QUANTITATIVE AND QUALITATIVE ANALYSIS OF PYRETHRINS AND PYRETHROSIN

BY

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for the Degree of Master of Science in the

University of Nairobi

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This thesis is my original work and has not been presented for a degree in any other university.

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..... : 28/11/95

This thesis has been submitted with our approval as university supervisors.

PROF. R.M. MUNAVU

.....

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.....

DEDICATION

Dedicated to my wife, children and parents.

ACKNOWLEDGEMENT

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ABSTRACT

Pyrethrins have been analysed by several analytical techniques e.g. HPLC, GLC, TLC, colorimetric, polarographic, etc. This work reports on the simultaneous quantitative HPLC analysis of pyrethrins and pyrethrosin using an ODS C_{18} HPLC column and aqueous methanol (90:10:v/v) as the eluent.

Analysis of a mixture of standards gave three peaks corresponding to pyrethrosin (retention time 2.40 minutes); pyrethrins II (retention time 4.00 minutes) and pyrethrins I (retention 6.00 minutes).

Statistical analysis of the data obtained revealed that there was no correlation between pyrethrins and pyrethrosin concentration.

This work shows that the commercial pyrethrum extract (pale extract) has a very low ratio of pyrethrosin to pyrethrins (0.50%: 18.10%). It also shows that amongst the clones studied, clone 107 is the best due to its high pyrethrins content (2.23%) and its relatively low pyrethrosin content (1.73%). Petals have a high concentration of pyrethrosin (2.05% to 3.38% and very low pyrethrins concentration (0;07% to 0.13%); therefore their removal before grinding of flowers and extraction could significantly reduce pyrethrosin concentration in the resulting extract without significantly affecting the pyrethrins concentration.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0 General

The use of pyrethrum powder as an insecticide originated in the Transcaucasus region of Asia at about 1800. It was introduced to Europe at an unknown date and its manufacture began at about 1828 (1).

Pyrethrins occur only in plants belonging to the genus *Chrysanthemum* (Pyrethrum), family Compositae. The two species which possess a significantly high toxic content to be used for the manufacture of insecticides are *C. cinerariaefolium* and *C. coccineum* (roseum), but the former is at present the only species of commercial importance (1).

The commercial pyrethrum plant is grown in Kenya, Tanzania, Ecuado, Japan, Rwanda, New Guinea, Brazil, Zaire, Indonesia, India, the former U.S.S.R., Taiwan, Zimbambwe, Yugoslavia and parts of South Africa (2). Most of these countries export ninety percent of their output to the United States of America and to Western Europe. Japan became the leading world producer during the first world war. The pyrethrum industry began to flourish in Kenya about 1932 and by 1940, Kenya overtook Japan and became the world's principal source, a position it still maintains (1). Kenya produces over 50% of the world output under the sound supervision of the Pyrethrum

Board of Kenya (2).

Kenyan pyrethrum flowers contain an average of 1.3% pyrethrins, reaching 3% in selected strains, Japanese flowers average about 1% and Dalmatian (Yugoslavian) flowers about 0.7% (1). The majority of the active constituents are present in the mature fully opened flower head, whereas the stem contains only about 0.1% as much. The achenes of the flower head contains about 90% of the pyrethrins in the flower. Pyrethrosin, a sesquiterpene lactone, has also been identified in various pyrethrum flower extracts.

Pyrethrum has been Kenya's third most important agricultural commodity after coffee and tea until recently when the export of horticultural products has gained marked significance (3). Kenya produced 93,000 tonnes of pyrethrum extracts in 1987 and exported 459 million tonnes of pyrethrum products in the same year, earning the country foreign exchange in the tune of K£103 million equivalent from the sales. The demand for pyrethrum extract in the world market is increasing due to the increasing pressure from environmental groups in the western world for a reduction in pollutants from synthetic insecticides.

Commercially, pyrethrum extract is obtained by solvent extraction of ground dry pyrethrum flowers using n-hexane. When n-hexane is employed as the extraction solvent, the product is a dark viscous oleoresin containing about 30% by weight of pyrethrins (4). This extract has unique properties such as:-

1) Ability to be highly synergised.

2) Repellent, knockdown, paralytic and toxic effects against a great variety of insects.

3) Almost complete harmlessness to man and other warm-blooded animals.

4) Rapid breakdown and no persistence of residues.

5) Hardly any build up of resistance in insect populations.

These properties permit the use of pyrethrins against insect pests in the house, even when a treatment is required just prior to harvest or stored food and livestock products.

Synthetic pyrethrum compounds have now been developed and are expected to compete with natural pyrethrum products to some marked extent in the future. However, in contrast to natural pyrethrins, synthetic ones are toxic to man and other warm-blooded animals and pose residue problems (5,6).

Because of the increasing consciousness of the risks associated with the widespread use of many synthetic insecticides such as toxicity to mammals, persistence of residues and insect resistance, the demand for pyrethrins has continued to grow.

Pyrethrins are distributed in various parts of the pyrethrum plant. The ovaries of the disc and ray florets of the capitulum, however, contain by far the highest and largest amount, while petals have the lowest amount (7). Distribution of pyrethrosin in the three different parts of the flowers had not been established.

1.1.1 Synthetic Pyrethroids

Amongst the various synthetic pyrethroids that have been developed in the laboratory, allethrin (figure 1) is the cheapest to produce and has been selected for commercial development.

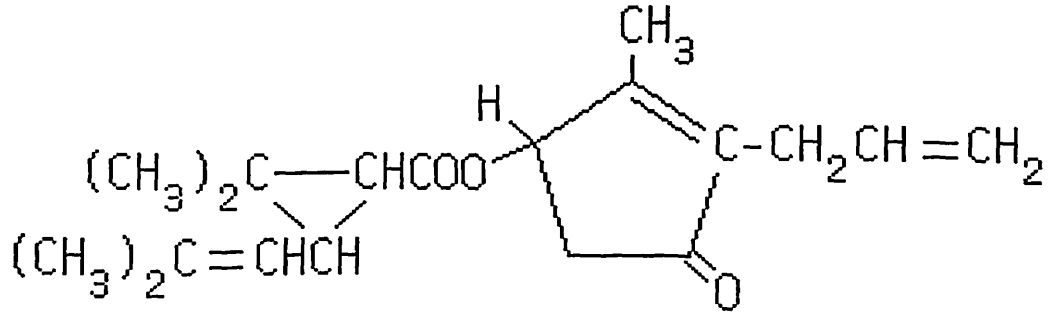


Fig. 1: Allethrin

The product as commercially produced is a clear brownish, viscous liquid containing 75-95% allethrin isomers. It is more stable than the natural pyrethrins upon exposure to ultraviolet light and heat (9,10). However, it is largely detoxified by hydrogenation of the double bonds of either acid or allyl side chain. It may undergo hydrolysis to form chrysanthemic acid and 2-allyl-3-methyl-2,4-cyclopentadienone which readily forms a dimer by the Diels-Alder reaction (11).

Its synthesis is unusually complicated for an industrial chemical and involves several steps as illustrated here below (8):-

1. $2CH_2=C(CH_3)-CH_2Cl \xrightarrow[2.\ Conc.\ H_2SO_4\ \Delta]{1.\ Mg} (CH_3)_2C=CH-CH=C(CH_3)_2$

2. $C_2H_5OOCCH_2NH_2.HCl + NaNO_2 \longrightarrow C_2H_5OOCCHN_2$

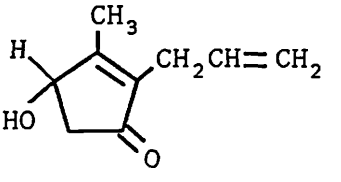
$$(CH_3)_2C=CHCH=C(CH_3)_2 \xrightarrow[\text{Copper Catalyst}]{} (CH_3)_2C(\text{—}CHCOOC_2H_5)CH=CH \xrightarrow{\text{hydrolysis}}$$

$$(CH_3)_2C(\text{—}CHCOOH)CH=CH \xrightarrow{SO_2Cl} (CH_3)_2C(\text{—}CHCOCl)CH=CH$$

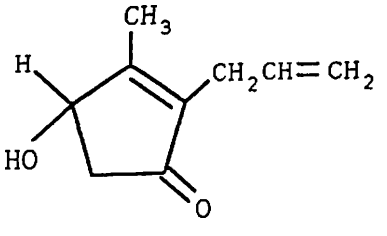
3. $CH_3COCH_2COOC_2H_5 \xrightarrow[NaOC_2H_5]{CH_2=CHCH_2Cl} CH_3COCH(CH_2CH=CH_2)COOC_2H_5 \xrightarrow{\text{alkali}}$

$$CH_2=CHCH_2CH_2COCH_3 \xrightarrow[2.\ NaOC_2H_5]{1.\ (CH_3CH_2O)_2CO} CH_2=CH(CH_2)_2COCH_2COOC_2H_5 \xrightarrow[2.\ CH_3COCHO]{1.\ KOH}$$

$$CH_3COCH(OH)CH_2COCH_2CH_2CH=CH_2 \xrightarrow{\text{alkali}}$$



4.



$+ \quad (CH_3)_2C(\text{—}CHCOCl)CH=CH \longrightarrow$ Allethrin

Furethrin or dl-3-furfuryl-2-methyl-4-oxo-cyclopent-2-enyl chrysanthemate dl-cis-trans-chrysanthemate (dl-furfurylrethronyl dl-cis-trans-chrysanthemate) is a new synthetic pyrethroid. The technical product is a pale yellow liquid (12,13).

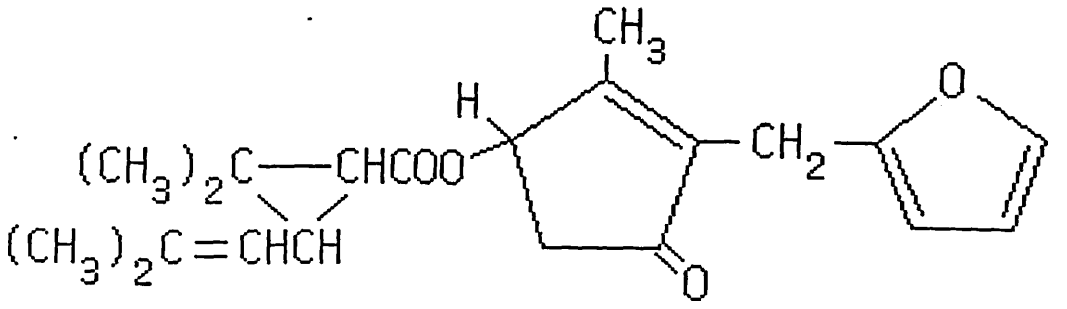


Fig. 2: Furethrin

1.1.2 Composition of Pyrethrum Extract

1.1.2.1 Pyrethrins

The pyrethrins are a mixture of six components. Although the relative proportions of these pyrethrins in extracts from different plants is variable (14), the insecticidal composition of the commercial pyrethrum extracts is relatively constant.

The pyrethrins are esters formed by a combination of two acids and three alcohols. The two acids are chrysanthemic acid and pyrethric acid. The three alcohols are pyrethrolone, cineralone and jasmololone. The esters of chrysanthemic acid are pyrethrin I, cinerin I and jasmolin I, respectively, and are together known as the pyrethrins I fraction (fig. 3-5). The esters of pyrethric acid, on the other hand, are pyrethrin II, cinerin II and jasmolin II, which represent the pyrethrin II fraction (fig. 6-8) (15,16).

