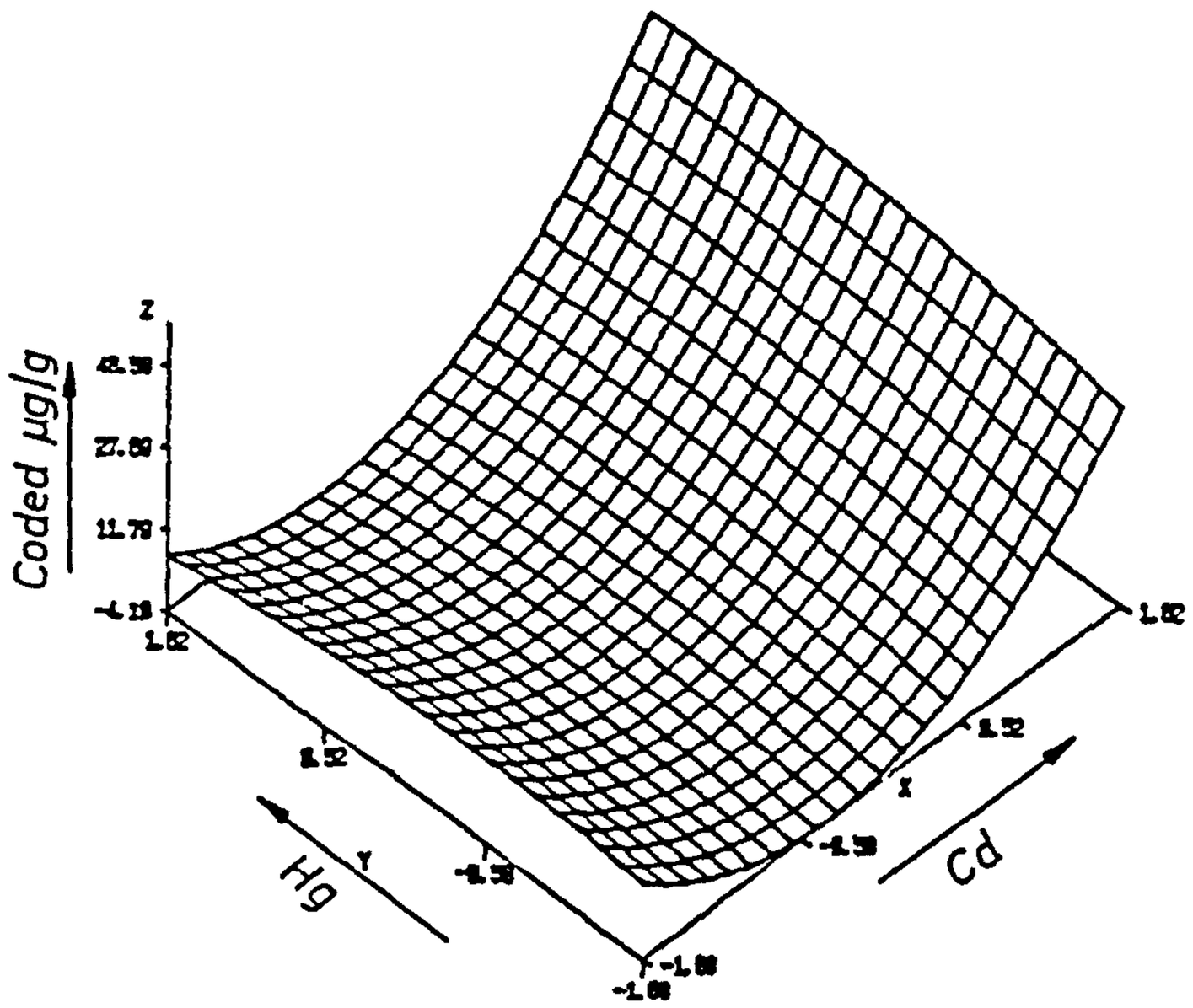
Clearly no interaction of any measurable significance takes place, especially bearing in mind the PROB [18] values, where only the Cd concentration of the nutrient solution is significant, while the other two variables (Se and Hg) are not significant. Frankly, this is somewhat surprising, because some interaction between the Cd and Hg was expected because of their very similar chemical nature. It was thought likely that they would compete for the "same sites" within the plant. Clearly, this is not borne out by the experimental results.

The response surfaces as shown in Figure 6.71 confirm these findings, there being no change across the Se or Hg axes.

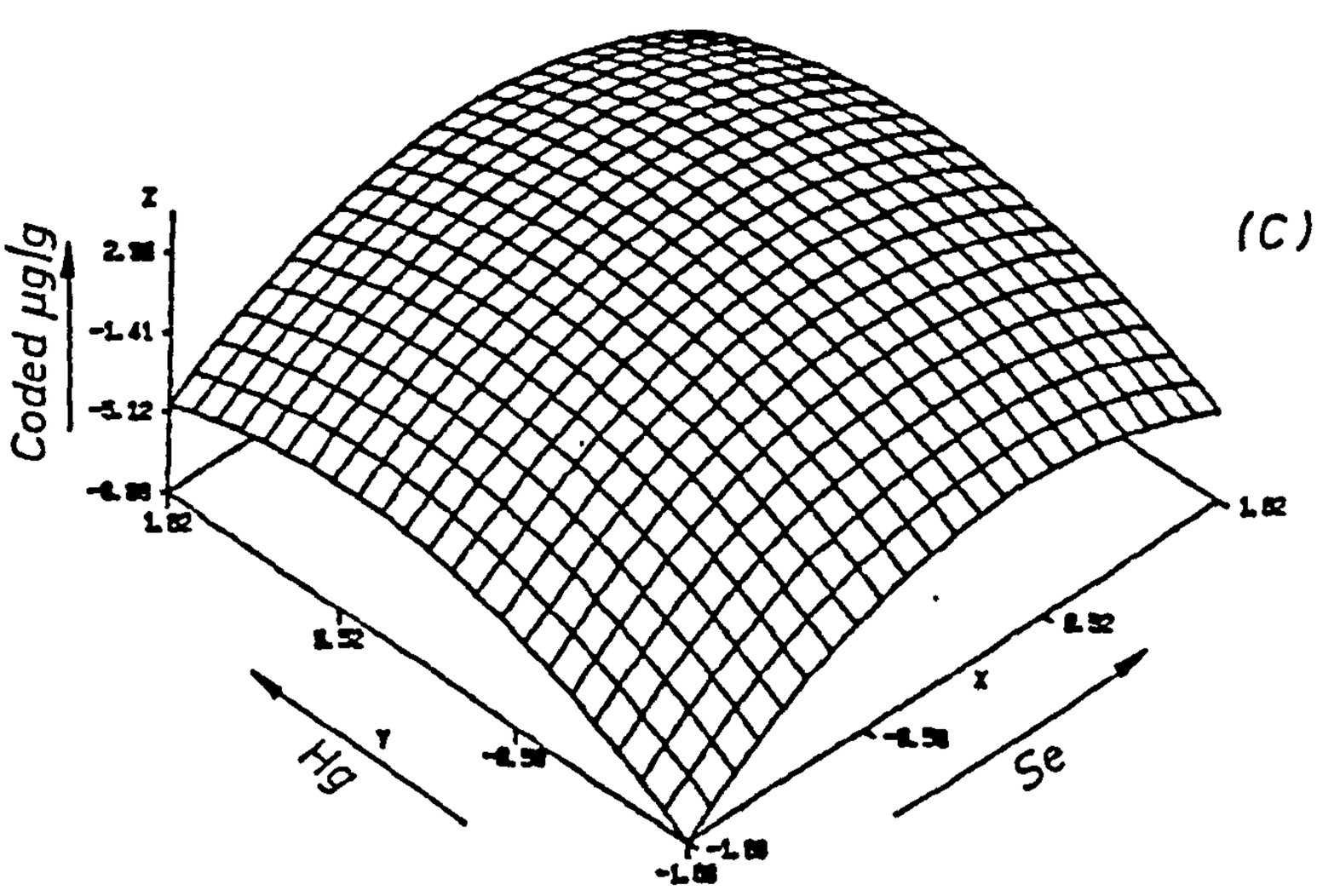
FIG. 6.71 RESPONSE SURFACES OF Cd UPTAKE BY SHOOTS OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se_1 Cd, Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



(A)



(B)



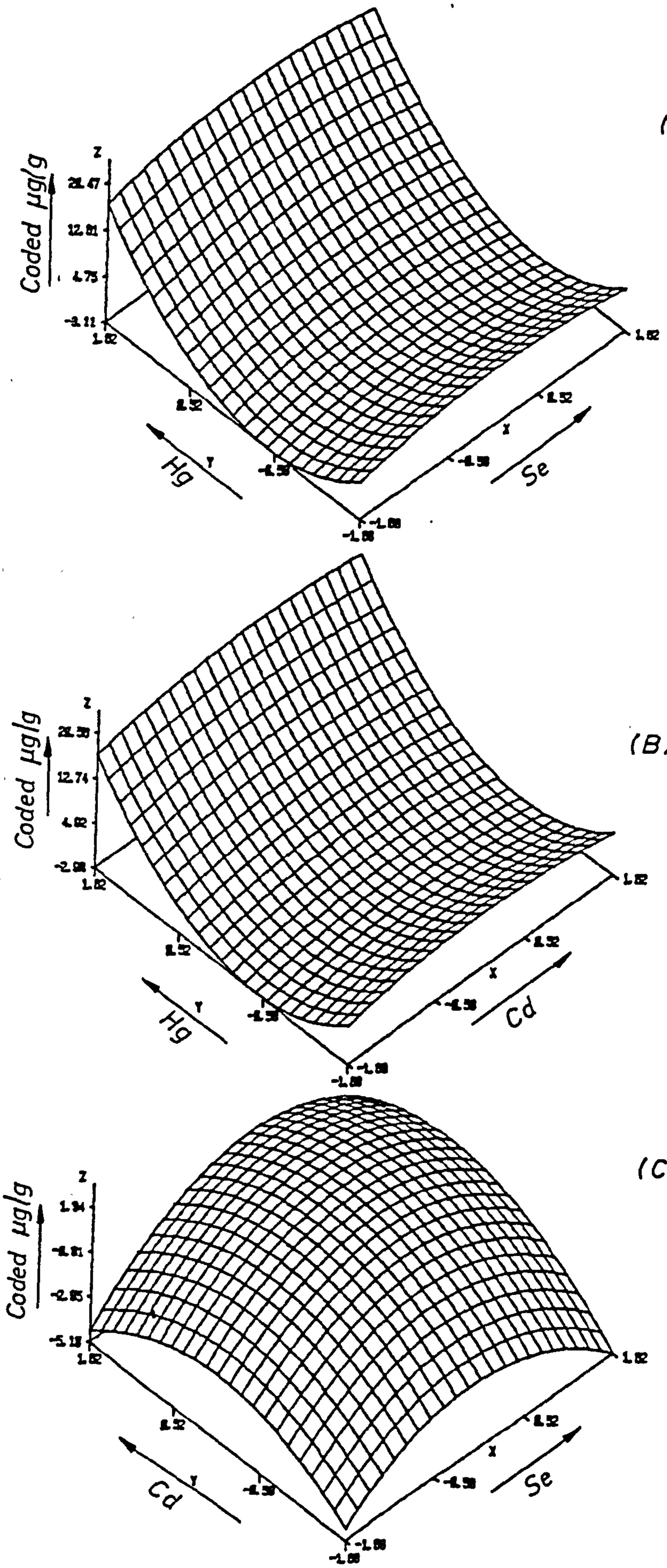
(C)

In an analogous manner to the Se(VI) and Cd(II) uptake, the mercury (II) uptake from the nutrient solution into the shoot system appears to be dependent only on the Hg(II) concentration (in the nutrient solution). Here, the levels of Hg in shoots are similar to those for the Cd in the shoots, being lower than in the roots, the exact opposite of the Se levels where Se is higher in shoots than in roots (see Table 6.4).

Note that the fit of R^2 is only 0.64, being lower than Se(VI) and Cd(II) values. Again, very similar values for the probabilities of each of the factors being involved in the process of uptake are noted, where the Hg linear term is significant and the Hg*Hg quadratic term is also significant but all the other terms are not.

Examination of the response surfaces in Figure 6.72 confirms these findings. As soon as the Hg(II) concentration in solution rises above a coded value of -0.5, then the concentration of Hg(II) in the tissue rises almost exponentially. Obviously the Se(VI) or Cd(II) concentrations have no effect on the situation. Comments similar to those made about the uptake of cadmium and possible competition with mercury are also relevant in this situation.

FIG. 6.72 RESPONSE SURFACES FOR Hg UPTAKE BY SHOOTS OF LOLIUM PERENNE SEEDLING. EXPERIMENT (Se,Cd,Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



Since the plants were divided into roots and shoots, a set of data similar to that describing the shoots was also available for the roots and was processed in a similar manner. However, because of the differing biological functions of roots and shoots, differences in behaviour towards toxic metals and hence in the form of the response observed is to be expected.

For roots in the same experiment (Se, Cd, Hg), Table 6.9 describes the response variable statistics for the roots, while Table 6.5 lists the root length, biomass and concentration levels of spike elements (Se, Cd, Hg) plus some essential metals such as Cu, Fe, Mn and Zn.

For root length response variable Y1, Table 6.9, also in terms of the three experimental variables, X1, X2 and X3, represent the concentration of Se(VI), Cd(II) and Hg(II) respectively. In this case, R^2 is only 0.608, which is due to the model rather than to random error. Thus the model used involving linear, quadratic and cross-product terms appears to be only a reasonable descriptor for the root length versus metal concentration.

From the probability terms [10], it is obvious that only the linear term is important, but not the quadratic or cross-product terms. The PROB values [17] seem to confirm that only Hg has a significant effect on root length, whilst both Se(VI) and Cd do not. Also the quadratic and cross-product terms are significant, signifying the greater toxicity of Hg to plants than Cd.