It has been shown that the structural requirements of the acid component

of a pyrethroid for knockdown are much less specific than for high toxicity (17). For toxicity, a ring system (preferably cyclopropane) α, 6 to the carboxyl group is essential and the activity is further dependent upon an isopropenyl, or, much better, an isopropylidene group attached to the cyclopropane ring. Stereochemical configuration is also an essential factor as the pyrethroids are among the most complicated of insecticidal molecules and their toxicity is readily destroyed by the action of heat, light and alkaline hydrolysis. Thus, the activity depends upon the intact esters whereas, chrysanthemic and pyrethric acids as well as pyrethrolone, cinerolone and jasmololone are not appreciably toxic (18). Effectiveness is dependent upon optimum geometric and optical isomerism. Toxicity in this case refers to the harm caused to insects, etc, which is ordinarily defined in terms of the lethal dose. Lethal doses are customarily expressed in milligrams of substance per kilogram weight of the subject (i.e. parts per million on body weight basis). Lethal doses are determined by introducing various dosages of the substances to be tested into laboratory animals or insects. That dosage which would be lethal to 50% of a large number of the animals or insects under controlled conditions is called LD_{50}. Thus if a statistical analysis of a large population tested showed that a dosage of lmg per kg was lethal to 50% of the population tested, the LD_{50} for this portion would be lmg per kg.

From toxicity data currently available, there is no evidence that pyrethrins are carcinogenic, teratogenic or mutagenic in test animals and by extrapolation, to man (19). The alleged allergenic properties of pyrethrins and other pyrethrum extracts have been tentatively shown to be caused by

impurities in the pyrethrum flower extracts. The main allergy causing agent has now been shown to be pyrethrosin (20).

The three components of pyrethrins-I fractions are given in figures 3-5 and pyrethrin-II fractions in figures 6-8 below:-

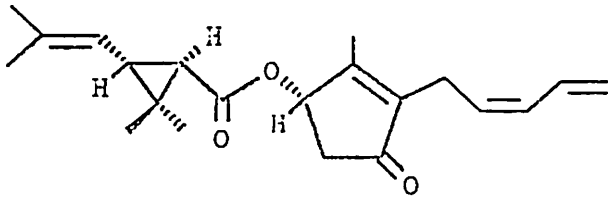


Fig. 3: Pyrethrin I

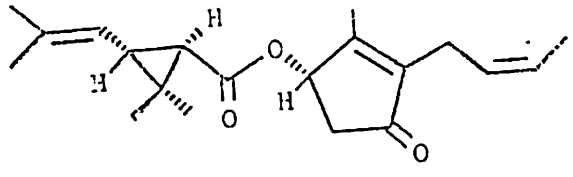


Fig. 4: Cinerin I

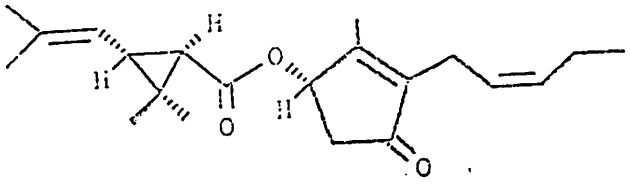


Fig. 5: Jasmolin I

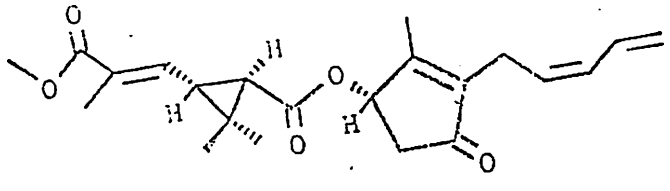


Fig. 6: Pyrethrin II

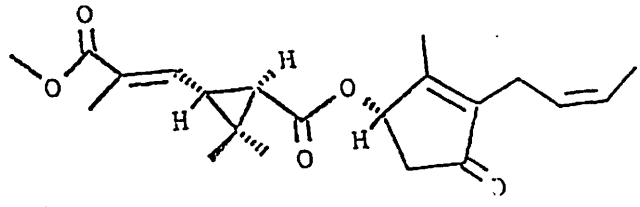


Fig. 7: Cinerin II

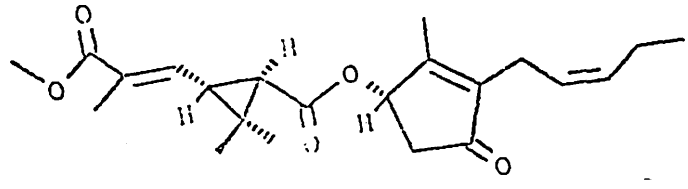


Fig. 8: Jasmolin II

1.1.2.2 Pyrethrosin

Pyrethrosin (Fig. 9) is a sesquiterpene lactone and was first isolated from pyrethrum extract by Rose and Haller (15), but its structure was fully elucidated by Barton and de Mayo (16,21). Pyrethrosin was also isolated by Ndalut (22). After soxhlet extraction of marc by n-hexane, the solvent is removed using a climbing film evaporator and the residue dissolved in warm acetone and filtered. The filtrate on cooling, deposits a white crystalline substance which on recrystallisation several times with acetone yields pure pyrethrosin crystals. Pyrethrosin was the first sesquiterpenoid shown to have a ten-membered carbocyclic ring and the ease of cyclisation pointed to its possible importance in the biosynthesis of related bicyclic sesquiterpenoids (23). Pyrethrosin has been reported to have a synergistic action on "pyrethrins" (24).

Extensive literature survey clearly indicates that chromatographic techniques available for the analysis of pyrethrum extracts have dealt at great lengths with

the six insecticidally active constituents, i.e. pyrethrins. Neither the quantitative analysis of pyrethrosin nor simultaneous qualitative and quantitative analysis of pyrethrins and pyrethrosin has been reported in the literature.

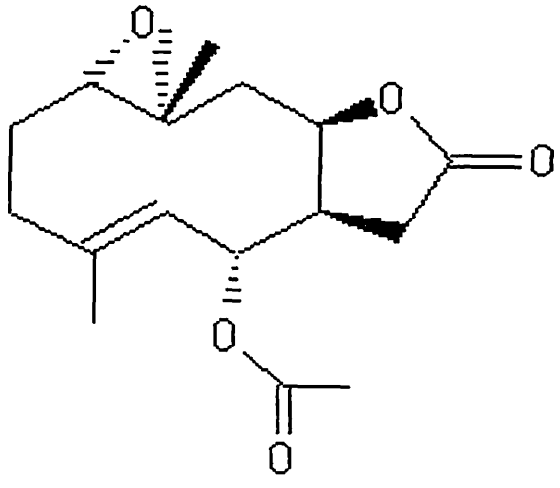


Fig. 9: Pyrethrosin

1.2 Background

Pyrethrum and its preparations are considered non-toxic to warm blooded animals and harmless to human beings. This view has been supported by many researchers who have experienced no ill-effects in the course of years of work on pyrethrins. As further proof of its safe use, it may be mentioned that pyrethrum has been employed in compositions for external use for not only insect repellent properties, but also in the control of body lice, scabies and miscellaneous skin affectations (25-27).

However, cases of dermatitis and allergic reactions in some of the workers handling pyrethrum or its extracts over long periods have been recorded from time to time (28,29). Discussing the incidence of dermatitis among workmen

in the pyrethrum industry of Kenya, Tonking (30) and Sequeira (31) reported that it is aggravated by hot weather and perspiration. Fortunately, only a limited number of individuals are susceptible to this form of dermatitis which is characteristic of rashes and eruptions caused by contact with an irritant (32). The areas most severely affected are between the fingers and in the folds of the skin around the neck and eyes. Reactions affecting the respiratory tract have also been reported (33) and sometimes, cross reactions have been observed between sufferers of pyrethrum and rag weed allergies (34). People suffering from allergies of the pollen of *ambrosia spp.* (ragweed) also react to unrefined pyrethrum extracts suggesting that similarities exist between allergens of the two species.

15 ppm though it does not appreciably dissolve in water (38). The nature of pyrethrosin's molluscicidal activity is worthy of further investigations.

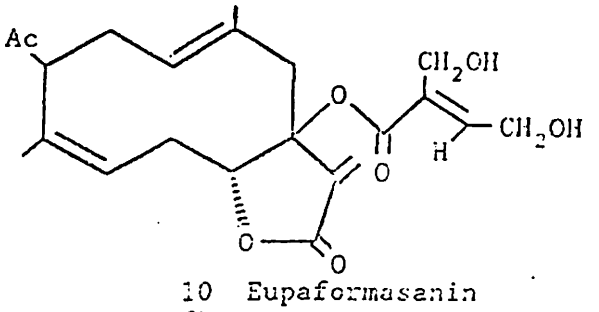
Nearly all sesquiterpene lactones have diverse biological activities. Many of these compounds are antifungal, antibacterial and antitumour, cytotoxicants and plant growth regulators (39). Various sesquiterpene lactones are known to be toxic to human and animal parasites, insects and vertebrates.

Most sesquiterpene lactones of compositae have been isolated from the shoots of the plants, mainly leaves and flowering heads, where they constitute upto 5% of the dry weight.

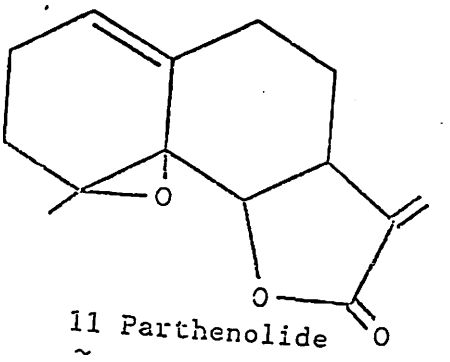
Germacranolides represent the largest group of sesquiterpene lactones. Considerable structural variety is recognized within the group mainly due to the unique configurational and conformational features and the reactivity of the cyclodecadiene (40). Pyrethrosin is an example of a germacranolide. Picman and other authors have established that the exomethylene group on the lactone is an essential requisite for cytotoxicity of sesquiterpene lactones (41-43). Some biologically active sesquiterpene lactones have been shown below.

Table 1: Some Biologically Active Sesquiterpene lactones isolated from various species of the compositae (40).

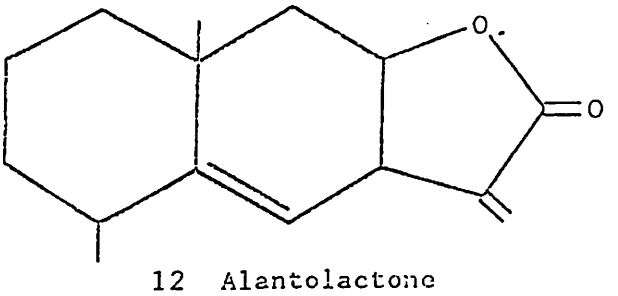
Plant species	Active Principal	Biological Activity
Eupatorium formosanum	Eupaformosanin	Cytotoxic, Antitumour
Chrysanthemum parthenium	Parthenolide	Antibacterial
Imula helenium	Alantolactone	Antifungal
Artemisia amuna	Artemisinin	Antimalarial
Ambrosia maritima	Ambrosin	Schistosomicidal
Chrysanthemum cinerariafolium	Pyrethrosin	Molluscicidal
Helenium autumnale var. montanum	Helenalin	Antihelminthic
Helianthus agrophyllus	Eupatolide	Insecticidal
Vernonia spp.	Glancolide-A	Antifeedant
Helenium amarum	Amerilin	Analgesic.



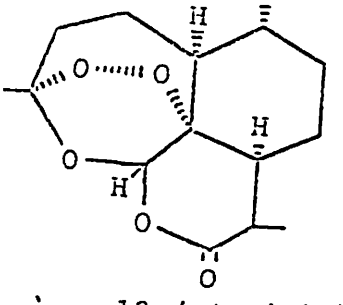
10 Eupaformasanin



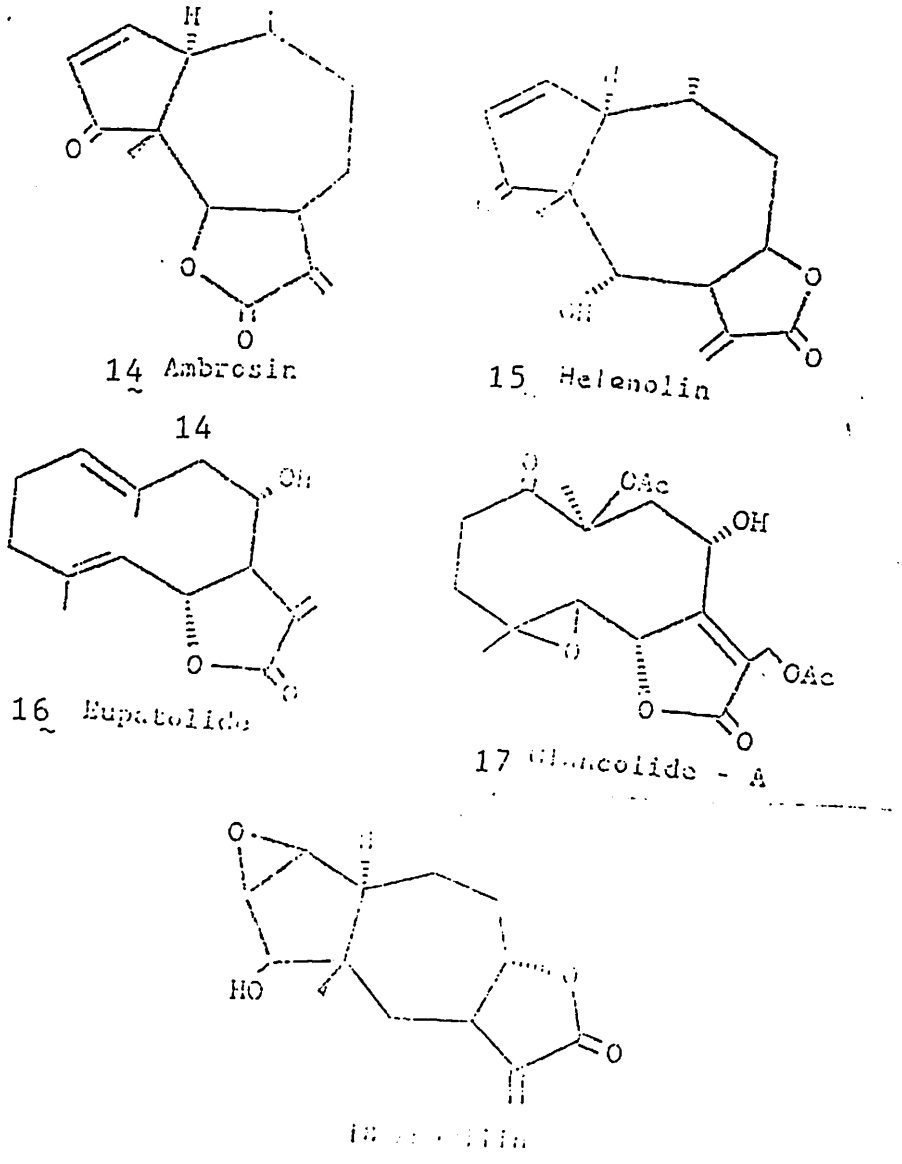
11 Parthenolide



12 Alantolactone



13 Artemisinin



Figs. 10-18: Structures of some Biologically Active Sesquiterpene Lactones

1.3 Isolation and Analysis of Pyrethrins and Pyrethrosin.

Pyrethrins have been extensively studied and very many methods of analysis reported. However, only little information is available on pyrethrosin analysis. No quantitative and qualitative method of analysing pyrethrosin and/or simultaneous qualitative and quantitative analysis of pyrethrins and pyrethrosin has been reported in the literature. Various analytical techniques of isolating pyrethrins are described in the next few paragraphs.

1.3.1 Column Chromatography using Adsorptive Charcoal.

This is one of the methods used for the separation and estimation of the four insecticidal constituents of pyrethrum (cinerins I and II and pyrethrins I and II). It is effected by elution from a column of adsorptive charcoal (44). The four pyrethrins separated are analysed by using I.R. spectroscopy. However, many methods for the separation of pyrethrins by column chromatography have been described. But most of this work was done before the advent of gas liquid chromatography with the result that jasmolins were not detected. Separation by adsorption chromatography either on alumina (45) or on a silica gel-plaster of Paris mixture is very useful in obtaining concentrates of pyrethrins I and II.

1.3.2 Displacement Chromatography

Pure pyrethrin I can be isolated from the pyrethrins I concentrate using a displacement chromatographic method (46). This method however, fails to give pure cinerin I or jasmolin I. Jasmolin II, cinerin II and pyrethrin II can be isolated from the pyrethrins II fraction by repeated chromatography on a silica gel-plaster of Paris column (47).

1.3.3 Liquid-liquid Partition Chromatography

A simpler method that has been described by Ricket (48) allows the separation of all six pyrethrins. In this method, liquid-liquid partition chromatography is employed.

1.3.4 Thin Layer Chromatography

The separation of small amounts of the individual esters can be achieved by thin-layer chromatography (T.L.C.) using a continuous development technique (49). Thin layer chromatography can also be used for detection of pyrethrins. In this method, pyrethrins I and II are separated on silica gel using ethyl acetate in n-hexane (1:4) for development.

1.3.5 Liquid-gel Chromatography

Liquid-gel chromatography has been used for analytical characterisation of pyrethrum extract (50). The use of lipophilic gel systems in the analysis of pyrethrum extract introduces many useful features not possessed by existing analytical procedures, many of which are based on chemical modification followed by separation and then analysis. Since all compounds are eluted eventually, the gel phase becomes available for re-use. This is a factor that contributes to the high degree of reproducibility of liquid-gel chromatography.

1.3.6 Titrimetric Method

A titrimetric method has also been used for the analysis of pyrethrins. The method has been referred to as the mercury-reduction method and was originally suggested by Wilcoxon (51). The method has been the most widely used for pyrethrins estimation and consists essentially of an alkaline hydrolysis of the mixed pyrethrin esters to give chrysanthemum mono- and dicarboxylic acids. Interfering fatty acids are eliminated as barium salts and the monocarboxylic acid is extracted by petroleum ether. This acid reacts with

Denige's mercury reagent. Iodometric titration of the mercurous salts formed, determines accurately the quantity of monocarboxylic acid. The dicarboxylic acid, insoluble in petroleum ether, is extracted by diethyl ether and subsequently determined alkalimetrically. The amounts of these two acids are expressed as pyrethrin I and II and together give the "pyrethrins" content. The association of Official Agricultural Chemists (A.O.A.C.) method uses sulphuric acid in a similar titrimetric analysis but gives low results.

1.3.7 Colorimetric Method

Pyrethrins have been estimated using a colorimetric method known as the "sulphur-colour" test. In this method, microquantities of an alcoholic solution of pyrethrins deposited on paper is heated to 70°C with a solution of sodium disulphide. A red brown colour develops whose intensity is proportional to the amount of pyrethrins present. In the "sulphur-colour test, described by Brown and others in 1956 (52), the method was based on measurements of the red-brown colour produced by heating pyrethrins with solutions of sulphur and lithium hydroxide. The method was useful for a period of about 5 years (1953 to about 1958) in estimation of small quantities of pyrethrins (3-5 mg). The method was discontinued due to accuracy problems.

1.3.8 Polarographic Method

Pyrethrins have also been estimated using a polarographic technique. Oiwa, and others established that allethrin could be estimated by polarographic

means (53). This method was also used in the separation of pyrethrins using as standards pure pyrethrins I and II separated by partition chromatography (54). They compared their method to A.O.A.C. method and stated that their method gave better results. Polarographic method has, however, not aroused widespread interest resulting in no corroborative work being done.

1.3.9 Gas Liquid Chromatography (GLC)

The separation of pyrethrins by gas liquid chromatography was first achieved by Donegan (55) and has proved to be the most valuable advance in pyrethrins analysis in recent years. The pyrethrins respond to electron-capture detector and the sensitivity is such as to provide a method suitable for residue determination. The relative response of the six components to the electron-capture detector appears to be proportional to that given by the flame ionization detector. The main advantage of the electron-capture detector is its specificity. However, its use in quantitative measurements requires considerable care as it is very sensitive to changes in operating conditions and its linear range is strictly limited. The hydrogen flame ionisation detector does not suffer from these defects; furthermore it allows the use of temperature programming (56). This detector is non-specific.

1.3.10 High Performance (Pressure) Liquid Chromatography H.P.L.C.

It is certain that recent advances in high-speed liquid chromatography has proved to be very valuable in pyrethrins analysis. Pyrethrins have been analysed by high pressure liquid chromatographic method and one such

method uses a zorbax ODS column of 25 cm x 4.6 um i.d. using acetonitrile-water (7:3) as the eluent, a flow rate of l ml/minute and detection at 240 nm (57). The following retention times were observed:- rotenone - 7 minutes, pyrethrin II - 11.5 minutes and pyrethrin I - 25.5 minutes.

The six pyrethrin esters have also been separated and quantified by high performance liquid chromatography using a double u-porasil column and a 1:1 mixture of anhydrous dichloromethane and water saturated dichloromethane as the mobile phase (58). The wavelength used for the detection was 254 nm.

Pyrethrins have also been analysed using normal-phase liquid chromatography (59). In this method, samples were dissolved in tetrahydrofuran and analysed on a 5 μm amino-column of 25 cm x 4.5 μm i.d. with hexane:tetrahydrofuran (9:1) as the mobile phase, a flow rate of 1.5 ml/minute and detection at 240 nm. Total elution time was 7 minutes.

High performance liquid chromatography has also been used for determining trace levels of pyrethrins and piperonyl butoxide down to 0.1 μg/litre in tap water. In this method, a 5 μm ODS C_{18} 25 cm x 4.6 μm i.d. was used. The mobile phase was methanol/water, 90/10, v/v (helium degassed), flow rate 0.8 ml/minute and the detection wavelength was 230 nm (pyrethrins) and 209 nm (piperonyl butoxide) (60). Under these conditions, retention times were as follow:-

Pyrethrins II ---------- 4.5 to 5.2 minutes

Piperonylbutoxide ---------- 6.0 to 6.8 minutes

Pyrethrin I ---------- 6.6 to 7.6 minutes

1.4 Comparison between GLC and HPLC techniques

HPLC yields separation efficiencies approaching those obtained by GLC at comparable elution times (61). However HPLC is superior due to the following reasons:-

a) Materials of low vapour pressure and thermal instability can be analysed.

b) Specific chromatographic systems and operating parameters may be selected for the resolution of complex mixtures.

c) Extensive clean up or derivatization are unnecessary.

d) Separations can be scaled up for preparative chromatography.

However, HPLC is less sensitive and selective than GLC since only few detectors are available.