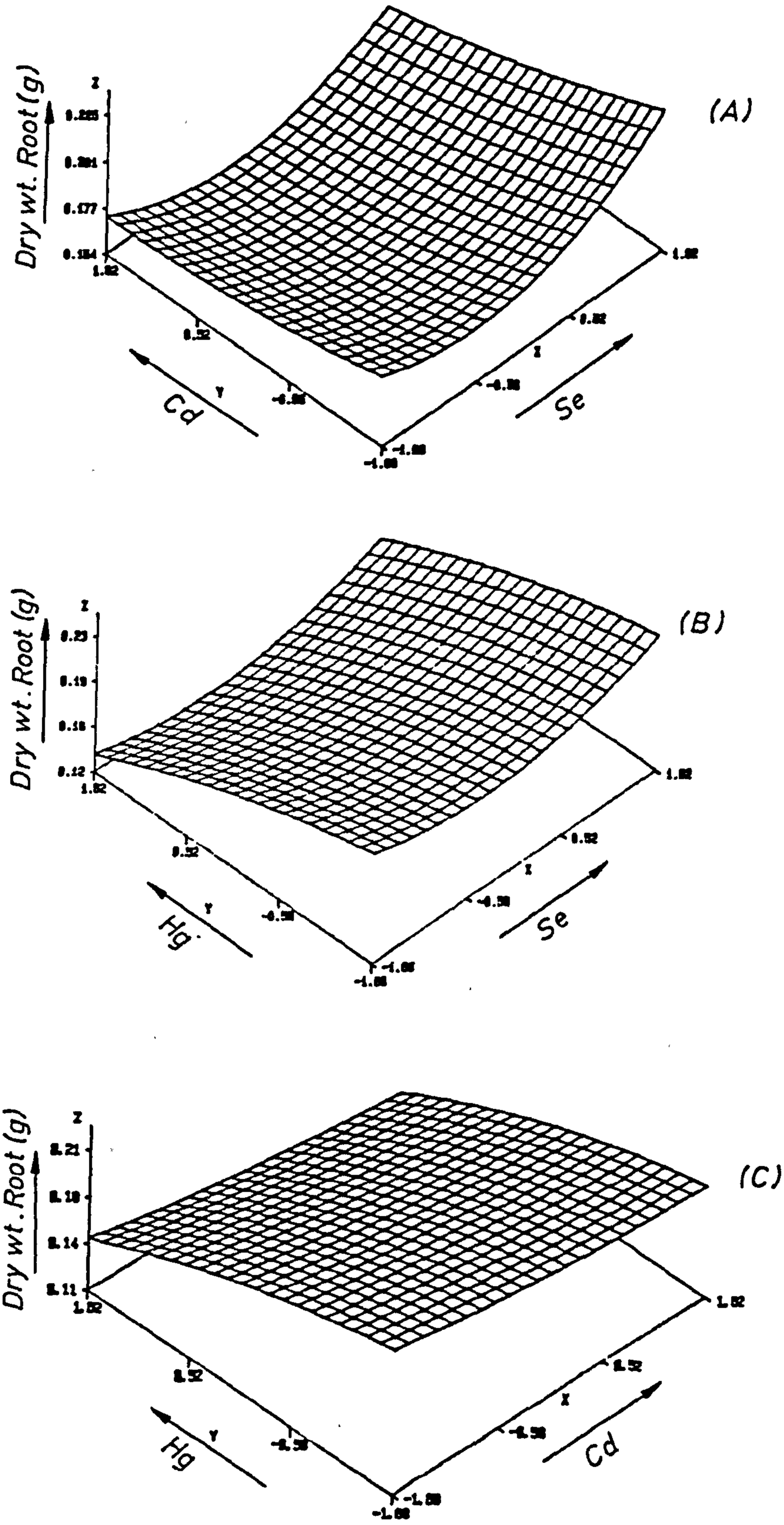
The factor test [18] suggests that only Hg has an effect on the root length response.

The response surfaces, Figure 6.73, confirm these findings, in this case increasing Hg(II) concentration has a marked effect on growth.

FIG. 6.73 RESPONSE SURFACES FOR ROOT LENGTH OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se Cd Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0

In the case of the dry weight of the roots as a response variable, see Y9, Table 6.5, while Table 6.9 presents the regression statistics for this response. The R^2 value being 0.14 is the lowest value among the nine variables obtained for the roots (cf. Experiment 2 where a value of 0.68 was obtained in a similar situation). The lack of fit is very significant with the pure error accounting for only about 5% of the total error. Thus the suggested model is not anywhere near an adequate fit for the data. Therefore, none of the terms, neither linear, quadratic nor cross-product, are significant to any degree. Looking at the probabilities of the parameters, none are also significant and the response surfaces confirm these findings. The surfaces (see Figure 6.74) are almost planar, as would be expected from an examination of the raw data in Table 6.5. Therefore, the levels of concentration of the toxicants in the nutrient solution were clearly insufficient to cause any change in the biomass of the roots, in spite of there being two well known toxic metals.

FIG. 6.74 RESPONSE SURFACES FOR DRY WEIGHT ROOT OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se, Cd, Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0

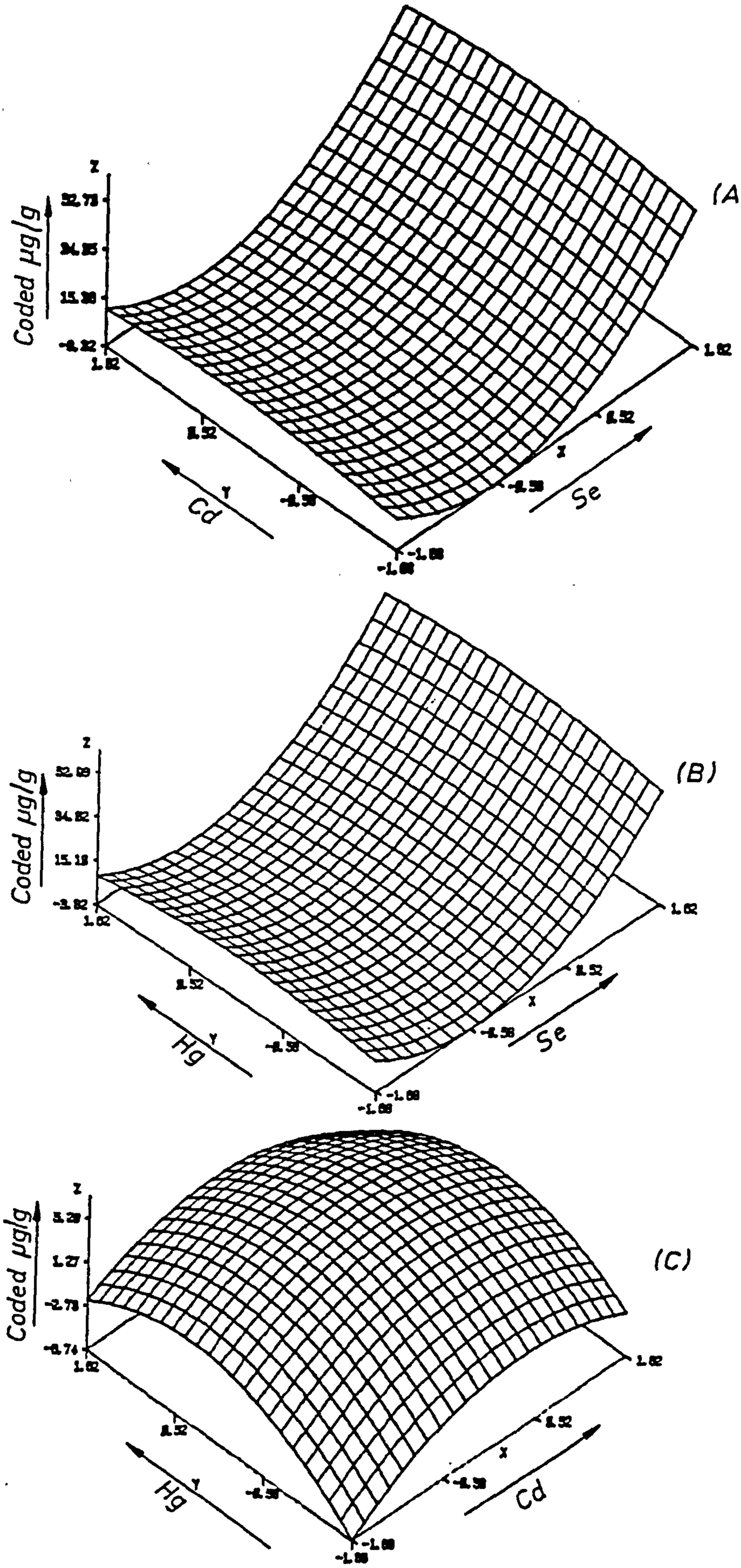


In a similar manner, following a discussion of root length and dry weights, let us examine the elemental analyses of initially the uptake of three added elements (by the plant). Thus Figure 6.75 illustrates the concentration of selenium in the tissue of the root. Notice in this case the pure error value is extremely low and most of the total error arises from the lack of fit of the model. In this case the R^2 value is 0.835, reflecting a reasonable fit of the data to the model being imposed on it. The probability of the regression values (see Table 6.9) in terms of linear and quadratic terms [10] for Se are very high, being greater than 99% that they are factors in the model. But notice that the probability of the cross-product term [10] is very small indeed, so once again no interaction terms are to be expected.

As commented previously for shoots, here the uptake of Se by roots is in a similar manner; the non-interaction between the Se level in the tissue and the Cd level in the nutrient solution is not surprising. However, a similar level of response is found in the case of increasing Hg(II) concentration where no interaction with rise in the Se content is found.

The response surfaces confirm these findings; Figure 6.75 depicts the response for the Se level in tissue at coded value = 0, i.e. Se = 0.05 µg/ml in solution with varying Cd and Hg concentrations. Notice the almost planar nature of the responses for Figure 6.75, A & B, in the direction of increasing Cd or Hg respectively. However, the level of Se is affected by a simultaneous rise in the concentration of Cd and Hg as shown in C. Here the plant is sufficiently stressed to try to overcome this problem by increasing the Se uptake.

FIG. 6.75 RESPONSE SURFACES FOR SELENIUM UPTAKE BY ROOTS OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se, Cd, Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



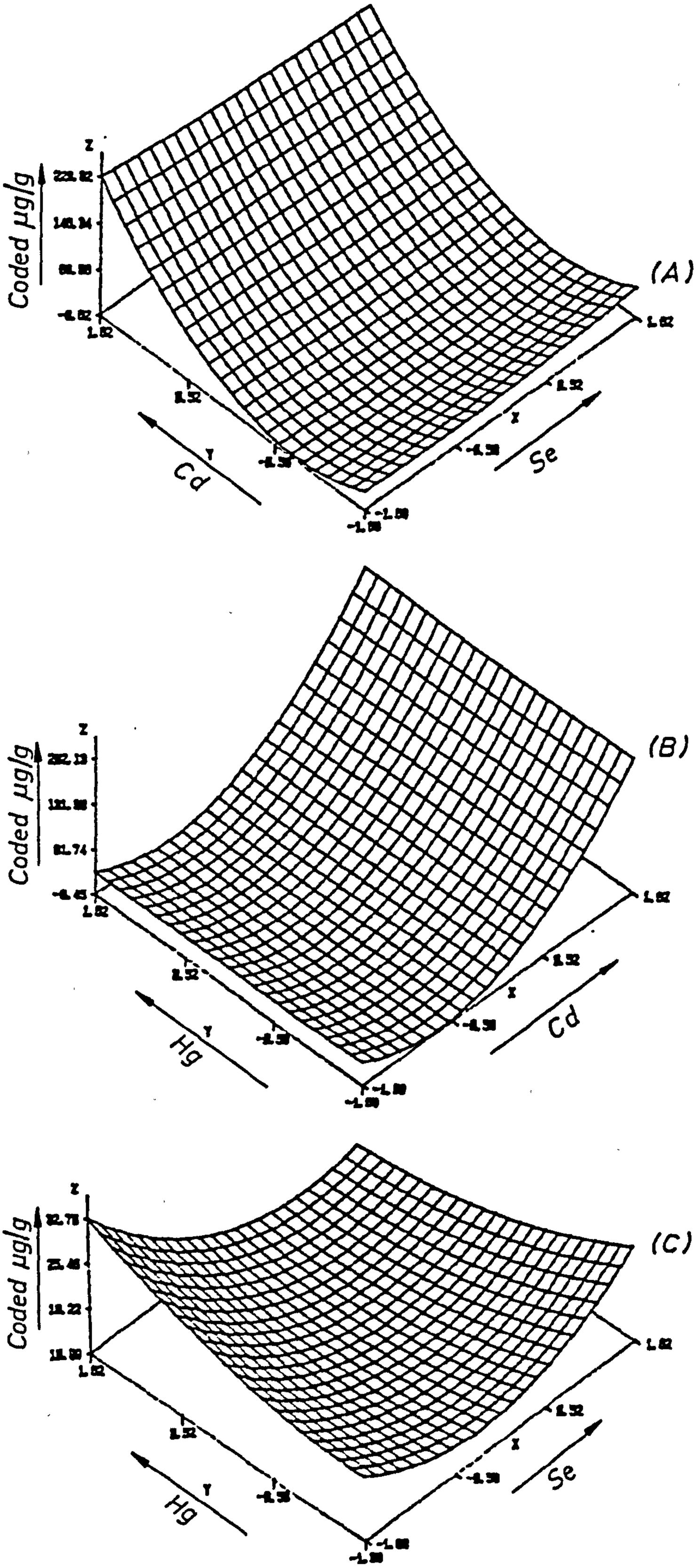
The cadmium uptake by the roots of the seedlings is similar to that just described for the selenium uptake. For the cadmium concentration as a response variable see response Y3, Table 6.5. The R^2 value of 0.98 is extremely high (the best R^2 value for Experiment 1), and suggests an almost perfect fit of the model to the data. However, notice that the pure error value is very low, some 5% of the total error, and so the lack of fit of the model to the data is also significant. The probability of the regression values [10] (see Table 6.9) in terms of linear and quadratic terms is also very high and both of them are important, but the cross-product term is not, so again no interaction terms are to be expected.

Examination of the PROB [17] shows only two variables are significant, Cd linear and Cd*Cd quadratic, while none of the other terms are significant.

Obviously, no such interactions are hinted at in the PROB [18] where only the Cd concentration in the nutrient solution is significant and highly so (as it should be), whilst the other two variables (Se and Hg) are not significant to any degree at all.

Clearly all the response surfaces are almost planar with respect to change - in either the Se(VI) or Hg(II) concentration, i.e. no change across the Se or Hg axes. In contrast, the Cd content increases dramatically as the Cd concentration in the added medium is increased; see Figure 6.76, A & B. Figure 6.76C shows the response for Cd uptake by the roots with varying Se(IV) and Hg(II) concentrations at a constant Cd concentration of 0.01 μg/ml.

FIG. 6.76 RESPONSE SURFACES FOR CADMIUM UPTAKE BY ROOTS OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se, Cd, Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



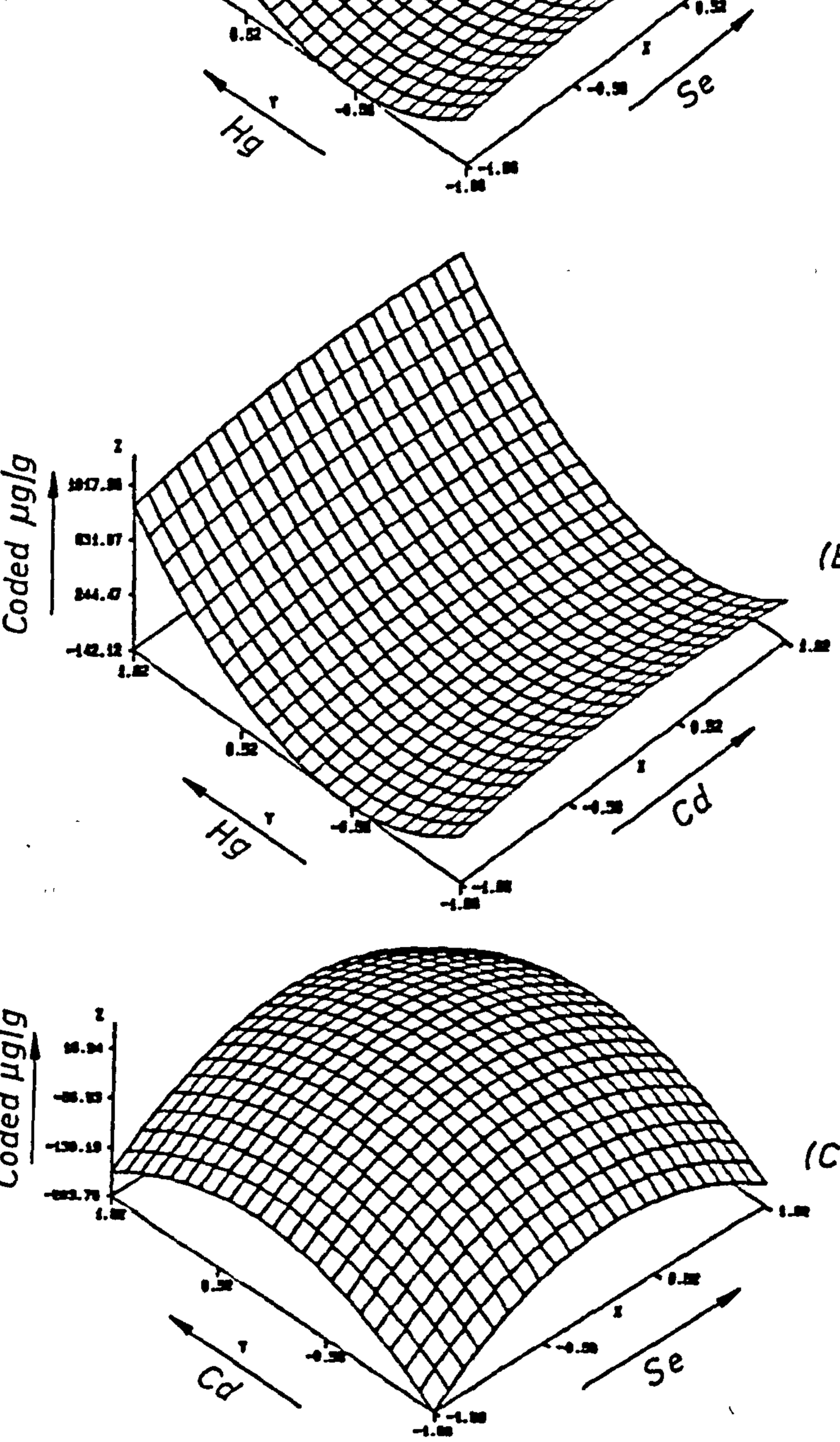
In an analogous manner to the selenium (VI) or cadmium uptake, the mercury (II) uptake from nutrient solution into the root system appears to be only dependent on the Hg(II) concentration in the nutrient solution. Table 6.9 describes the response variable statistics for this uptake.

Note that the fit of R^2 is only 0.7507, being a lower fit than for Cd uptake by roots, but allowing an R value of about 0.866, showing the extreme sensitivity of the R^2 value to slight changes in the fit of the model. The probability of regression values [10] in terms of linear and quadratic terms is very high and important, but the cross-product terms are very small indeed; so once again insignificant interaction terms are to be expected.

The only variable of importance is the Hg(II) content of the nutrient solution, there being no effect due to the concentrations of Se(VI) or Cd in the nutrient solution. An inspection of the PROB values [17] confirms these suggestions, where only the linear Hg(II) and quadratic Hg(II)*Hg(II) parameters are deemed significant.

The mercury content of the roots is very much higher than in the shoots, see Tables 6.4 and 6.5. The lack of fit of the model is deemed highly significant because of the extremely small value of the Type 1, SS for the pure error values, being less than 1 in 50,000. Examination of the response surfaces confirms these concepts, the mercury level increases markedly when the mercury concentration in the nutrient solution exceeds coded 0.0 (0.05 µg/ml), but is almost planar with respect to increasing Se or Cd concentrations. See Figure 6.77 A & B, while Figure 6.77C depicts a dome shape with apparent toxicity of Cd and Se. However, the low R^2 value must render further interpretation open to great doubt.

FIG. 6.77 RESPONSE SURFACES FOR Hg UPTAKE BY ROOTS OF LOLIUM PERENNE SEEDLINGS. EXPERIMENT (Se, Cd, Hg) WITH THIRD ELEMENT AT CODED LEVEL = 0



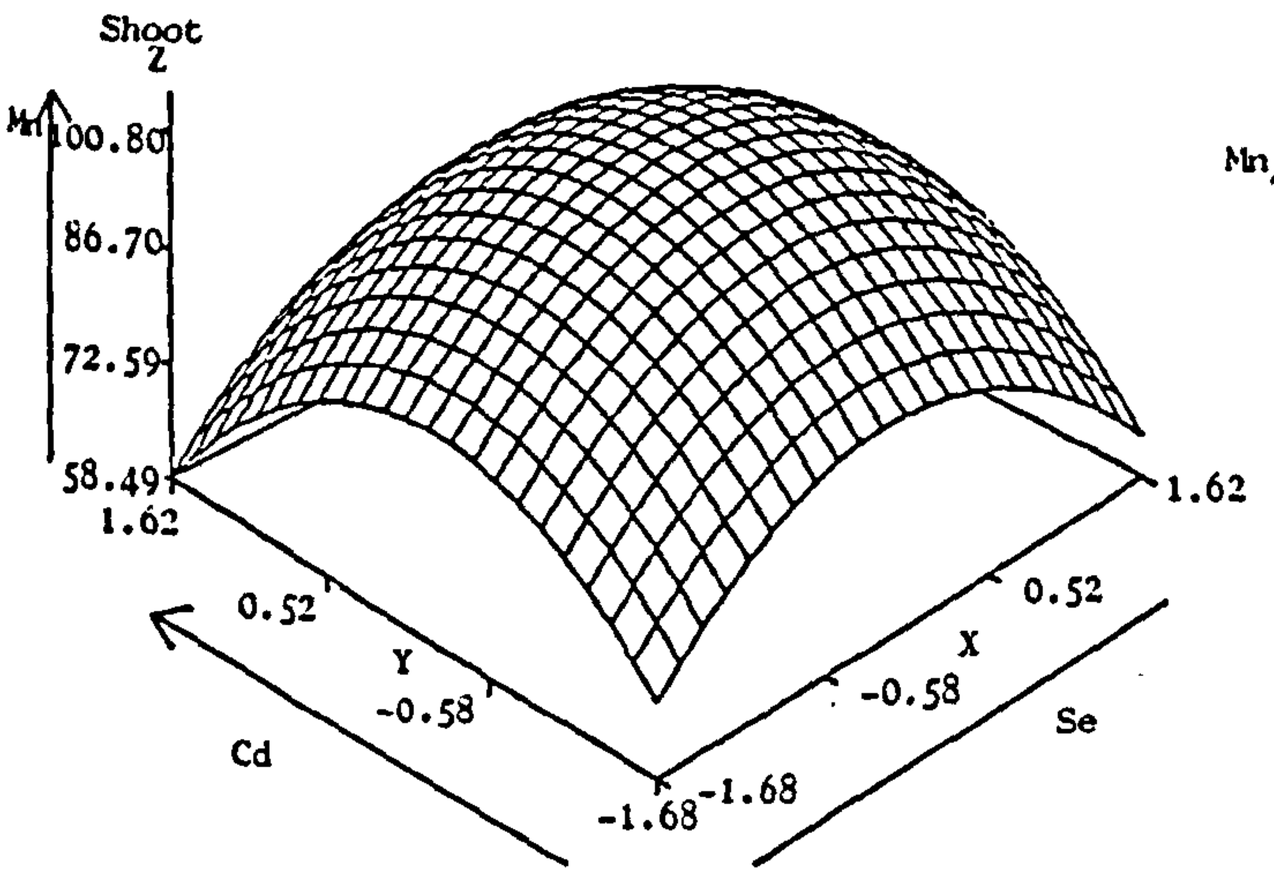
In an analogous manner to Experiment 2 [Se(VI), Te(VI), Tl(I)], the levels of the essential metals (Cu, Fe, Mn and Zn) in the plant tissue were also determined to see if they were affected by interaction through the agency of the added "spike" metals on the nutrient solution. The essential metals were present at normal concentrations in all the nutrient solutions involved. The first example, the uptake of manganese for both roots and shoots of Lolium perenne seedlings, is shown in Figure 6.78.

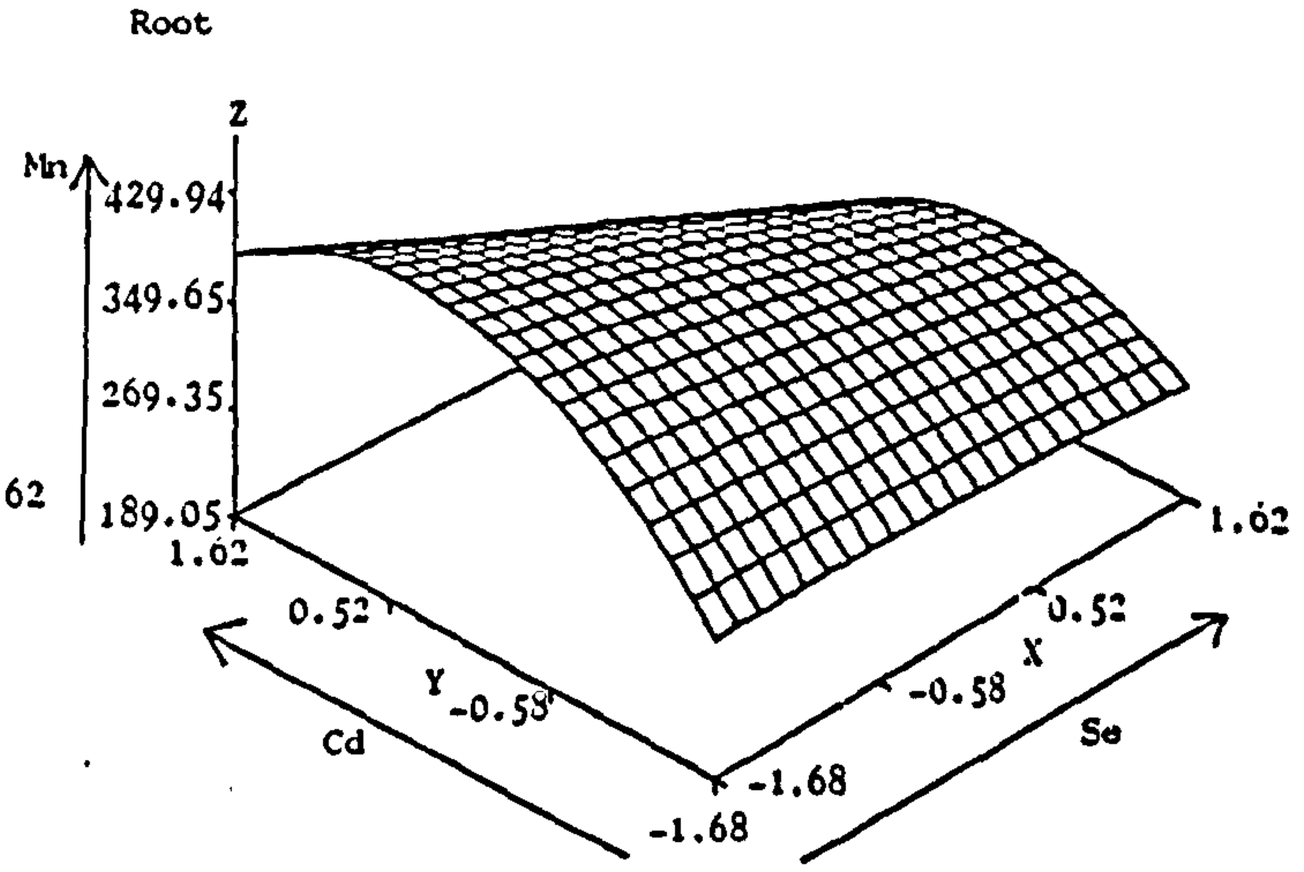
For the roots, regretfully no parameter value approaches significance, the R^2 value is very low (see Table 6.9), although all three surfaces (as shown in Figure 6.78) are of the saddle point type.

Clearly, the levels of manganese are higher in the roots than in the shoots. The case is opposite for Experiment 2 (Se, Te, Tl), thus changing the identity of the spike metals yields different results for manganese uptake. For all surfaces, the Se(VI) level does not greatly affect the level of Mn(II) present. However, the twin toxic elements of Cd and Hg(II) produce a very clear effect, with increases in the Mn uptake mirroring the rise in concentration in the nutrient solution of Cd(II) or Hg(II).