1.5 Objectives and Reasons for the Study.

1.5.1 Introduction

Before going into the objectives listed below for carrying out the study, it should first of all be noted that the work and conclusions drawn thereof may appear contradictory due to the two approaches that can be made viz:-

(i) Pyrethrosin, a sesquiterpene lactone has molluscicidal activity and hence is a desirable compound (20). Most sesquiterpene lactones of compositae have diverse biological activity (38,40) meaning that there is a possibility of pyrethrosin having other beneficial biological activities. More research should be conducted to establish whether pyrethrosin has other biological uses plus

pyrethrosin should be commercially used as a molluscicide.

(ii) Pyrethrosin has also been reported to cause dermatitis (22); hence, its presence in pyrethrum products, e.g. pale extract, oleoresin, etc is undesirable. Research should be conducted to establish the levels of pyrethrosin that may cause dermatitis so that maximum allowable levels of pyrethrosin in pyrethrum products may be established. To commercially exploit pyrethrosin, a selective method of extraction or separation from pyrethrins should be developed.

1.5.2 Objectives

The objectives of this study include:-

1) Developing a HPLC technique for simultaneous qualitative and quantitative analysis of pyrethrins and pyrethrosin . HPLC technique of analysis is superior to GLC in the sense that it offers high resolution without the possibility of pyrethrins degradation so often experienced in GLC techniques. In this technique, quantitation can be achieved either by area comparison with a standard or by U.V. measurements on the collected fractions.

2) To determine the concentration (amounts) of pyrethrosin and pyrethrins in different pyrethrum clones (cultivers) with a view to establishing the best clones to grow. Assuming that pyrethrosin is undesirable, the best clones to grow should be those with high pyrethrins content and low pyrethrosin content. However, in view of pyrethrosin's possible uses, clones with high concentrations of both pyrethrins and pyrethrosin would be the best to grow.

It was also necessary to establish a correlation if any, in the concentrations of both compounds.

3) To establish how the pyrethrosin content varies in the three different parts of the pyrethrum flowers viz:- Ray Florets, Petals and Sepals. Pyrethrins content in the three different parts of the flowers has been established. It was therefore necessary to establish whether pyrethrosin content follows the same pattern as pyrethrins content, and to determine a correlation in the two compounds if any.

4) To determine the pyrethrins and pyrethrosin content in the following randomly selected samples:-

i) Pale Extract

ii) Oleoresin

iii) Two types of mosquito coils.

These three samples were analysed for the following reasons:-

Oleoresin was analysed to establish whether pyrethrosin is extracted together with pyrethrins. Pale extract was analysed to establish whether clean-up of oleoresin removes any or all of the pyrethrosin, if found to be present in oleoresin.

Two types of mosquito coils were analysed to show whether they contain any pyrethrosin.

CHAPTER TWO

2.0 EXPERIMENTAL

2.1.0 Materials and Chemicals

Analar grade acetone, ethyl acetate, silica gel and celite were supplied by Kobian Kenya ltd.; analar grade chloroform was supplied by Alpha Chemicals; activated charcoal was supplied by Zeta Chemicals, while HPLC grade methanol was from Aldrich Chemical Company. Double distilled, de-ionized water normally meant for X-Ray fluorescence analysis was used as the eluent in the HPLC. The chart papers used were Kipp and Zonnen BD41.

Pyrethrum Board of Kenya kindly donated Whatman cellulose extraction thimbles of single thickness, 30 mm internal diameter by 100 mm external length, 32 mm external diameter by 100 mm external length. They also donated a varian micropack ODS C_{18} analytical HPLC column of 5 mm internal diameter by 5 cm; four vials of world standard pyrethrins; two batches of 10 gm of pyrethrosin dust from which pyrethrosin standard was prepared; one batch of 50 ml and two batches of 20 ml each of pyrethrum extract (pale extract); three batches of 10 gm each of oleoresin. The flower samples were picked from Pyrethrum Board's research station at Molo on three different seasons from different sections and fields in the same research station. Employees of pyrethrum Board of Kenya assisted in the picking.

Two types of mosquito coils, namely, Mos. Kill mosquito coil and Doom perfumed mosquito coil, were purchased from a leading supermarket

chain and analysed for their pyrethrins and pyrethrosin content. Both were indicated as being pyrethrins/pyrethroids based. The procedures for their extraction and analysis were similar to the procedures employed in the case of pyrethrum dust samples. Three batches of mosquito coils for each type were purchased on three different occassions.

2.2.0 INSTRUMENTATION

A Beckmann Gradient Liquid Chromatograph Model 332 coupled with two Beckmann Model 110A pumps was employed for the analysis. It was equipped with an Altex Model 420 instrument controller and Model 210 sample injector, both from Beckmann. The sample injector had a 20 ul sample loop. A varian micropack ODS C_{18} analytical HPLC column, 5 mm internal diameter by 5 cm was used together with a check pre-column. The U.V.-Vis variable spectrophotometer used was a Hitachi Model 100-40 fitted with an analytical flow-cell.

For normal data analysis, a Beckmann Kipp and Zonnen BD 41 recorder was used and was equipped with Kipp and Zonnen BD 41 chart papers.

Most of the data was collected and analysed using a BBC model B micro-computer. It was connected to the instrument through a simple interface comprising of a circuit with a Zener diode and a resistor which had been earlier constructed; and computer accessed through the analog port which contained an on-board analog to digital converter ADC. Interfacing and

operational software was then developed for chromatographic analysis. The output voltages of the detector had been tested for compatibility with the micro-computer input and found to be within the recommended range that is sufficient for detection and not of a magnitude to damage the computer. As an added precaution, the protective circuit was designed to "cut off" at + 2.3 volts and -0.3 volts.

A series of programs were then fed into the computer to do the following:-

1) Collect data from the output of the detector into the memory of the computer. Sampling of the detector output was done at the rate of ten points per second, but only an average of every five were taken; thus, only two points had to be conserved due to its relatively small size. These were found to be sufficient data points from which to reconstruct a continuous chromatograph.

2) Data from electrical equipment is contaminated with electrical and electronic random noise which had to be removed before the chromatograph could be analysed. A routine to perform this task 'smoothing' afterwards was included in the data collection program.

3) After entering the data and subsequent 'smoothing', the spectra was saved on diskette, from where it could be recalled and analysed.

4) Also included was a program to display the chromatogram on screen or paper complete with retention times and with the ability to expand or compress the scale. The scale had options of 2-10 whereby a scale of 2 gave the largest peak in the options and a scale of 10, the smallest. Un altered

scale however gave the largest peak but in most cases, the peaks were found to go out of the scale of the paper unless the above scales were used. To display the chromatogram on paper, an EPSON FX-85 printer was used.

2.3.0 METHODOLOGY

2.3.1. Preparation of Pyrethrosin Standard

A few grams of pyrethrosin dust, supplied by Pyrethrum Board of Kenya, were dissolved in warm acetone and filtered. The filtrate on cooling forms small brownish white crystals. The crystals formed were redissolved in warm acetone and filtered. This filtrate appeared yellowish in colour. The procedure was repeated until the filtrate lost the yellowish colour and became colourless. After repeating this procedure five times, the colourless filtrate was allowed to cool and form crystals. Clear white bipyramidal crystals resulted which were stored in the refrigerator awaiting analysis. The melting point of these crystals was 197-198°C which is within the reported range of 193-200°C (48). The sample was analysed by HPLC under various conditions and only a single peak was observed. This clearly indicated that the sample was pure.

2.3.2 Standardisation of Extraction Procedure

Since chloroform easily dissolves both pyrethrosin and pyrethrins, it was selected as the extraction solvent. The samples were therefore hot extracted with chloroform at 68°C. In order to determine the total number of hours required to extract all the prethrins and pyrethrosin, 10 gm of

pyrethrum grist were soxhlet extracted with 250 ml of chloroform at 3 hour intervals. After every 3 hours interval, the chloroform was evaporated by a rotatory evaporator and the extract dissolved in the HPLC solvent system i.e. methanol: water (90:10; v/v). Aliquots of 20 microlitres were then injected into the HPLC machine and the machine run for 8 minutes. If peaks appeared where either pyrethrosin or pyrethrins were expected to appear, it meant that extraction was incomplete. Another 3 hour extraction interval was then carried out. This process was repeated until no peak appeared where pyrethrins and pyrethrosin were expected.

After repeating this procedure thirteen times, i.e. the 37th to 39th extraction hours, it was observed that no peaks appeared. This meant that, if all the samples to be analysed were extracted for the same length of time, all other conditions being the same, one would safely assume that any pyrethrins and pyrethrosin that might have remained must have been below the detection limit of the HPLC machine. It was then decided to extract all the test samples for a minimum of 40 hours.

2.3.3. Removal of Co-Extracted Plant Materials

Pyrethrum samples have been cleaned up by adsorption on a florisil column followed by elution with acetone-hexane (15:85) before analysis with a GLC. (62).

The best way so far devised of isolating the active constituents (pyrethrins) from the unwanted compounds is the nitromethane extraction method of Berthel, et al. (63). The method is more suitable for small

quantities and is tedious. However, as earlier mentioned (section 1.4), extensive clean-up of samples before analysis by HPLC technique is not necessary as is the case with GLC (61). It is advisable to remove most of the co-extracted plant materials before injection into the HPLC machine. The presence of co-extracted plant materials usually clog HPLC column leading to a build-up of back pressure and thus interferes with results obtained. To safeguard the column, five clean-up procedures were tried. The clean-up method adopted was that by which the least amount/if any, of the standards was lost during clean-up.

In each of the five methods tried, 10 mg of world standard pyrethrins and 10 mg of pyrethrosin were simultaneously dissolved in the eluting solvent. A small glass column was packed with 1 gm of one of three substances:- silica gel, celite or fine activated charcoal.

In all of the five methods, the eluting solvent was passed through the column so as to completely wet the solid support (charcoal, celite or silica gel) before the dissolved standards were passed through the column. The column was not allowed to dry before finishing the clean-up. After the solution containing the dissolved standards was passed through the column, the container was rinsed twice with fresh solvent and this was also passed through the column.

The solvent was then completely evaporated by a rotatory evaporator. The temperature at which this was done depended on the solvent. In the case of ethyl acetate, 78°C was the temperature chosen. The cleaned standards were then dissolved in 50 ml of the HPLC solvent system (Methanol: water,

90:10; v/v) and after analysis, peak areas were compared with those obtained when 10 mg of pyrethrosin and 10 mg of world standard pyrethrins were directly dissolved into 50 ml of HPLC solvent system and analysed.

The following combinations constituted the five clean-up procedures:-

1) Column packed with activated charcoal. Ethyl acetate was used as the eluting solvent.

2) Column packed with celite. Ethyl acetate was used as the eluting solvent.

3) Column packed with silica gel. Ethyl acetate was used as the eluting solvent.

4) Column packed with silica gel. Chloroform was used as the eluting solvent.

5) Column packed with celite. Chloroform was used as the eluting solvent.

6) This was the control, i.e. no adsorbent and solvent were used. Both standards (Pyrethrosin and pyrethrins) were directly dissolved in the HPLC solvent system and analysed by HPLC.

Table 2: Results of five clean up procedures

Method	Adsorbent	Solvent	Peak areas		
			Pyrethrosin	Pyrethrin I	Pyrethrin II
1	Activated charcoal	Ethyl acetate	9516.20	13732.50	10624.80
2	Celite	Ethyl acetate	15154.30	16575.90	14286.40
3	silca gel	Ethyl acetate	12185.10	13148.60	8782.10
4	Silica gel	Chloroform	3046.20	6829.60	6470.60
5	Celite	Chloroform	1348.50	1632.90	918.80
6	CONTROL		15256.20	16625.60	143595.50

The second clean up procedure i.e. column packed with celite and ethyl acetate used as the eluting solvent, gave the highest values of peak areas. The peak areas obtained in this case compared very well with those observed when 10mg of both standards were directly dissolved in the HPLC solvent system and analysed as can be seen from the control (method 6). The difference in peak areas for pyrethrosin is 0.7%, that for pyrethrins I is 0.3% whereas that for pyrethrins II is 0.5%. This resulted in the choice of this clean up procedure for all the extracted samples analysed.

2.3.4 Collection and Preparation of Pyrethrum Flowers

The flowers were picked at the Pyrethrum Board of Kenya's Molo Research Station by workers of the Pyrethrum Board who are experienced in picking flowers for research purposes. The flowers were then dried at ambient temperatures, spread on raised sieves to avoid fermentation which would otherwise lower the pyrethrins content. Separation of the three different parts of the flower i.e. sepals, petals and ray florets either before or after drying does not have any effect on the pyrethrins or pyrethrosin content. However, it was found easier to separate them after drying instead

of when they were green. Thereafter, the flowers were ground using a BS 410 mesh and the dust obtained stored in cotton paper bags awaiting extraction.

2.3.5 Extraction

In all the cases, 10 gm of the dust sample was weighed into a cellulose extraction thimble which was then put into a soxhlet extractor. The soxhlet was then attached to a half litre flat bottomed flask containing 300mls of chloroform and some boiling chips added. A condenser already connected to a source of tap water was attached on the upper side of the soxhlet. All the glassware used had quickfit joints. The extractions were done at 68°C for a minimum of 40 hours each.

Most extractions were undertaken at the laboratories of Pyrethrum Board of Kenya - Nakuru where twenty samples were extracted simultaneously. The resultant solution (extract) was then filtered through cotton wool and chloroform evaporated by a rotatory evaporator. The extract was then allowed to cool after which it was weighed to determine the yield. These weights were important in calculating the percentage concentrations. the extracts were then put in small sample vials, labelled and stored in the refrigerator to await clean up and analysis. For each sample, including mosquito coil samples, three extractions were performed.

2.3.6 Sample Preparation

In the case of extracted samples, 10 mg of the extract was weighed and

then dissolved in ethyl acetate in a 50 ml beaker. A small glass column, 5 inches long and blocked at the tapering end with cotton wool was packed with approximately 1 gm of celite. A small amount of ethyl acetate was first passed through the column to completely wet the celite before clean up could commence.

The extract (10 mg) in approximately 5 ml of ethyl acetate was then passed through the column and collected into a 50 ml pear shaped flask. Care was taken to avoid the celite drying up. After all the solution in the beaker passed through the column, the beaker was rinsed twice with about 20 ml of ethyl acetate and this also passed through the column. The column was also rinsed twice with 2 ml of ethyl acetate to remove any pyrethrins or pyrethrosin which may have remained on the celite. Ethyl acetate was then completely evaporated by a rotatory evaporator with the temperature thermostatically set at 78°C.

The HPLC solvent system, i.e. Methanol: Water; 90:10 v/v was then used to dissolve pyrethrins and pyrethrosin from the pear shaped flask. This solution was carefully transferred to a 50 ml volumetric flask and the volume made up to the 50 ml mark with Methanol: Water; 90:10. After thorough shaking, the solution was filtered through cotton wool and a little amount of the filtrate transferred to a 5 ml sample vial. The sample vial was labelled and then stored in the refrigerator awaiting HPLC analysis.

For each extract, the above steps were repeated three times resulting in three cleaned samples.

2.3.7 HPLC Analysis and Operating Conditions

The HPLC column was always kept in methanol. Before starting the analysis, methanol was first pumped through the system for about an hour with the detector on till stability was achieved. Methanol and water was then introduced on a linear gradient in three minutes until the ratio of methanol: water was 90:10; v/v.

With the detector still on, the HPLC machine was left with a flow rate of 2 ml/minute to stabilise again over a period of about 30 minutes. After achieving stability, the flow rate was reduced to 0.8 ml/minute before starting the analysis. The detector was then zeroed after achieving stability. This zeroing of the detector meant that the pen of the chart recorder could now start at the baseline.

The HPLC machine used had a magnetic stirrer which is switched on when two different solvents are used for analysis. It thoroughly mixes the two solvents to give a homogenous solvent system.

Aliquots of 20 microlitres were injected into the injection pot for all samples analysed and the sample loop then connected to the column through the sample injection system. This operation and that of the data collection by the computer were synchronised and elution was complete in six to eight minutes in nearly all the cases by which time all the pyrethrins and pyrethrosin had passed through the system.

At the end of each analysis, the data collected by the computer was passed through a "smoothing" routine to remove random electronic noise and then stored or saved as digital spectrum on diskettes for future analysis. The

system was then ready for the next analysis.

The three peaks of interest were identified by comparison of retention times with those of known standards and where ambiguity arose, the samples were spiked with a known standard. An increase in peak height confirmed the identity.

For each of the prepared (cleaned) samples, at least three injections were made, after which the average peak area was calculated. The average peak area was used for calculating the percentage concentrations.

When the computer was not used for data collection and analysis, the chart speed of the recorder was adjusted at 1 cm/minute and retention times determined as explained above. All the analysis was done at a wavelength of 230 nm.

2.3.8. Calculations and data analysis

Two formulae were derived and used for obtaining percentage concentrations. The first formula was used in calculating percentage concentrations for samples that were extracted with chloroform in the laboratory i.e. all flower samples for the different clones, mosquito coils and the two dust samples supplied by Pyrethrum Board of Kenya and was in the form of:-

$$\% \ Concentration = \frac{concentration\ of\ standard.\ A_1\ .\ Y}{X\ .\ A_2}$$

where,

The concentration of pyrethrins I in the standard used was

given as 13.60% and that of pyrethrin II was 9.44%. The concentration of pyrethrosin standard which was previously prepared in the laboratory was 100%

X was the weight in gms of the extracted dust.

A_1 was the peak area given by the sample.

A_2 was the peak area given by the standard, and

Y was the weight in gm of the extract obtained from X gm of the dust sample.

The second formula was used in the analysis of those samples that did not have to be extracted as they were supplied in a ready-for-analysis form i.e. pale extract and oleoresin. These two were extracted by Pyrethrum Board of Kenya using n-hexane as the extraction solvent and calculation formula was in the form of:-

$$\% \ Concentration = \frac{A_1 \ . \ 100}{A_2 \ . \ concentration \ of \ standard}$$

where,

A_1 was the peak area given by the sample,

A_2 was the peak area given by the standard, and standard concentration was as in the first formula except in the case of pyrethrosin where this was unity.

Similar formulae as the above have been used in the analysis of n-docosyl ferulate in *P. africanum* extract by HPLC (64) and also the analysis of pyrethrins by HPLC (65) with the only difference being that the concentration of the standard is replaced by standard purity.

The results obtained from the study have been arranged in various tables with samples bearing the close resemblance to each other being grouped together.

2.3.9 Use of Microcomputers in Chemical Analysis

The demands of modern research are such that precision and accuracy are of prime importance, while at the same time, great emphasis is laid on speed and efficiency. Consequently, analytical instruments have had to grow in sophistication and invariably the volume and complexity of data generated has increased proportionally. In order to meet the high standards and to achieve the set objectives in research, the modes of handling data have had to change accordingly.

This process has been greatly aided by the development of microprocessors. The microprocessor has made it possible for low-cost computers to be available for aquiring and processing complex data sets thus enabling more effective analysis than was previously possible (66). Most modern analytical instruments now have on-board microprocessors or computers for performing functions such as instrument control, data logging, data analysis etc. Use of computer systems on analytical instruments has greatly simplified the task of chemical analysis and has enabled researchers to make more effective use of several steps in chemical determination than was previously practical. Chromatographic analysis is one such area where computers have found great applicability and especially in the analysis of complex mixtures.

Though many modern materials for column chromatography that are capable of separating a wide range of compounds, including isomers of one compound, have been developed, rarely does separation occur with 100% resolution. Other variables such as temperature, electrical fluctuations, solvent composition etc. add to the complexity of the spectra obtained thus making it difficult to interpret. A computer system can very effectively be employed to resolve peaks of complex spectra by mathematical method (67, 68) and at the same time keep track of the changes or drift in the baseline. Further, the system can obtain heights, peak areas and retention times of various components and calculate their concentrations. The system may obtain other parameters if instructed and it can be used to control the numerous functions pertaining to the instrument and hence reduce human intervention and hence errors, increase reproducibility and save time. In the study reported in this thesis, a microcomputer (BBC microcomputer) was interfaced with the HPLC mainly for the purpose of data collection and analysis. It was thus possible to acquire data, remove random noise, store data on disc, analyse for peak areas and retention times, and resolve merged peaks, print results etc.

CHAPTER 3

3.0 RESULTS AND DISCUSSION

3.1.0 Obtaining Retention Times and Peak Areas

Quantification in chromatography requires the determination of the size of the peak either in units of area or weight, both of which are related to concentration and in some instances, peak heights are used. These determinations are sometimes complicated by the inability of the chromatographic system to fully resolve all the components in a sample, thus resulting in poorly defined merged peaks and further, by the instability of the baseline.

Since the introduction of computers in chromatography, various methods have been developed for peak area determination. These range from the simple ones such as the height times width (at half height) and the trapezium methods, to elaborate gaussian curve - fitting methods (67, 68).

In this study, a fairly simple method was employed to obtain peak areas and to resolve merged peaks. This involved the summation of consecutive heights along a curve and after substraction of the baseline, the values obtained were directly proportional to the area of the peak. As this method follows the actual curve, all points along it are taken into consideration and is hence more accurate than either the trapezium method or the height times width (at half height) methods.