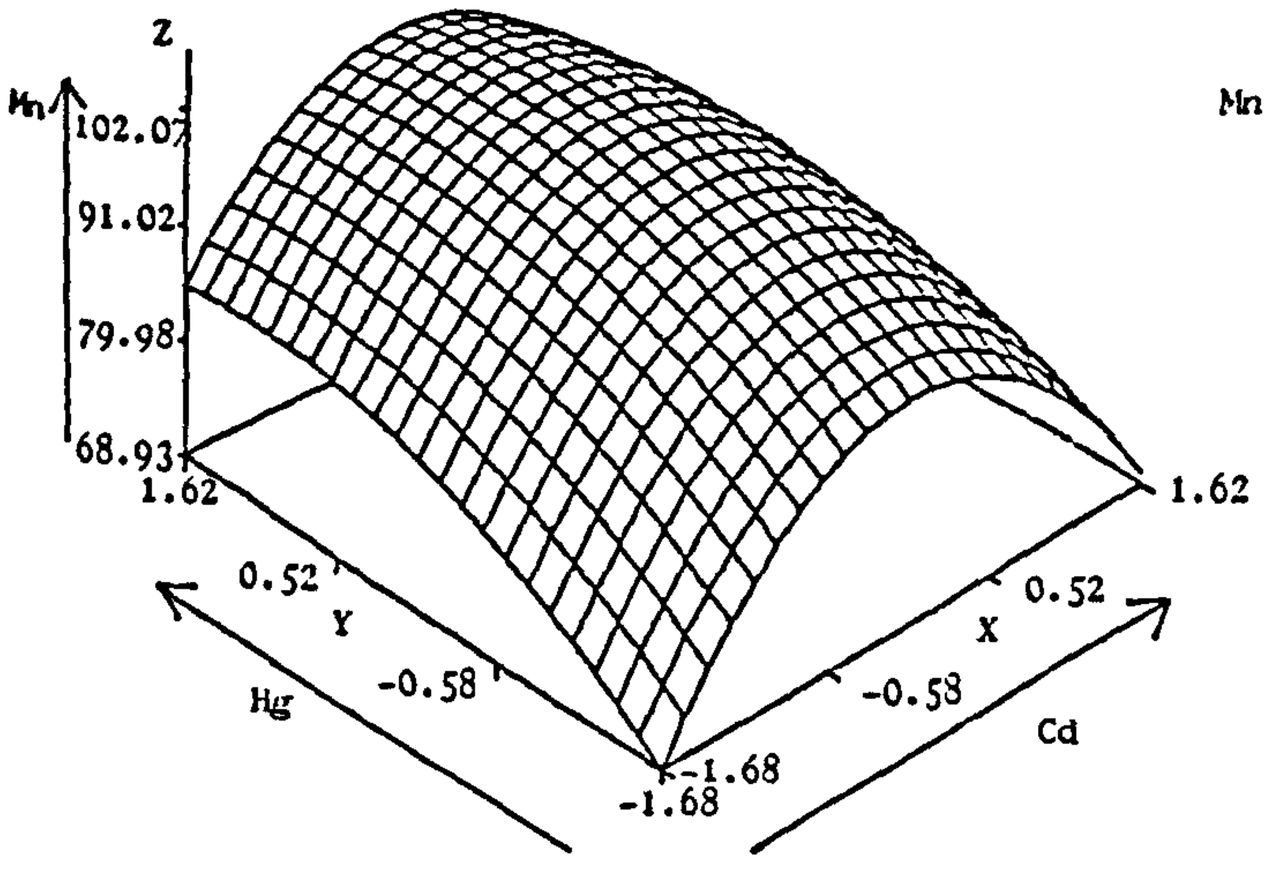
For the shoots in the same Experiment, the uptake of manganese is shown as response Y5, Table 6.6, while Table 6.10 represents the data treatment values. In this case, the R^2 value of 0.7429 is high (relative to the essential metals). The probability of the regression values [10] in terms of quadratic terms is also very high and important. But there are no significant values in the linear or cross-product terms. An examination of the PROB values [17] will confirm these findings, there being no significant experimental variables except

Figure 6:78. Response Surface for Manganese taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment I (Se, Cd, Hg) with third element at coded level = 0

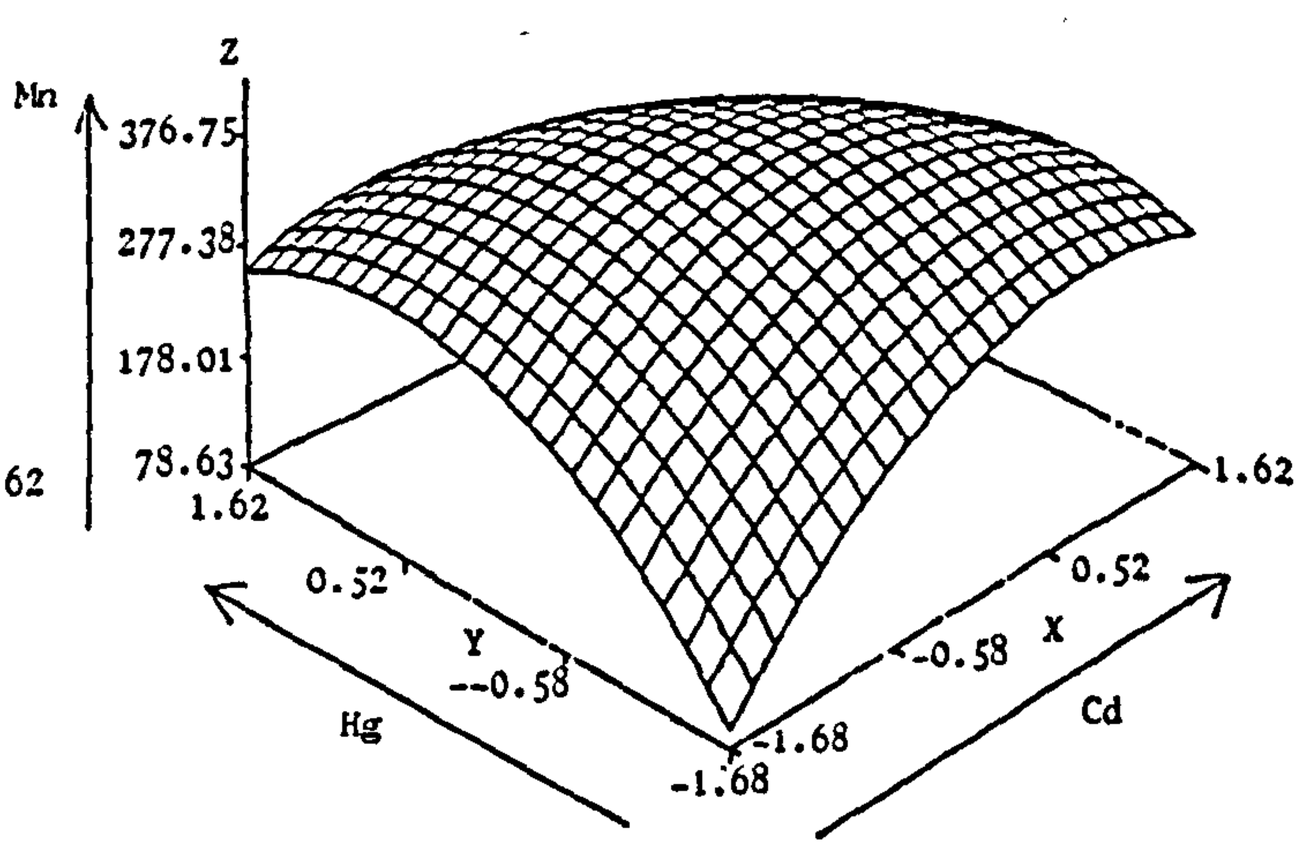




Shoot



Root



for the quadratic Se(VI)*Se(VI) and Cd(II)*Cd(II) terms, where both terms are highly significant and have an effect on the Mn(II) uptake response. All the surfaces in Figure 6.78 describing the shoots are similar in shape, being the maximum type; the levels of Se(VI) and Cd affect the shoots levels of Mn to a greater extent than does either Hg or uptake by the roots.

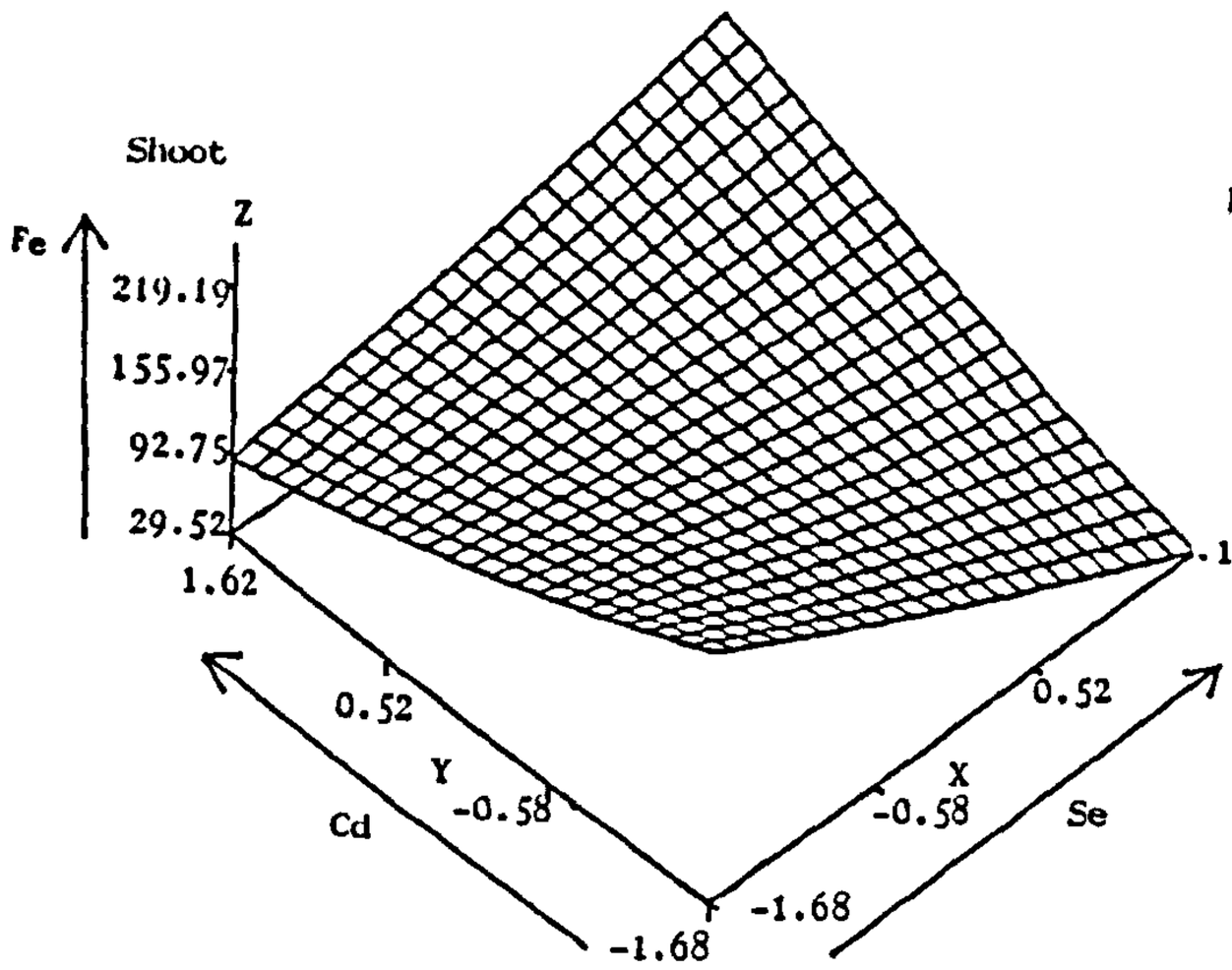
The uptake of iron by Lolium perenne seedlings in the presence of the spiked toxic elements for both roots and shoots is listed in both Tables 6.4 and 6.5 as response Y6.

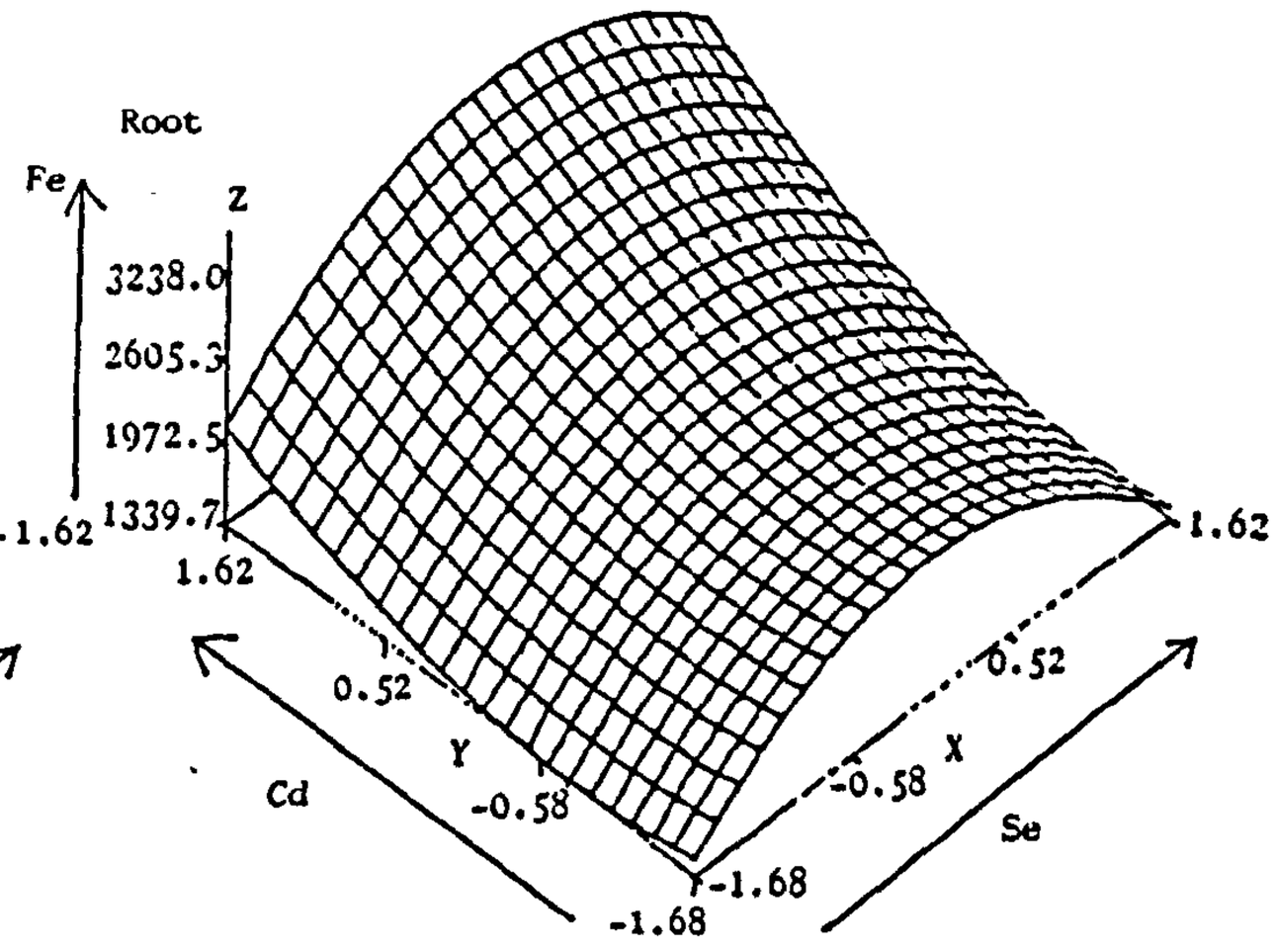
For the roots case, the fit of R^2 is unexpectedly low being only 0.359, so the model proposed is an extremely poor fit for the data. The pure error only accounts for less than 0.5% of the total error, so making the lack of fit parameter an important guide. With this level of error in the lack of fit to the proposed model, it is not surprising that none of the parameters for roots are significant, i.e. there appears to be no support for the hypothesis that the level of iron in the roots is affected by the presence of Se(VI), Cd or Hg(II) when they are present over a very large concentration (up to 5 µg/ml, 1 µg/ml, 1 µg/ml respectively) range.