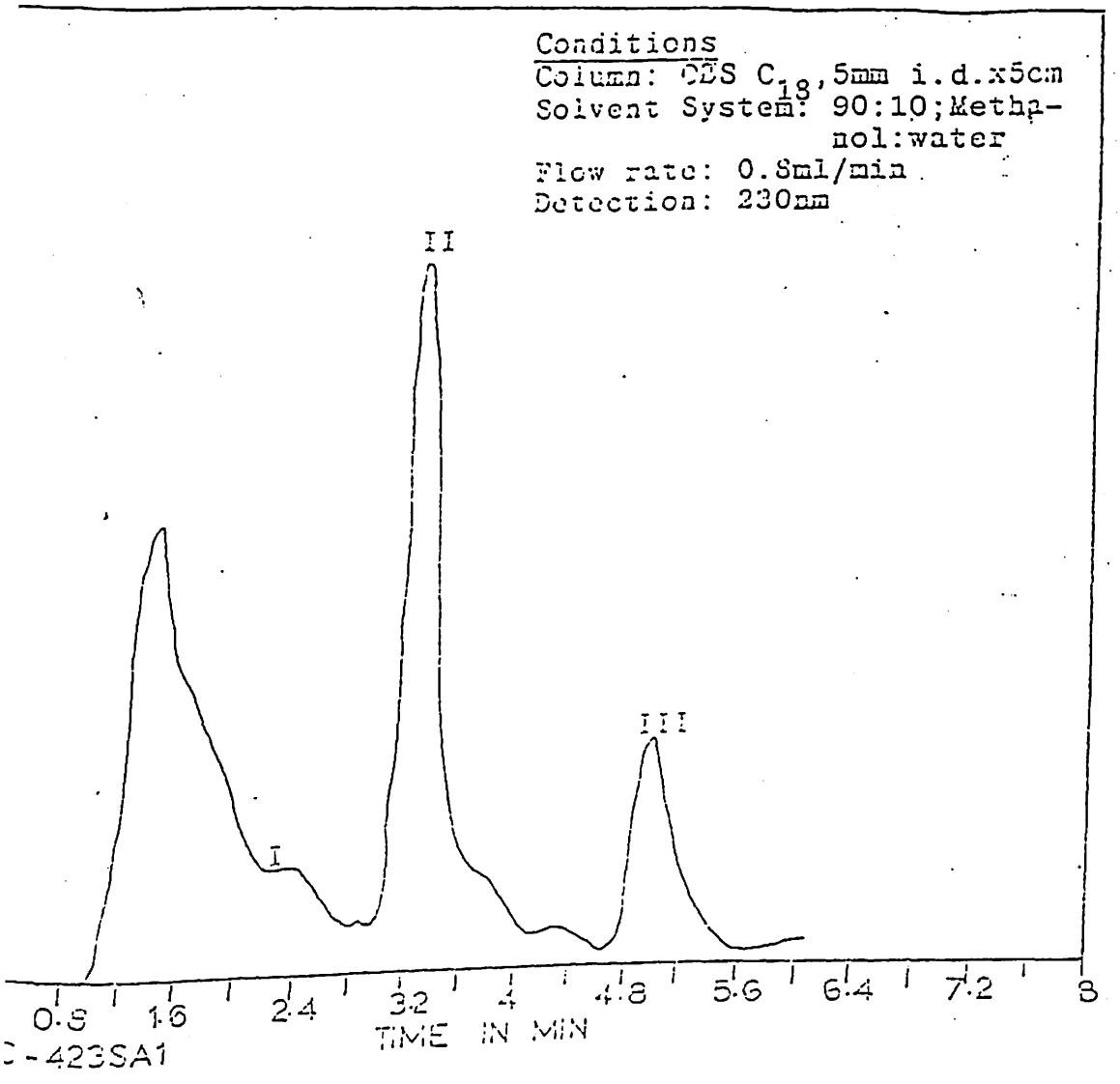
It was also possible to obtain areas of peaks that on an ordinary

chromatogram chart would be considered out of range and hence not quantifiable. With the computer system, peaks exceeding the maximum peak height of the chart could be stored in memory and later reconstructed for the purpose of analysis or display.

Identification in chromatography relies on the location of the peak in the chromatogram, i.e. the retention time which is a factor of the polarity of the substance. Retention times were easily determined even for broad peaks as the program could pick out slight changes in the gradient. Where ambiguity arose in the identification of a peak, the sample was spiked with the suspected compound and an increase in height confirmed the inference. This is illustrated in figures 19 and 20. Figures 19 and 20 represent chromatograms of the same sample with the only difference being that in figure 20 the sample was spiked with pyrethrosin.

The retention times for pyrethrosin ranged from 1.72 minutes to 2.52 minutes, that for pyrethrins II from 3.04 minutes to 4.00 minutes, while that for pyrethrins I ranged from 4.27 minutes to 6.00 minutes for the standards and samples analysed. In all samples analysed, the shortest retention times observed were those for sepal samples whereas some of the longest retention times observed were for the standards. It should however, be noted that in the case of similar samples or extracts, the variations in retention times were very slight.

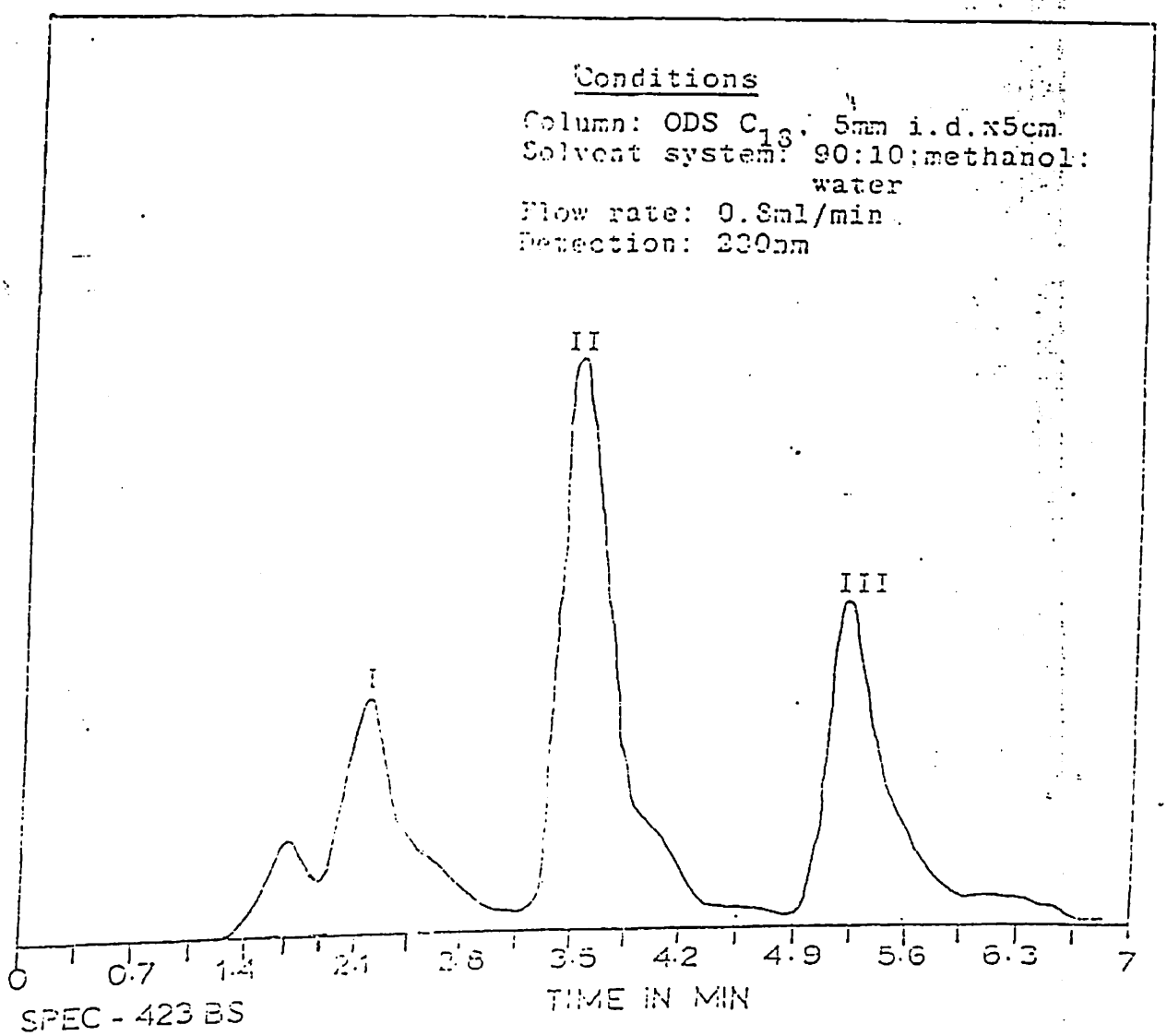
Fig. 19 Chromatogram of the extract of Clone 423



Identification of peaks:

I	pyrethrosin
II	pyrethrins II
III	pyrethrins I

Fig. 20 **Chromatogram of the extract of Clone 423, Spiked with Pyrethrosin Standard.**



Identification of peaks:

I	pyrethrosin
II	pyrethrins II
III	pyrethrins I

Variations in retention times have also been observed in the analysis of pyrethrins and piperonyl butoxide (60). They have also been observed in the analysis of polycyclic aromatic hydrocarbons in roast meat and smoked fish (69). These variations in retention times observed while analysing for the same compounds in different samples is due to matrix effect (70). This means that retention times for the same compound can vary depending on the chemical environment and concentrations in which the compound is found. Hence, though sepals and ray florets contain pyrethrosin and pyrethrins, the chemical environment in which these exist is definitely different since both contain other compounds some of which are different. These other compounds interact with the compounds of interest, the stationary phase and the mobile phase in different ways hence causing the phenomena of matrix effect.

3.2.0 Resolution of Peaks

In some of the sample chromatograms, e.g. figure (21), the peak for pyrethrosin has not been well resolved. This is a common problem associated with isocratic development where the resultant chromatogram of a multicomponent mixture shows uneven degree of separation between the components of the mixture (71). That is, the front of the chromatogram shows a series of bunched and poorly resolved peaks whereas the middle portion shows good separation and the final portion shows good separation but sometimes long diffuse peaks which if continued would be difficult to detect (71). This is a general elution problem and is common in all forms of

chromatography.

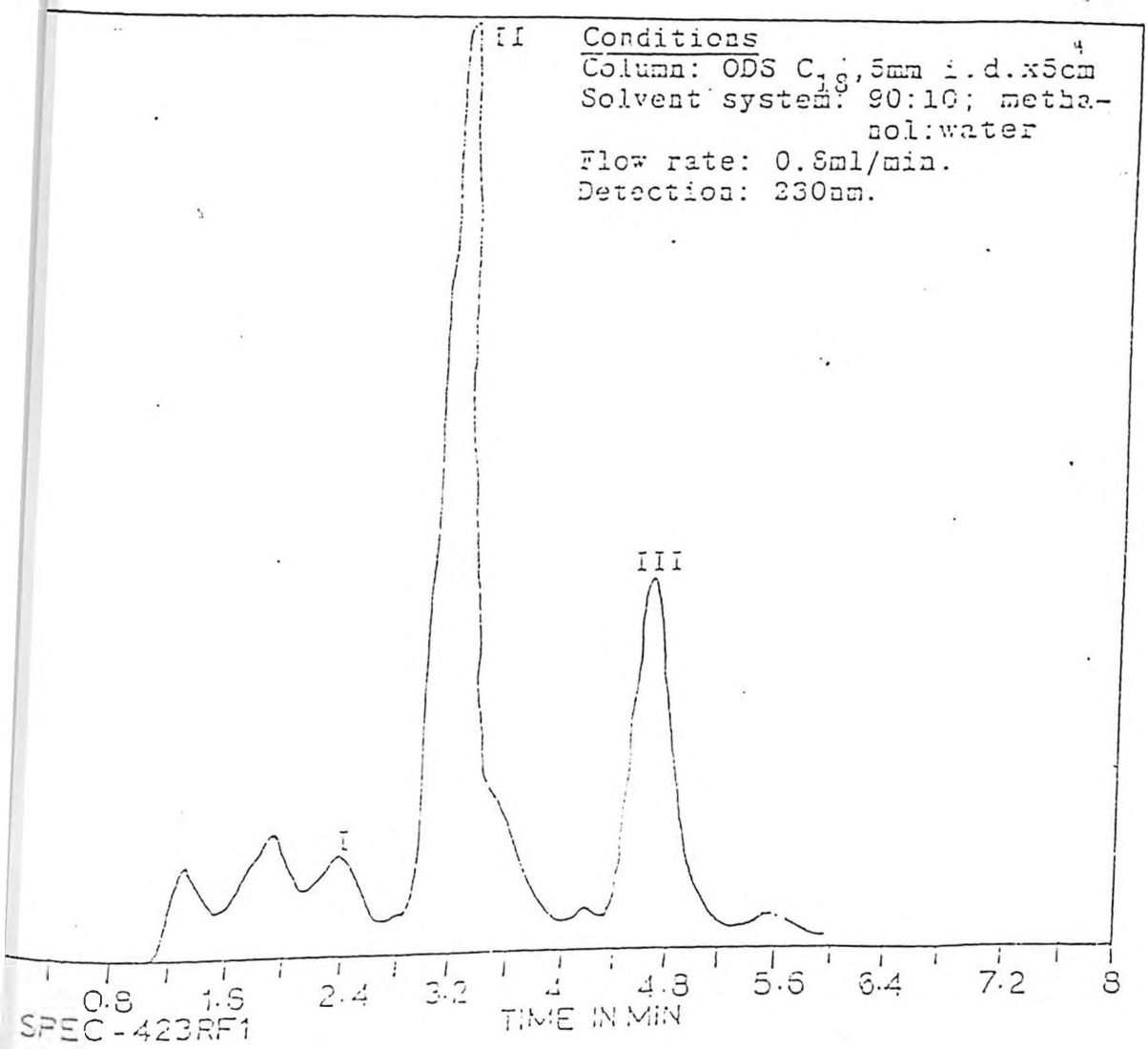
Variation in retention times may be minimized by instituting (a), temperature programming; (b), Flow programming and (c) coupled columns. Temperature programming and flow programming are not efficient in solving this problem and have little effect in capacity factor. Temperature programming is also inappropriate for routine analysis since too much time is required to equilibrate (71). Coupled column technique (same or different columns) has many advantages especially in routine analysis (71). To resolve this problem, the computer used for the analysis was equipped with a program for resolving merged peaks, i.e. the perpendicular drop method (68).