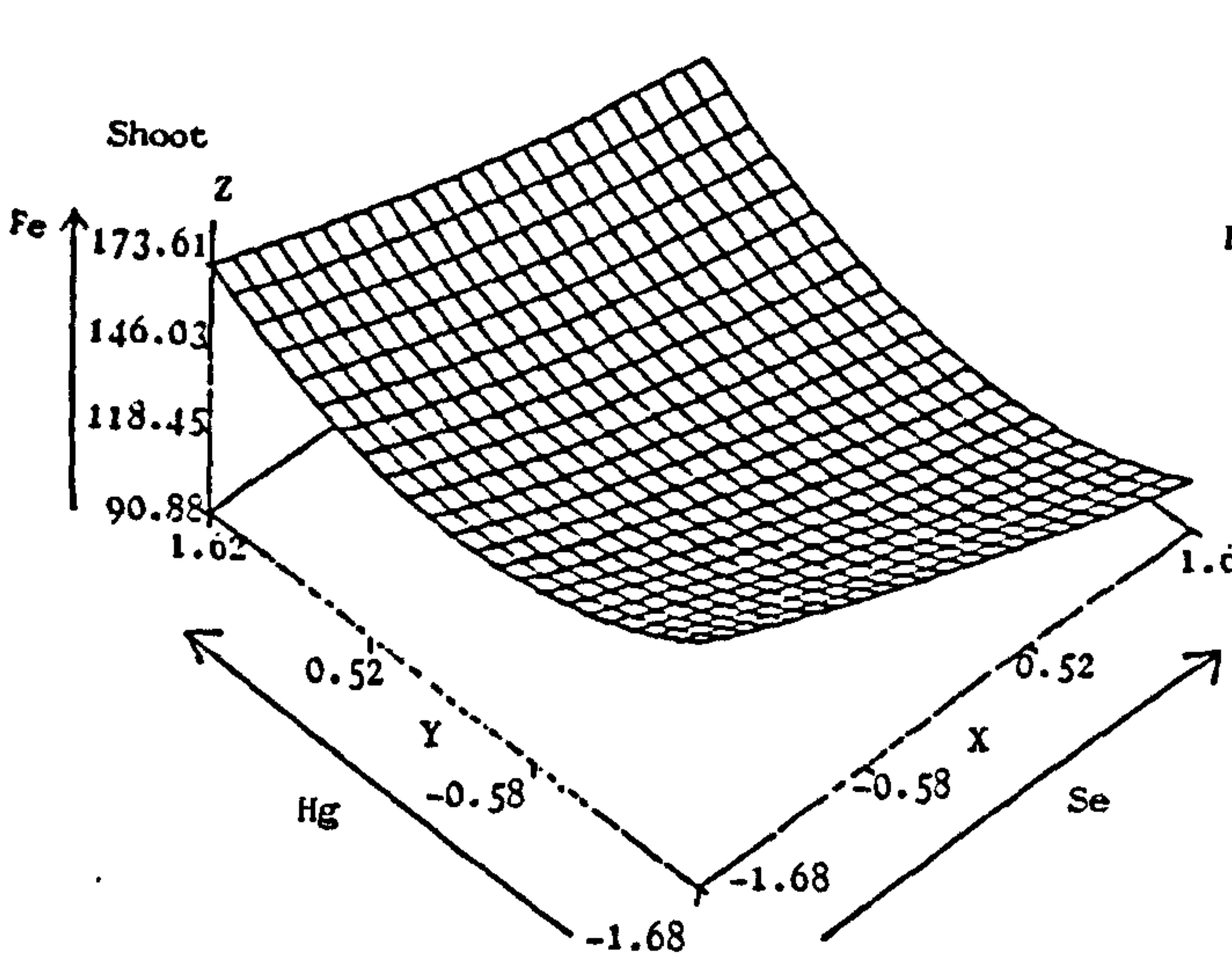
Thus it is not unexpected that the response surfaces in Figure 6.79 shown are the saddle point type.

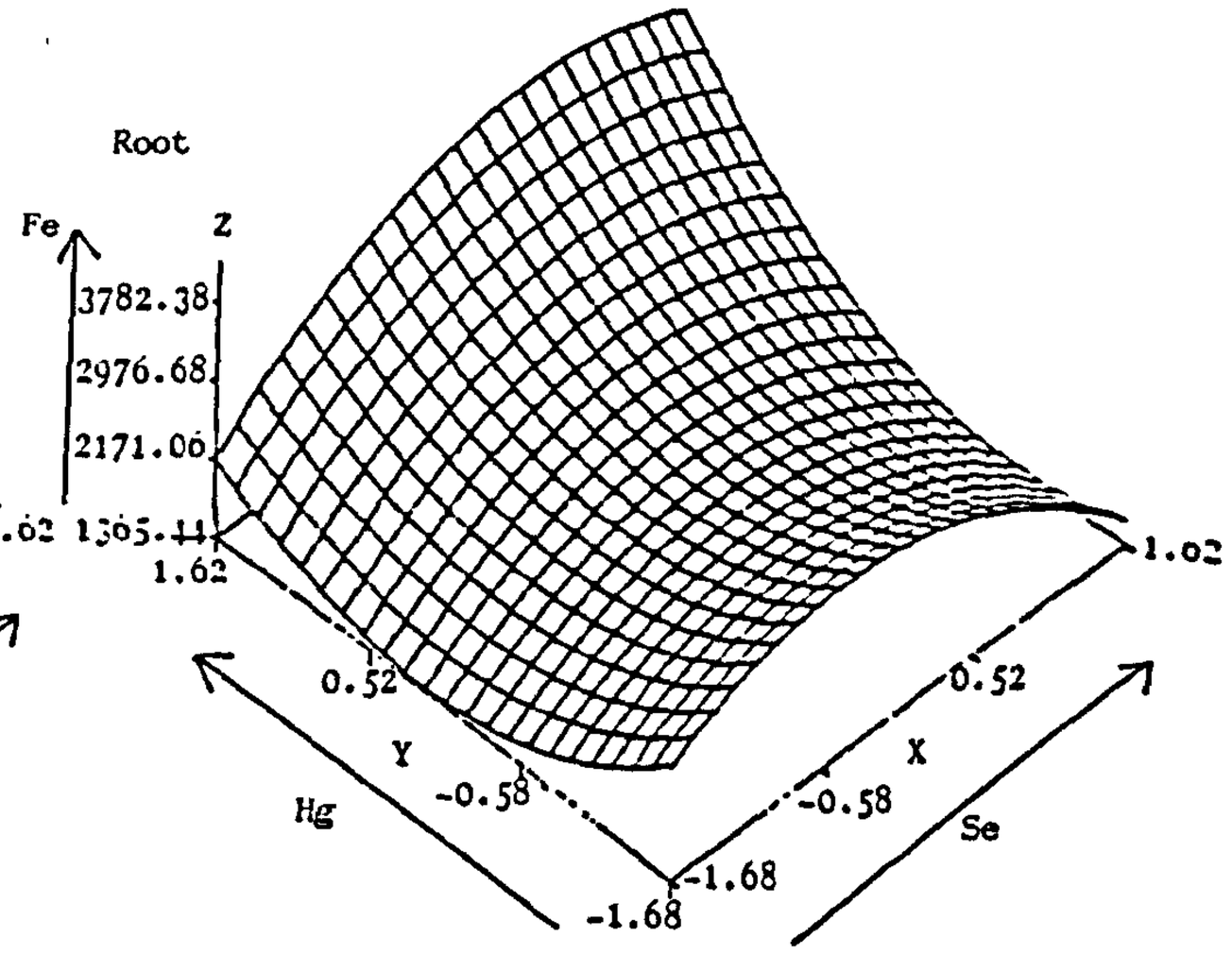
Generally, at these levels of the spiked elements it can be said that levels of iron in the roots appear not to be affected to any significant extent by the levels of these particular toxicants in the nutrient solution.

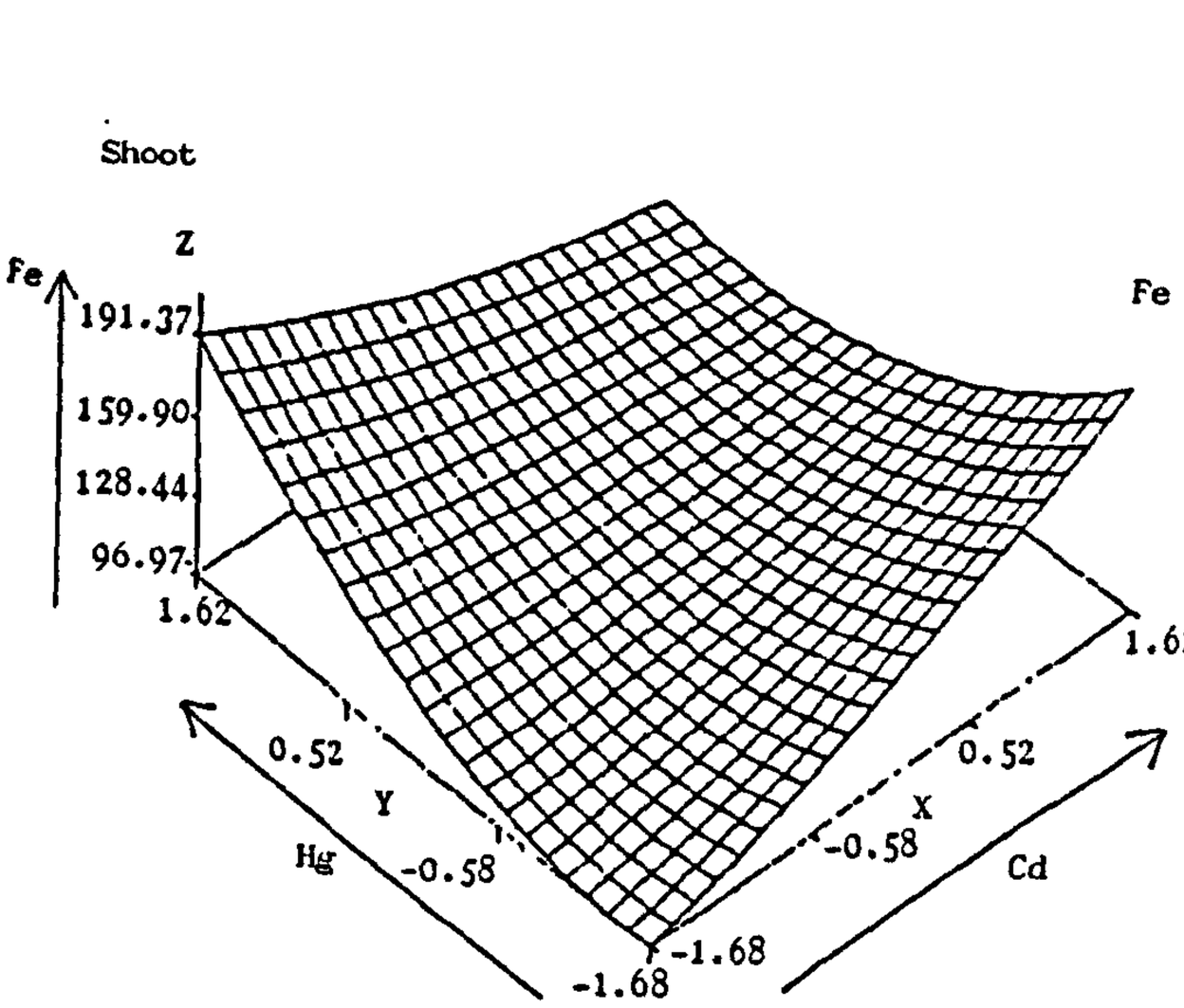
Figure 6:79. Response Surface for Iron taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment I (Se, Cd, Hg) with third element at coded level = 0