3.3.0 Determination of Concentrations

As mentioned in section 1.3.10 pyrethrins have previously been analysed at various wavelengths i.e. 230 nm, 240 nm, 254 nm etc. The wavelength of this analysis was chosen as 230 nm since a u.v. scan of pyrethrosin confirmed that pyrethrosin absorbs between 225 nm and 237 nm.

The conditions used for the analysis were such that the detector was more sensitive to pyrethrins than to pyrethrosin with the result that very small peak areas due to pyrethrosin represented higher percentage concentration than higher peak areas due to pyrethrins. This can be overcome by the use of photodiode-array detector which is not available in Kenya. This can be seen in the case of the standards chromotagram (fig. 23) which is a tracing of the chromatogram from the chart paper.

Fig. 21 Chromatogram of the Extract of Ray Florets of Clone 423



Identification of peaks: I pyrethrosin
II pyrethrins II
III pyrethrins I

The chromatogram was obtained when 10 mg of pyrethrosin and 10 mg of the world standard pyrethrins were both dissolved in 50 ml of the solvent system (methanol: water; 90;10, v/v) and analysed. The pyrethrosin concentration in the prepared standard was 100 percent, that for pyrethrins II in the world standard was given as 9.44 percent whereas that for pyrethrins I was given as 13.60 percent (PBK method). Hence, in the calculations, any peak area due to pyrethrin II for example was calculated for percentage concentration using the value 9.44 percent in the equation etc.

In figure 22 peak I is due to pyrethrosin, peak II due to pyrethrins II and peak III due to pyrethrins I. This is a tracing of the chromatogram from the chart paper. STDS 1 on figure 23 is another chromatogram of the same standards but in this case printed directly from the computer program used for most of the work. All the chromatograms appearing in the chapter with the exception of figure 22 were obtained directly from the computer. Hence, in order to have standard chromatograms for comparison purposes, the same scale has been used throughout though the computer program had the ability to compress or enlarge the peaks as explained in section 2.2.0.

Since the same procedures were followed when analysing the samples, similar formulae were used for calculating the percentage concentrations. These formulae are given in section 2.3.8.

3.4.0 Statistical Treatment of the Data Obtained

The results obtained have been arranged in various tables with each table containing data of results for samples that bear a close resemblance to

each other. For example, the first table in this chapter represents the results obtained when thirteen pyrethrum clones were analysed.

In analysing the data obtained, two different statistical methods have been employed depending on the number of samples to be compared. For the first two tables of this chapter, the student's t-test would not have been appropriate for the analysis since we were dealing with multiple comparisons and hence would have been compelled to repeat the treatment very many times which is a very tedious exercise.

Various methods of analysis for this kind of data can be employed but the analyst selects the one that is most appropriate for the data collected e.g. Duncan's new multiple range test (MRT), Turkey's test (usually referred to as HSD [honestly significant difference] test) etc. This is also known as the F-test and makes use of a single value against which all differences are compared. In this work, Turkey's test was chosen as it was found to be the most appropriate for the analysis of data for the first two tables.

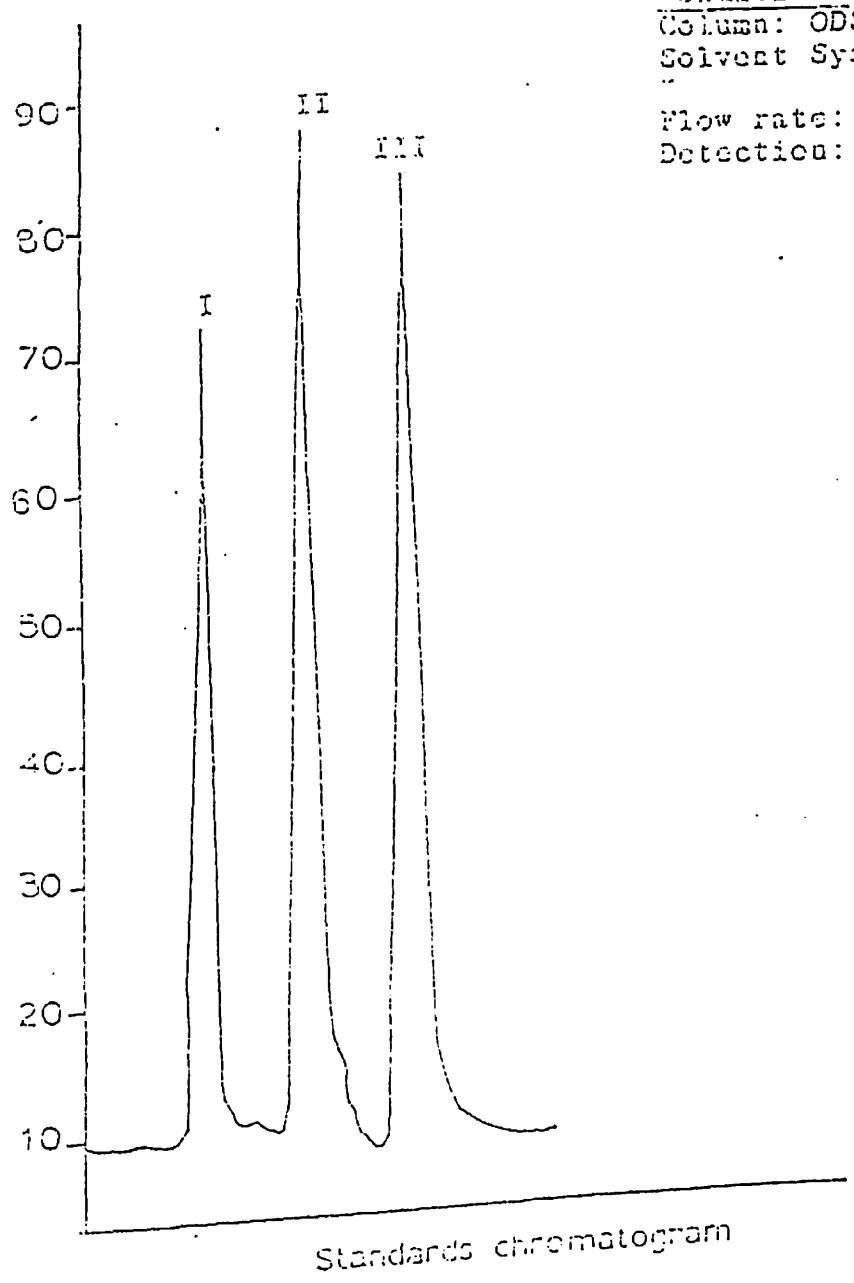
HSD is given by:-

$$HSD = q_{\alpha, K, N-K} \sqrt{\frac{MSE}{n}}$$

where,

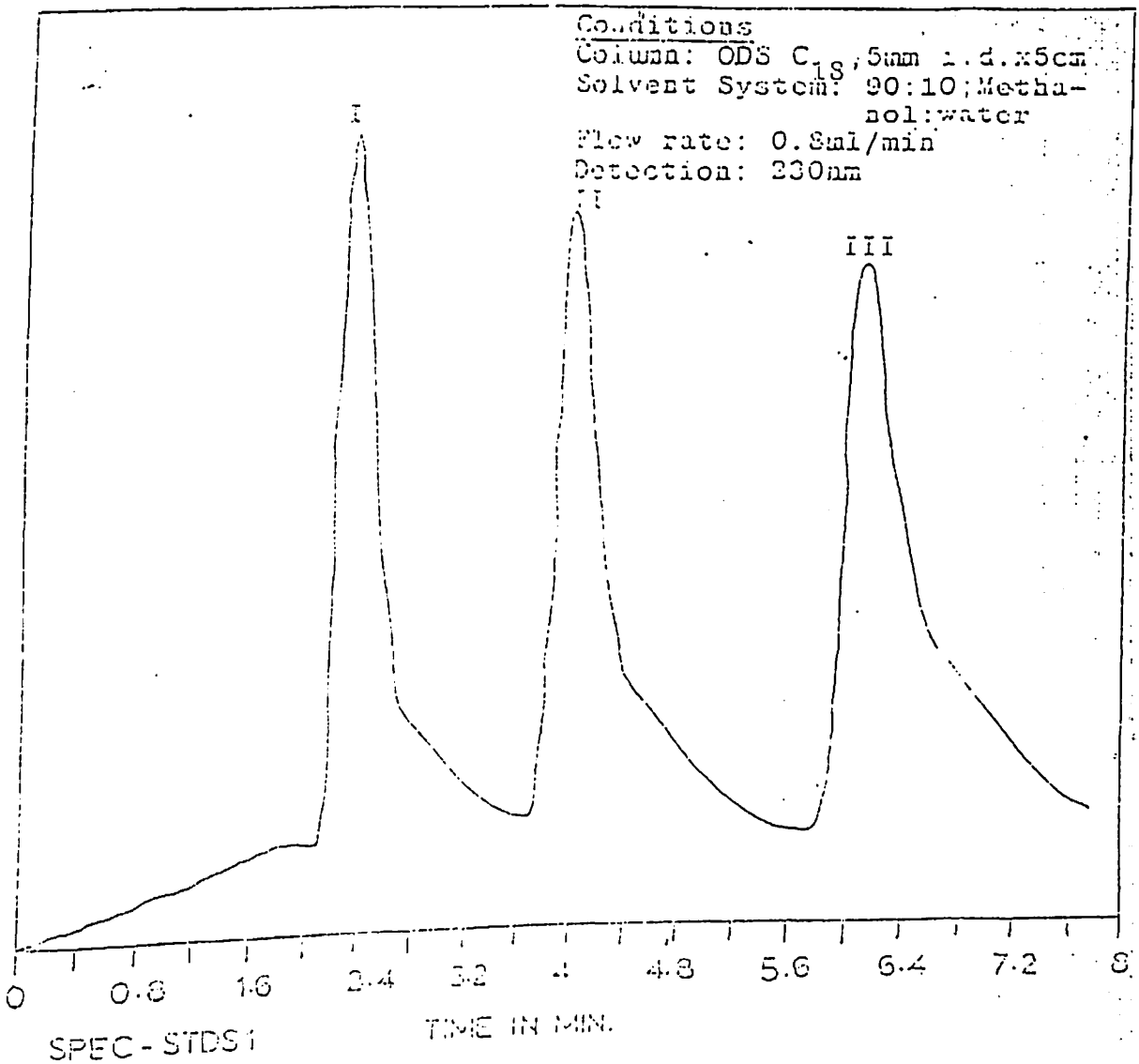
α	-	chosen level of significance
K	-	number of means in the experiment
N	-	total number of observations in the experiment.
n	-	number of observations in a treatment
MSE	-	error mean square from the ANOVA table and
q	-	is obtained by entering appendix table H with α, K and N-K.