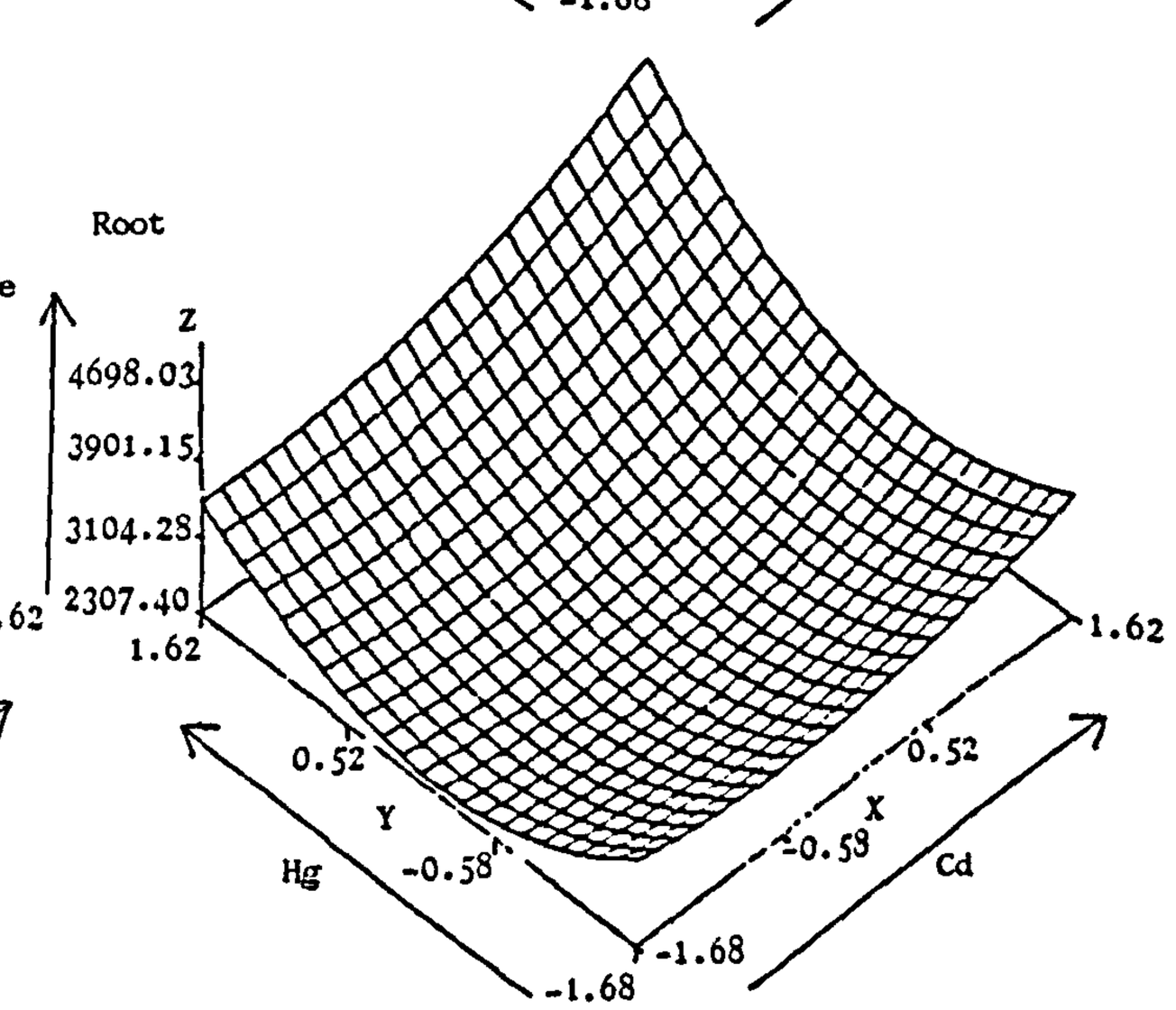












For the shoots, the R^2 value obtained of 0.4608 is a better fit than for the roots. Although the model is a very poor fit for the data, the pure error accounts for less than 10% of the total error, so making the lack of fit parameter important. However, the levels of iron in the shoots are much lower than in the roots.

Examination of the PROB values [17] seems to confirm that there is no significant value except for the cross-product value of Se*Cd, i.e the result is that there is an interaction between Se(VI) and Cd, the first such interaction in any of the Experiments.

All the response surfaces confirm this fact, that when the Se(VI) concentration in the nutrient solution increases, the uptake of iron by the shoot decreases, with Se(VI) having a greater effect on iron uptake than the other toxicants. Since this experiment presents the only data suggesting any interaction between elements, the experiment must be repeated to be assured of the validity of the result. However, a clue as to the positive nature of the interaction is to be seen in the low values recorded for the linear Se(VI) parameter. Regretfully, the same is not true for the linear Cd(II) parameter, so much further work is called for.

The uptake of copper as a function of the added elements, for both shoots and roots of Lolium perenne seedlings are listed in Tables 6.4 and 6.5 respectively as response Y7.

Regretfully, examination of the roots data shows that via the R^2 value is an extremely poor fit for the data. Again, the pure error figure is low so the lack of fit figure is highly significant. As expected, the levels of copper in the roots are higher than in the shoots. On examination of the PROB values [17], as expected no parameter is significant. The response surfaces seem to confirm this fact, that the level of Cu in the roots is not affected by the presence of Se(VI), Cd or Hg(II), even when they are present over a large concentration range (see Figure 6.80). However the fit is so bad as to render detailed interpretation almost meaningless. Thus (like iron), the uptake of copper by the roots seems to be unaffected by the levels of these three particular toxicants.

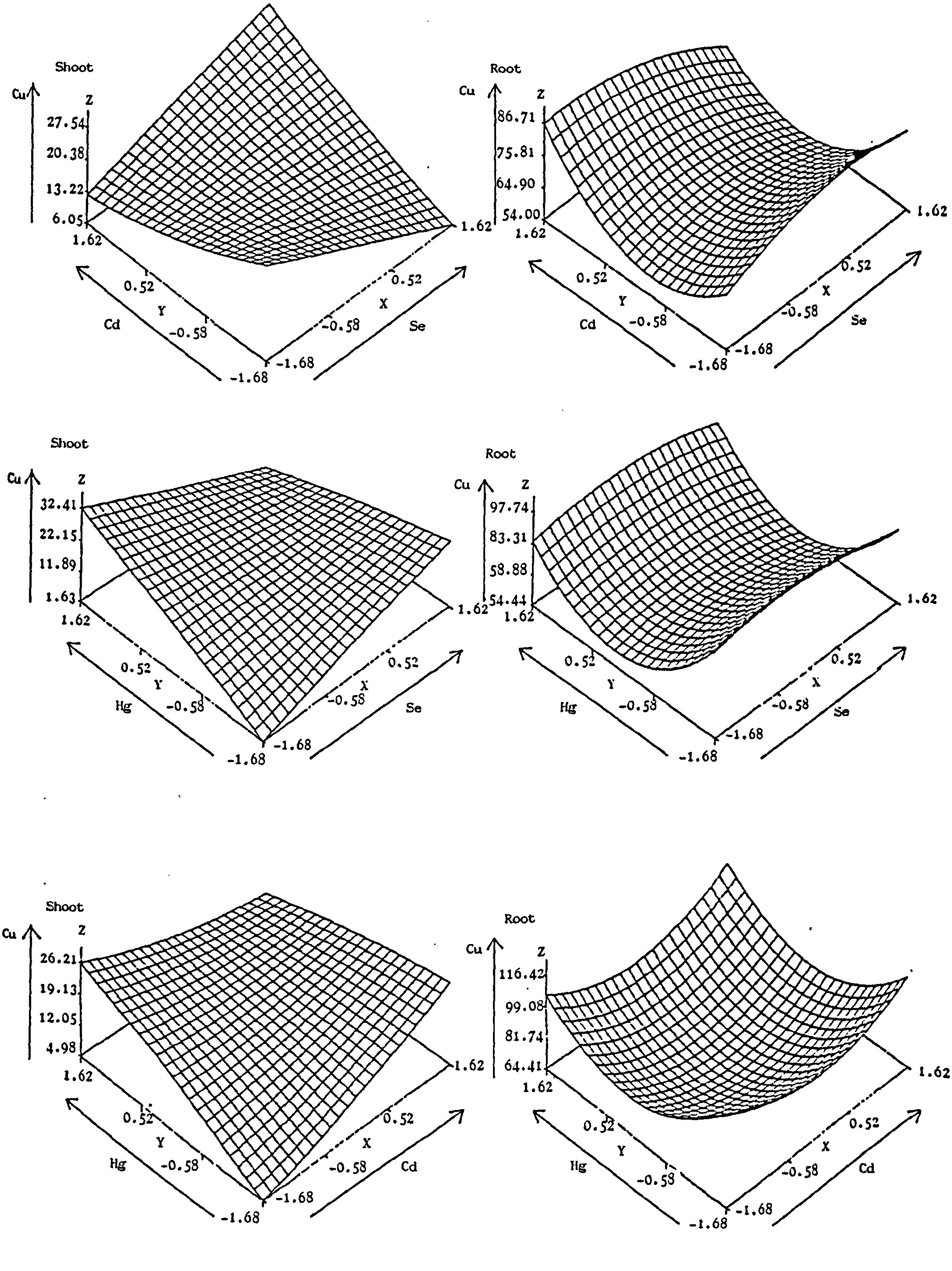
However, for the shoots the fit is much better, the R^2 value in this case being 0.7327, so it is to be expected that some significance may be attached to the data.

With the very significant probability terms [10], it is obvious that the cross-product term is important, while the linear and quadratic terms are not. Thus result is of some importance because it is the only case where this term is significant while the others are not. Examination of the PROB values [17] seems to confirm these findings, that all the cross-product terms are significant, but with no significant value for linear or quadratic terms.

The factor test [18] produces a greater than 90% probability that all the experimental variables have an effect on the Cu uptake by shoots. Figure 6.80 illustrates the response surfaces of Cu uptake

by shoots. The result is surprising because it seems to indicate that the translocation of copper from the roots (where its uptake is unaffected by these elements) is dependent on the concentrations of cadmium, mercury and selenium. Since it has been shown in Figure 6.80 that the uptake of Cd, Hg and Se from the toxicant solution into the shoots is only dependent on the concentration of the respective elements in the nutrient solution without any interactions, some form of linear relationship between the Cu content of the shoots and the toxicant element concentration of the shoots is to be expected. Thus it is probable that the translocation of say Cd to the shoots causes a change in the mobility of the copper into the shoots. In this case, increasing Cd concentrations cause a decrease in the Cu content of the leaves.

Figure 6:80. Response Surface for Copper taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment I (Se, Cd, Hg) with third element at coded level = 0



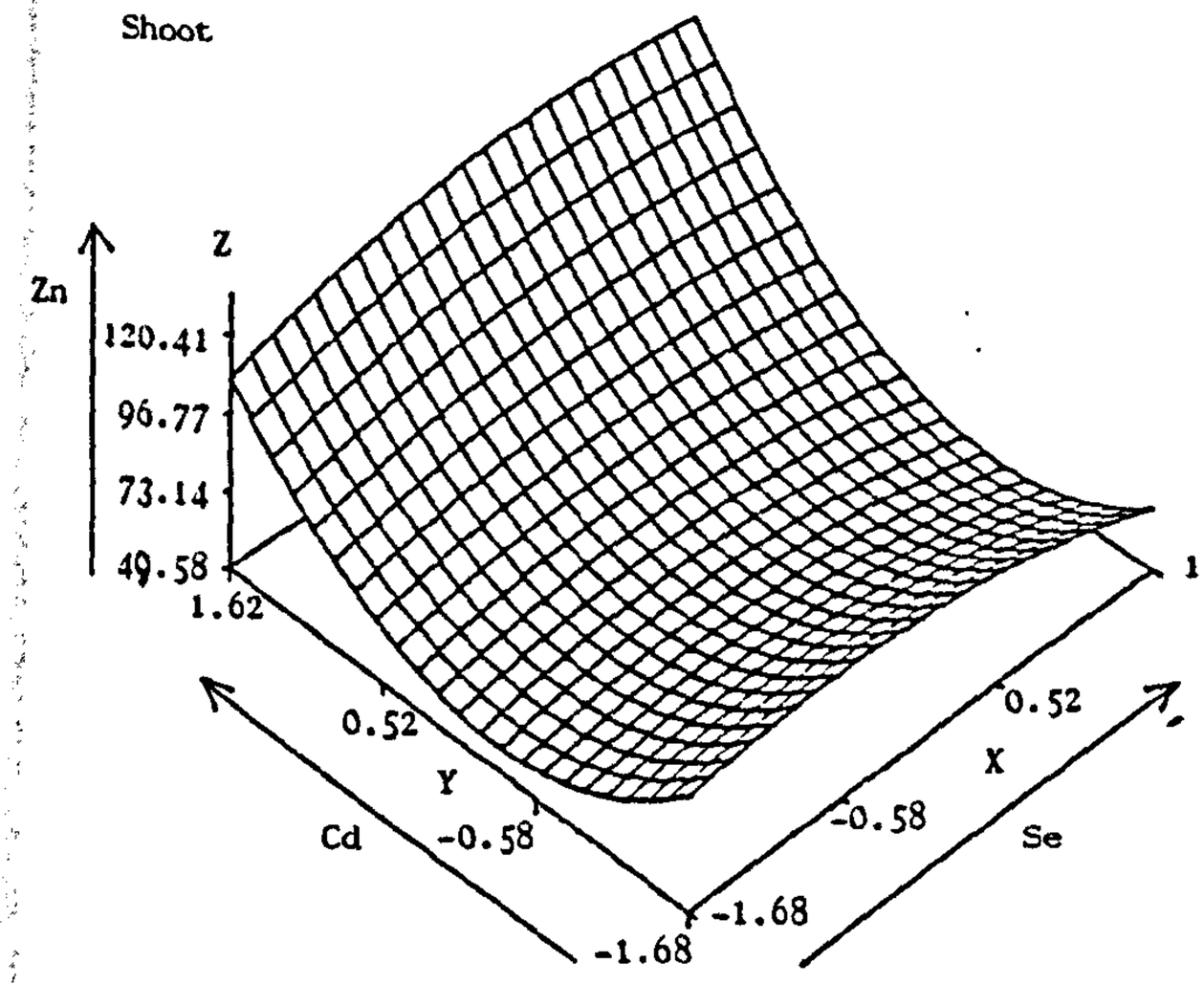
The last example of the possible effect of the added toxicants on the level of essential metals is that of the uptake of zinc by both shoots and roots of Lolium perenne are listed in Tables 6.4 and 6.5 as response Y8.

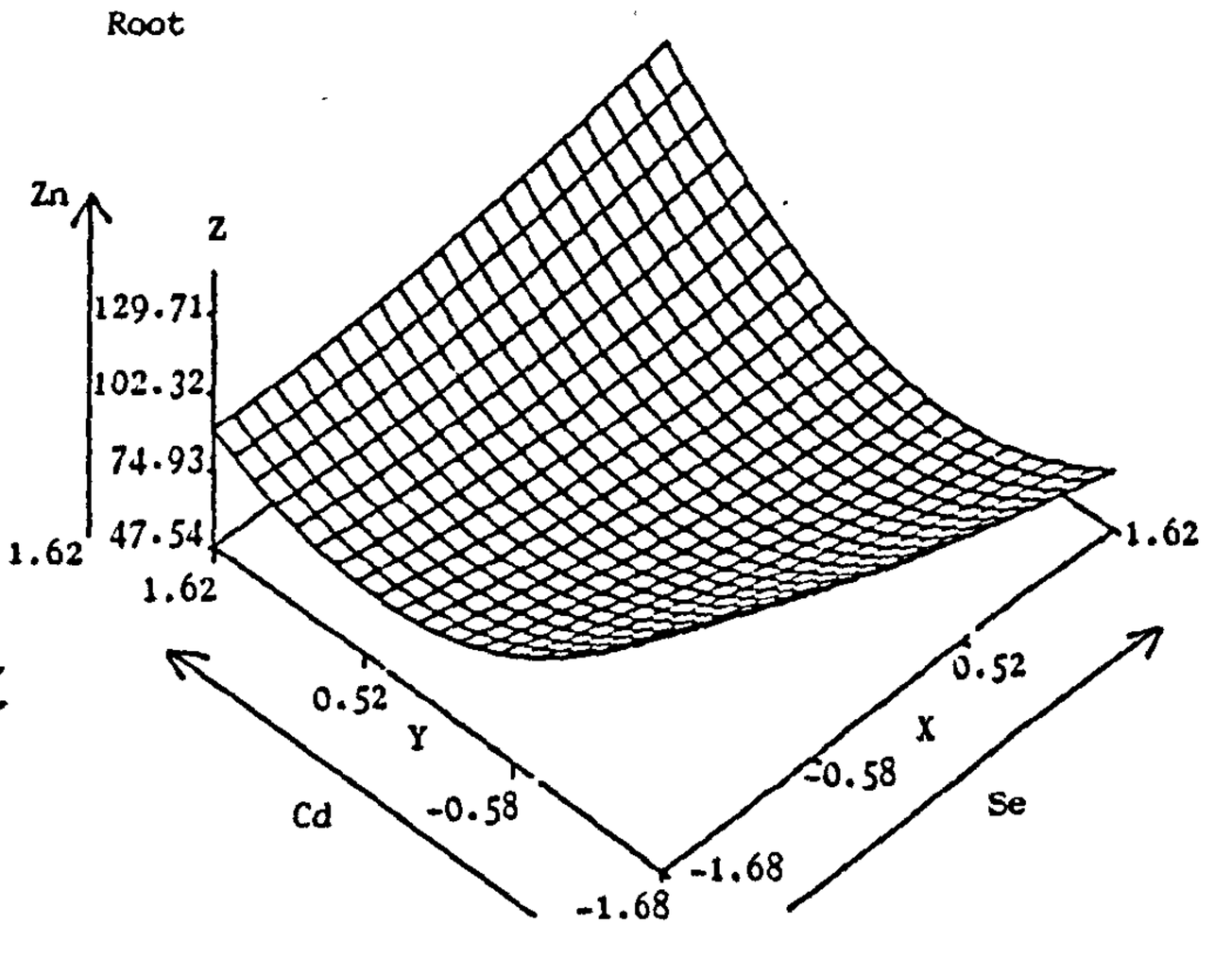
For the roots, the R^2 value is 0.784, being better than for the Mn, Fe and Cu uptake by roots. Thus for the regression characteristics [10], it is obvious that all terms, linear, quadratic and cross-product, are important results, which is the first time that all three factors are deemed significant in any of these experiments. The result is a welcome one but probably can be explained by the similarity of zinc to both cadmium and mercury, they are all members of the Group IIB elements. Thus some form of competition for the active sites of necessary zinc metabolism or storage within the roots is to be expected.

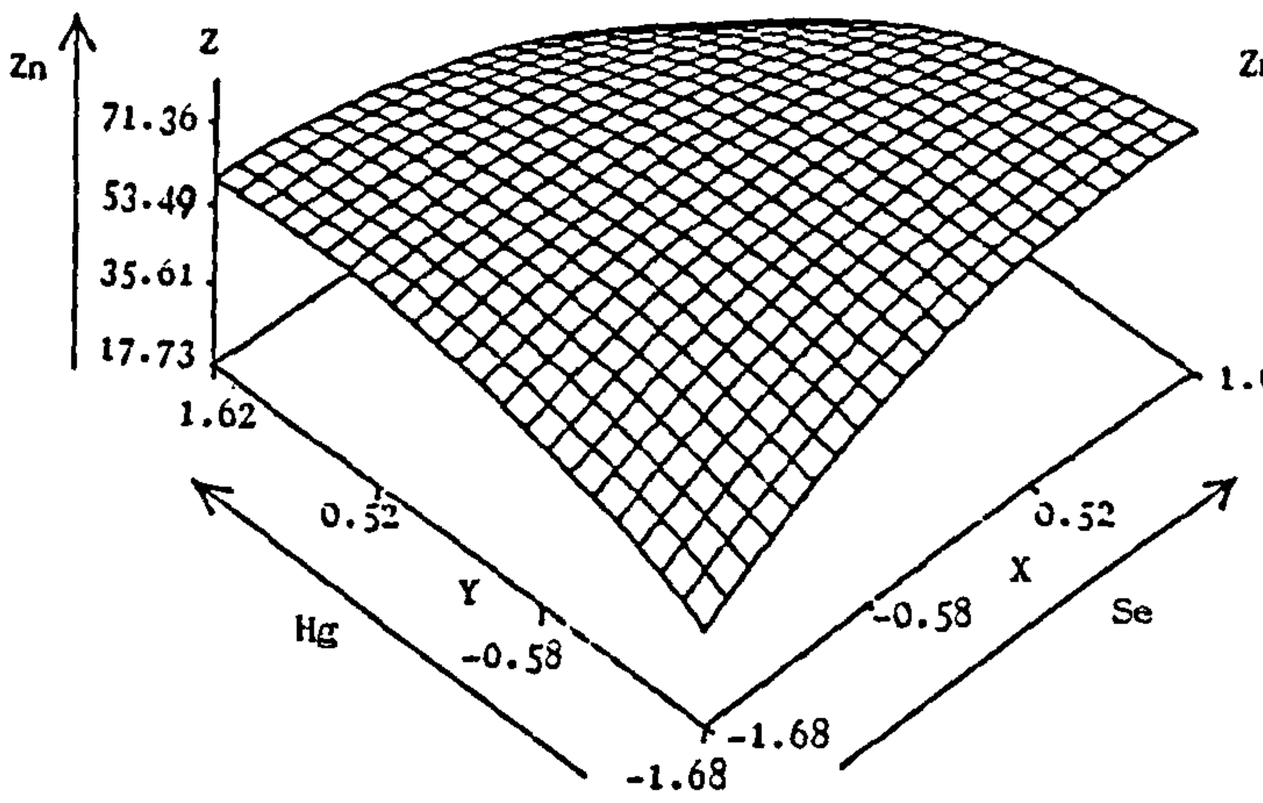
Also, examination of the PROB values [17] confirm these findings, where the Se(VI) and Hg(II) of linear terms are significant, the Cd*Cd quadratic term is significant and Se(VI)*Hg(II) cross-product term is important, the latter being a rare example of the possible interaction between Se(VI) and Hg(II). Obviously, such interaction is hinted at in the PROB [18] values, where the Se(VI), Cd and Hg(II) concentrations of the nutrient solution are all significant.

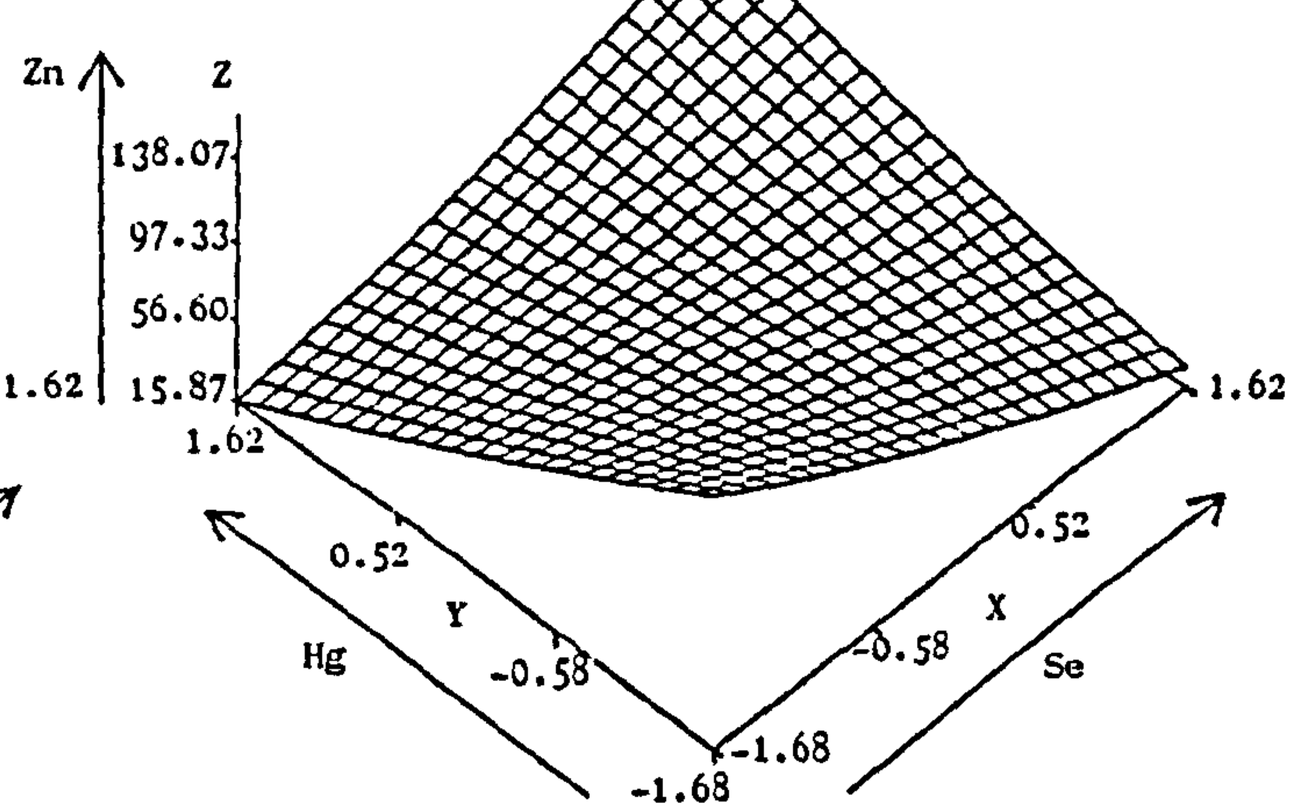
Clearly all the responses are almost planar with respect to change in all the spiked elements concentrations as shown in Figure 6.81.

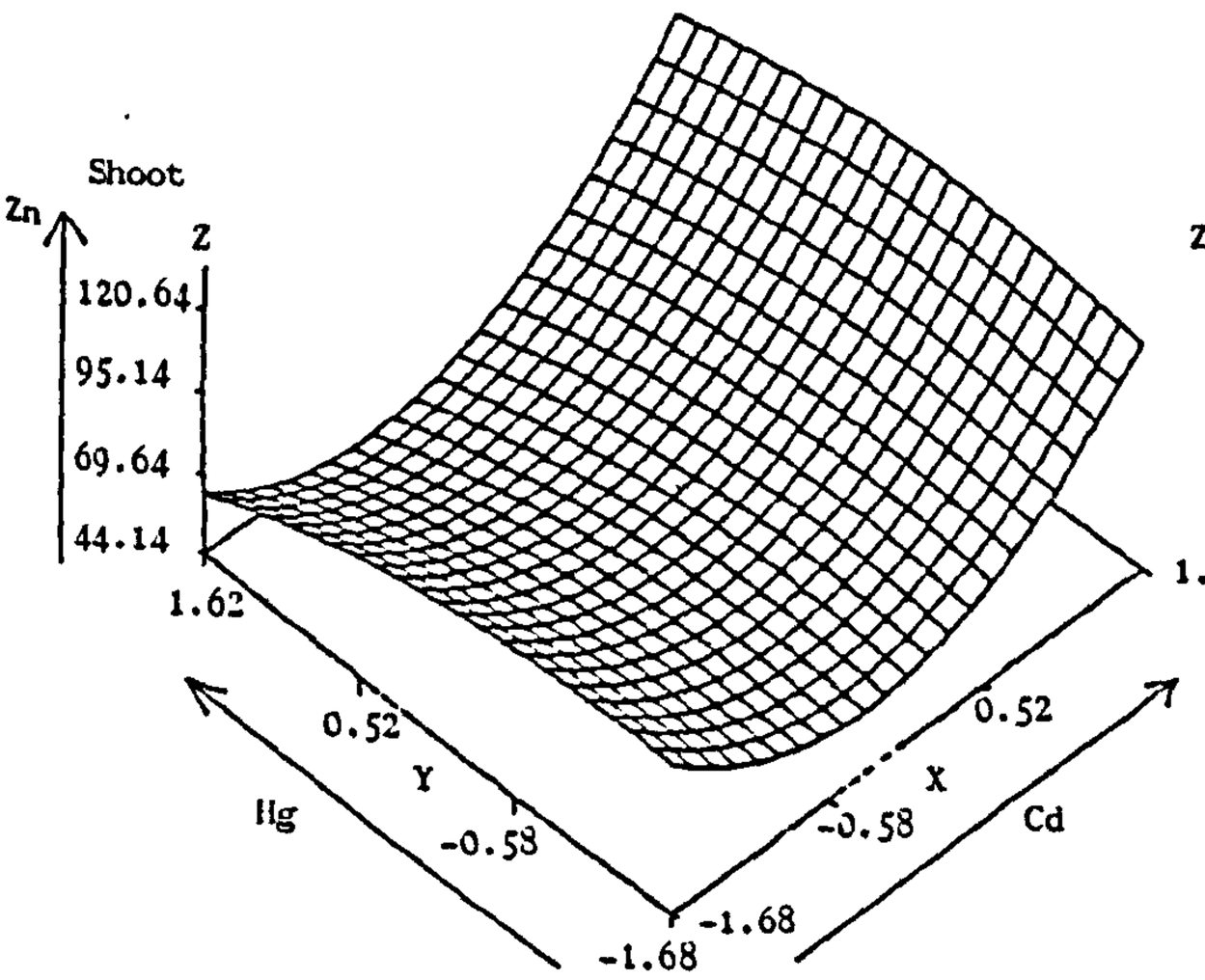
Figure 6:81. Response Surface for Zinc taken up by Shoots and Roots of Lolium perenne seedlings.
Experiment I (Se, Cd, Hg) with third element at coded level = 0

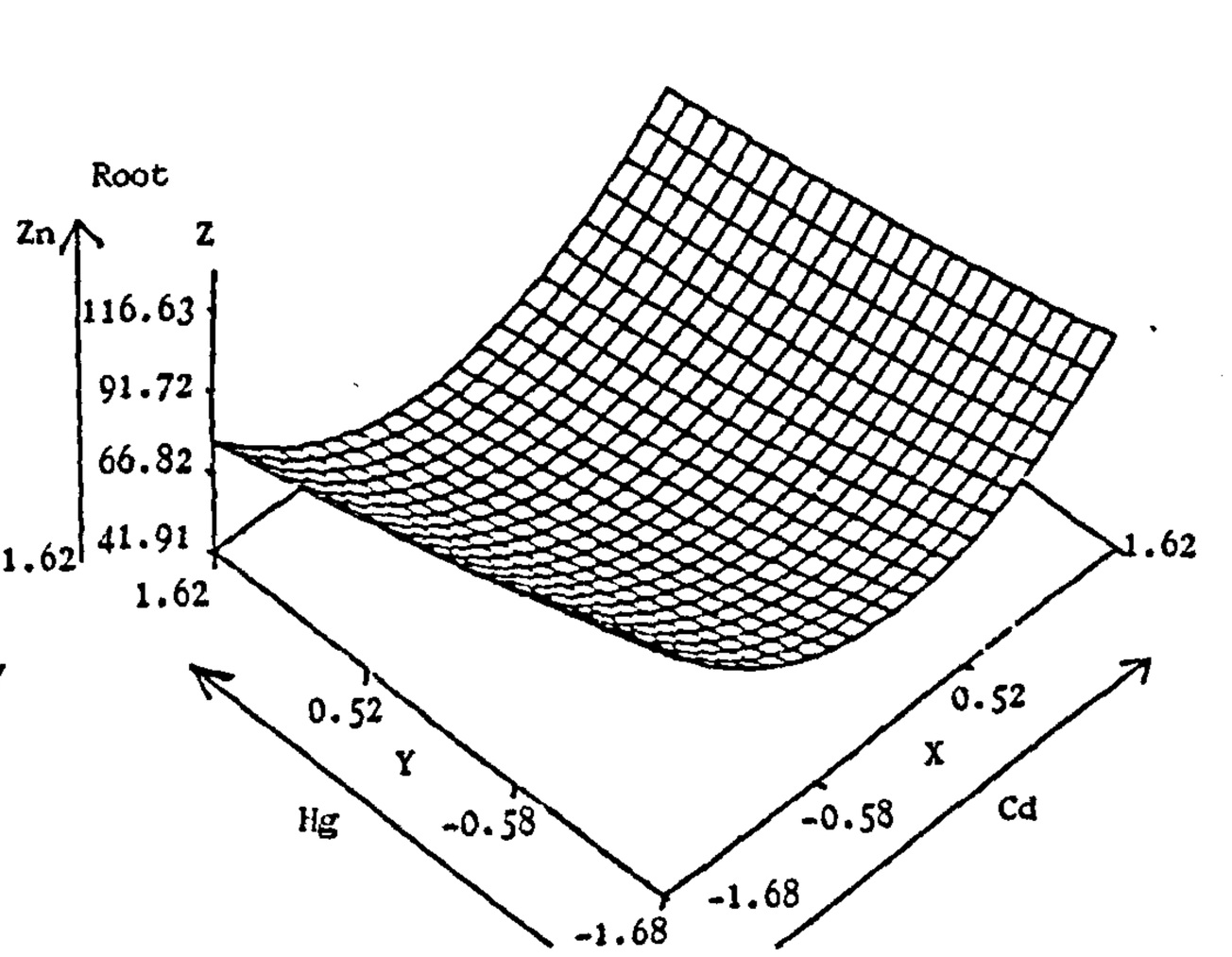












Although a similar situation appears to hold for the level of zinc in the shoots, in this case the R^2 value is 0.726. As expected, the regression probability terms [10], it is obvious that linear and quadratic terms are significant but the cross-product terms are not, a different situation from the roots.

Examination of the PROB [17] values shows that only the Cd term is significant of the lower parameters, as is the Cd*Cd term. Neither the linear or quadratic Se(VI) or Hg(II) terms are significant, similarly no cross-product terms are of importance. The factor test [18] indicates that only the Cd concentration within the nutrient solution have an effect on the zinc content of the shoots. Since the uptake of Cd into the shoots depends almost linearly on the level in solution, a similar relation between Cd in the shoots and Zn in the shoots is to be expected. What is interesting is that the competition between Cd and Zn must be of greater severity than between Hg and Zn. In one aspect slightly surprising because of the greater toxicity of Hg over Cd. On the other hand, chemically Cd is closer to Zn than Cd to Hg and therefore the result can be interpreted in this manner. Clearly much further work by better experimental design has to be carried out in this region of interest looking for such interactions in more detail, possibly through the restricted range of concentrations.

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CHAPTER 7

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

1,2-Diaminobenzene derivatives react quantitatively with Se(IV), but not with Se(-II, 0 and VI) in acidic solution to form piazselenols, complexes which allow the easy determination of selenium.

The Se (-II, 0 and VI) can be determined by conversion to Se(IV) with suitable oxidation or reduction reactions, thus the oxidation state of the Se in water samples can be detected by reaction with Br + Se(0) $\rightarrow$ Se(IV) or HCl for Se(VI) $\rightarrow$ Se(IV). By judicious choice of conditions the speciation of Se in water can be ascertained, but further work to develop a robust method is needed.

A great deal of synthetic work on the preparation of new substituted 1,2-diaminobenzenes was carried out in order to prepare, if at all possible, more satisfactory reagents for the determination of selenium. Following synthesis, the reagents were characterised by the usual spectroscopic procedures and an extensive study of the gas chromatographic properties of each piazselenol was performed. The best reagent found so far for the determination of Se(IV) by GLC + ECD is 1,2-diamino-3-bromo-5-trifluoromethylbenzene which is much more sensitive and has a lower retention time (2.6 min) relative to the now classically accepted 3,5-dibromo-compound (11.3 min).

Digestion for plant samples using Teflon vessels with nitric acid (alone) at 150°C gave high recoveries. For the clean-up and purification of the Se(IV) in the extracted samples, a (1:1) perchloric acid water mixture is the best reagent but again more work is needed on a great variety of matrices.

The GLC + ECD method with the new ligand is extremely sensitive; a detection limit based on regression analyses of around 5 pg is obtained, the method requires no specialised skill but is slow when

compared to GFAAS.

In the second Experimental chapter samples collected from the River Avon were found to be contaminated with selenium, copper and zinc, reflecting either industrial or sewage work sites. In water samples, the selenium was found to contain 15-828 ng/L of Se(IV) and 52-2958 ng/L as total (dissolved) Se. The percentage of Se(IV) in waters varied from 11.27 to 53.55% of the total Se, which seemed to decrease as distance from the original point of injection increased, the conversion of Se(IV) to Se(VI) probably reflecting the oxidising nature of the river.

Sediments from the River Avon were found to be highly contaminated with zinc, copper and cadmium, while only the Reybridge sample was highly contaminated with selenium, again emphasising the location of this site near to industry. The sediments also contained Cr, Ni, Pb and Tl within the range expected for such material.

Selenium was taken up by Lolium perenne seedlings as Se(VI). The element critical levels were found to be 82.1 and 116.6 μg/g of Se for roots and shoots respectively for this species, expressed on a dry weight basis. Therefore, work must be carried out to investigate:

i) how selenium is taken up by plants;

ii) whether selenium is essential for plant growth;

iii) what the relationship is between selenium and toxic metals such as Cd, Hg and Tl;

iv) how selenium acts to detoxify these metals.

However, the determination of Se in plant tissue was satisfactory. It is obvious that selenium uptake in the root and shoot increases with increase in selenium concentration in the nutrient solution,

up to a level of around 25 µg/ml of Se(VI); beyond that concentration the plant dies very quickly. The correlation coefficient between the two Se-concentrations was 0.949 for roots and 0.9919 for shoots. At the same concentration (30 µg Se/ml) the plant appeared to take up more of the essential metals like Mn, Fe, Cu and Zn, probably via a mechanism to overcome the selenium toxicity. Thus the Lolium perenne seedlings directly reflect the value of the selenium concentration fed to them, the species being neither an accumulator nor an excluder of selenium.

A general excursion in the viability of the use of DPASV for the determination of those metals forming amalgams with mercury was carried out. The values obtained were to be compared with the more usual procedure of GFAAS. Samples of water were taken from various sites along the River Avon, where the zinc was always above the median concentration for such a river system. A similar situation arises for Ni which is also higher than the range reported for fresh water. With these two exceptions, all other metal levels were as expected, thus the Cr concentrations of 0.15 to 4.55 µg/L were within the recommended range (0.1-6 µg/L). Similarly, the levels of Cd, Cu and Pb were well within those expected for fresh water. But the analytical results obtained for Cd seem to differ for the two techniques employed, with the DPASV results being higher than for GFAAS, so further work is suggested to solve this problem. Nevertheless, at the same time, both methods demonstrate an increased level of Cd at Reybridge, Kellaways and Keynsham, each being downstream of industrial areas.

However, for Cu and Pb the levels reported by both techniques are very close together. Cu was present in all samples but over a wide

concentration range, being the opposite of Pb levels which appear to occupy a rather narrow range of concentrations. As expected, Staverton, Newbridge (Bath) and Keynsham exhibited higher levels of copper, each being downstream of an urban area, in particular sewage works.

In order to prepare for a study of metals on plants, mercury in such samples was determined by CVAAS and GFAAS. The results obtained seem to differ for the two techniques employed; with the CVAAS results being higher than for GFAAS, also the CVAAS method was sensitive, more precise, faster and cheaper, and is the method recommended.

Thallium was determined in sediment samples by DPASV with HMDE, while for plant samples thallium was determined by DPASV and GFAAS, again the level of thallium with DPASV was higher than reported by GFAAS.

The estimation of the critical levels of cadmium, mercury, thallium and tellurium indicated the toxicity of these metals to Lolium perenne seedlings was as follows:

Hg > Cd > Tl > Te > Se (in shoots)

Hg > Tl > Te > Cd > Se (in roots)

Further work must be carried out to investigate the mechanism of toxicity of each metal.

To study the possible interactive effects between the three elements (selenium, tellurium and thallium) and (selenium, cadmium and mercury), factorial experiments based on a rotatable design have been executed. The grass seedlings (Lolium perenne) were grown in Hoagland's nutrient solution spiked with varying concentrations of metals and metalloids. The resultant metal concentrations were determined by digestion with nitric acid in a Teflon vessel, heating

at 150°C for 60 minutes and measurement by GFAAS.

Studies of the interactive effect of combinations of toxic elements upon the yield of plants and uptake of elements by plants have been shown and can be represented by a second-order polynomial to a first approximation. A detailed comparison between two software packages, SAS and GINO, was made. However, the responses so far measured have been very basic ones, macro in nature, e.g. dry weight, total concentration of metal taken up and detailed explanations have been proffered. What is clearly required is a whole series of well-designed experiments involving more biochemical responses of plants to such stresses.

An associated suggestion is to be ready to adapt or modify the concentrations of the toxicants used in such experiments, especially bearing in mind the results available from any univariate experiments. Thus in Experiment 1 the upper levels of the toxicants were insufficient to stress the plants sufficiently to produce interactive effects between the elements. The problem with Experiment 1 was that insufficient care was exercised in choosing the experimental limits for the added toxicants and hence the final results were not as useful as they might have been. The results highlight the need for very careful attention to detail when designing such large experiments.

A further problem is that the levels of selenium chosen were not sufficiently low to help indicate whether or not selenium is an essential element for plant growth.

APPENDICES

APPENDIX 1 (GINO)

a) Production of Regression Equation

```
Minitab -log "filename"
-- read 'filename' C1-C8
-- let C11=C1*C1
-- let C22=C2*C2
-- let C33=C3*C3
-- let C12=C1*C2
-- let C13=C1*C3
-- let C23=C2*C3
-- regress C4 9 C1-C3,C11-C13,C22,C23,C33
-- end
-- stop
```

b) Generation of Surfaces

Three dimensional response surfaces were produced by using the "C. Hein 3-D Program" while contour plots were generated from a program developed by C. Scott called "hex2.fortran".

APPENDIX 2 (SAS)

a) To print data file e.g. Khaled

Type Table 6.4 as example

	x1	x2	x3	y1	y2	y3	y4	y5 as Dry Wt.
1	-1	-1	-1	30.00	1.30	1.73	1.04	1.1910
20	0.0	0.0	0.0	23.70	3.62	2.21	1.11	0.6295

From Trial No. 1 to Trial No. 20, this file calls data Khaled.

b) Production of Regression Equation

```
data Kahled;
infile metal;
input x1-x3 y1 y2 y3 y4 y5;
proc print;
run;
proc sort; by x1-x3;
proc rsreg;
model y1=x1-x3/lackfit;
proc rsreg;
model y2=x1-x3/lackfit;
proc rsreg;
model y3=x1-x3/lackfit;
proc rsreg;
model y4=x1-x3/lackfit;
proc rsreg;
model y5=x1-x3/lackfit;
run;
```

Give this file name PL08.sas;1

c) To get read out e.g. Table 6.4(a) to 6.8(a)

Type SAS PL08.sas;1
The read out is called PL08.LIS;1

d) Generation of 3-D Plot of Response Surface

```
data Khaled;
do  x=-1.68 to +1.68 by 0.15;
do y=-1.68 to +1.68 by 0.15;
z = 20.67 - 180*x  3.10*y - 3.23*x*x - 2.52*y*y + 1.75*x*y
z = b0 + b1x + b2y + b11x*x + b22y*y + b12x*y
output; end; end;
proc g3d;
plot y*x = z/tilt = 45 rotate 45;
```

APPENDIX 3 (for Graphs and Writing Using SAS)

a) Graphs

i) Data file must be name Cush.Dat;1

x3	y3
1	12.50
2	13.34
3	12.05

ii)
```
goptions nocharacters;
dataa;
infile sesh;
input x y;
data b;
infile mnsh;
input x1 y1;
data c;
infile fesh;
input x2 y2;
data d;
infile cush;
input x3 y3;
data e;
infile znsh;
input x4 y4;
data all;
merge a b c d e;
proc gplot;
plot y*x y1*y1 y2*y2 y3*y3 y4*y4 / overlay;
symbol1 c=green v=star i=join;
symbol2 c=blue v=plus i=join;
symbol3 c=red v=diamond i=join;
symbol4 c=blue v=0 i=join;
symbol5 c=red v=star i=join;
```

b) Writing

```
goptions nocaps;
proc gslide;
title1 " ";
title2 " ";
title3 F=titalic c=red h=3"CHAPTER 1";
title4 " ";
title5 f=titalic c=black h=2"GENERAL INTRODUCTION";
```

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