Fig. 22 **Chromatogram of Mixture of Pyrthrosin and Pyrethrins Standards (Chart Paper)**



Identification of peaks: I pyrethrosin
II pyrethrins II
III pyrethrins I

Fig. 23 **Chromatogram of Mixture of Pyrthrosin and Pyrethrins Standards (Computer Print)**



Identification of peaks:

I	pyrethrosin
II	pyrethrins II
III	pyrethrins I

All possible differences between pairs of means are computed and any difference which yields an absolute value that exceeds HSD is declared to be significant.

In my case, the level of significance α was taken as $\alpha = 0.05$ (meaning 95% confidence level).

In all the other tables in this chapter, the statistical method of analysing the data chosen was the student's t-test.

3.5.0 Pyrethrosin and Pyrethrins Concentration in the Thirteen Clones Studied.

In all the tables appearing in this chapter, the pyrethrins concentration given represents both pyrethrins I and II.

In table 3 below, each of the mean percentage concentrations referred to represent the mean of three means obtained earlier as explained in the experimental section. For example, the mean pyrethrins concentration for clone 423 indicated is 1.16 which is obtained from three means i.e. (1.1522 + 1.2785 + 1.0613) ÷ 3 = 1.1640. This is the data used in the statistical analysis. Each of these three means represent the mean of the results obtained from one picking season (see experimental section). This same steps have been followed for the data shown in tables 3 and 4 of this chapter.

Figure 24 is a bar chart representing the pyrethrins and pyrethrosin concentration in percentage for the thirteen clones studied.

Table 3 gives the mean pyrethrins and the mean pyrethrosin concentrations in percentage, for the thirteen clones studied.

Table 3: Mean Pyrethrins and Pyrethrosin Concentrations for Thirteen Clones Studied

Clone	Mean pyrethrosin concentration % w/w			Mean pyrethrins concentration % w/w		
		S.D.	C.V.		S.D.,	C.V.
423	1.97	± 0.1210	6.1	1.16	± 0.1091	9.4
442	2.50	± 0.0086	0.3	1.07	± 0.0196	1.8
336	2.01	± 0.0020	0.1	0.75	± 0.1754	23.4
4743	2.80	± 0.0000	0.0	0.88	± 0.0080	0.9
122	2.44	± 0.1057	4.3	1.07	± 0.0316	3.0
313	5.64	± 1.0597	18.8	1.34	± 0.1240	9.3
6	2.50	± 0.1045	4.2	1.41	± 0.2957	21.0
96	5.03	± 0.2130	4.2	1.48	± 0.0092	0.6
1013	3.40	± 1.0945	32.2	2.04	± 0.0164	0.8
223	1.70	± 0.2135	12.6	0.82	± 0.0305	3.7
107	1.73	± 0.0000	0.0	2.23	± 0.0001	0.0
4331	1.61	± 0.0073	0.5	1.65	± 0.0088	0.5
64	2.04	± 0.0018	0.1	0.94	± 0.0187	2.0

n= 3

After statistical analysis of the data, it was observed that HSD = 0.0865 This meant that any two means whose difference is equal to or more than 0.0865 in respect to pyrethrins concentration differ significantly. The following means were found to be the same:- 442 and 122, 313 and 6, 6 and 96, 336 and 223, 4743 and 223 and 4743 and 64. All other means are different. Overlap of means was found in the case of clone 223 whose mean is not significantly different from that of clone 336 and clone 4743. It was also observed in the case of clone 6 whose mean is not significantly different from the means for clone 313 and clone 96. Clone 107 had the highest pyrethrins concentration followed by clone 1013 whereas the lowest pyrethrins

concentrations were observed in the case of clones 336 and 223 followed by clone 4743.

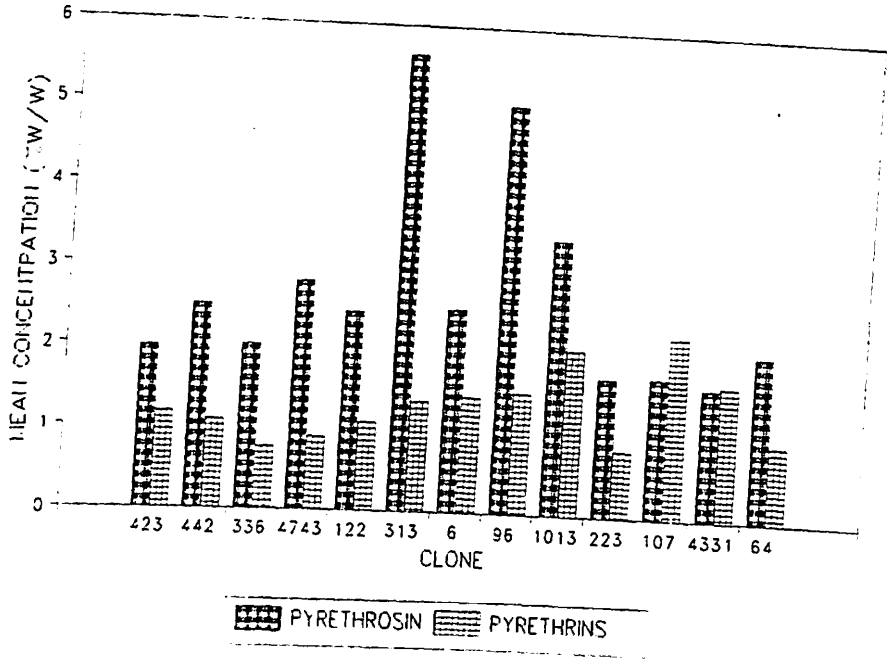


Fig. 24: Bar Chart for the Pyrethrins and Pyrethrosin Concentration for the Thirteen Clones Studied

Table 3 also shows the mean pyrethrosin concentration for the thirteen clones studied. Figure (24) represents the same data in bar chart form. It is clear from fig. (24) and table 3 that clones 313 and 96 stand out distinctly from the rest of the clones in their pyrethrosin concentration by having a very high pyrethrosin concentration.

Statistical analysis of the data in this case shows that any two means whose difference is greater than or equal to 0.3524 are significantly different. Hence, the following clones have means that do not differ significantly in respect to pyrethrosin concentration :- 423 and 336; 442 and 4743; 442 and 123; 442 and 6; 336 and 6; 4743 and 6; 122 and 6; 423 and 223; 423 and

107; 223 and 107; 223 and 4331; 107 and 4331; 423 and 64; 223 and 64 and means for clones 107 and 64. All other mean pairs differ significantly. A lot of overlap is also observed in this case.

3.5.1 Correlation between Pyrethrosin and Pyrethrins Concentration in the Thirteen Clones.

If a correlation exists between the pyrethrosin concentration of a flower and the pyrethrins concentration, then for every value of pyrethrosin obtained, a corresponding value for pyrethrins would be obtained. This relationship could either be direct or inverse. However, computation of the variance ratio of the data for pyrethrins and pyrethrosin lies outside the critical region. This means that there is no correlation between the pyrethrosin and the pyrethrins concentration. You cannot therefore use the results of either parameter to predict the other.

Inference

Since there is no correlation between the pyrethrins and pyrethrosin content, in a breeding programme, neither of these compounds can be used to assess the other for selection purposes. Thus, in breeding new strains of pyrethrum that are high in pyrethrins content and low in pyrethrosin content, specimens for propagation would have to be selected for these two characteristics independently of each other. In such a programme, one can select clones which show the desired qualities then propagate them or else two lines may be established: one for increased pyrethrins content and the other for reduced pyrethrosin content. These two lines are then crossed after a number of generations when significant improvements in the desired traits are observed. Lack of correlation in the two compounds indicates that the two do not influence each other's synthesis in any way. Hence their production could be autoregulatory or else, influenced by other factors other than each others' presence.

It should be noted that if other positive uses of pyrethrosin (besides molluscicidal activity) are discovered since nearly all sesquiterpene lactones have diverse biological activity (section 1.2) or if pyrethrosin's molluscicidal activity could be commercially exploited, then, a high concentration of pyrethrosin in the pyrethrum flower would be more advantageous than it would be disadvantageous. However, if the advantages of pyrethrosin are disregarded, then clones with high concentration of pyrethrosin would be considered as being disadvantageous since pyrethrosin cause dermatitis (section 1.2). Hence, clone 107 would be the best as it has the highest

pyrethrins content and has one of the lowest pyrethrosin content. If both parameters are however, considered, then those clones with high amounts of both would be considered as being more advantageous e.g. clone 1013 which had the second highest pyrethrosin content and also the second highest pyrethrins content (3.40% and 2.04%, respectively).

The standard deviation for pyrethrosin in the thirteen clones studied are quite low and so are the coefficients of variation. 77% of the samples analysed for pyrethrosin have coefficients of variation ranging from 0.0% to 6.1% showing that concentration of pyrethrosin does not vary much with the seasons. A few clones showed high coefficients of variation e.g. clone 313 with 18.8% and clone 1013 with 32.2%. This could probably be due to the picking intervals being different for the three seasons that samples were collected. In the case of pyrethrins, standard deviations are again low with 85% of the samples analysed having coefficients of variation ranging between 0.0% to 9.4%. This again shows that pyrethrins concentration does not vary seasonally. Clone 336 and clone 6 had coefficients of variation of 23.4% and 21.0% respectively which are reasonably high. This could also be due to the same reasons above. It is a phenomenon that is sometimes observed in analysis [69].

3.6.0 Pyrethrins and Pyrethrosin Concentration in the Different Parts of the Flowers in the Clones Studied.

A pyrethrum flower has three parts. These are the ray florets, sepals and petal. Four clones were selected for the study.

Table 4 shows the mean pyrethrosin and pyrethrins concentration for the different parts of the flowers for the clones studied.

Table 4: Mean Pyrethrins and Pyrethrosin Concentration for the Different Parts of the Flower for the Clones Studied

Clone	Part of the flower	Mean pyrethrosin concentration in % (w/w)			Mean pyrethrins concentration in % (w/w)		
			S.D.	C.V.		S.D.	C.V.
107	Ray florets	2.81	± 0.0236	0.8	1.82	± 0.0153	0.8
	Petals	2.68	± 0.1591	5.9	0.08	± 0.0132	16.5
	Sepals	1.96	± 0.2404	12.3	0.93	± 0.0291	3.4
64	Ray florets	2.82	± 0.3899	13.8	2.17	± 0.0424	2.0
	Petals	2.05	± 0.0943	4.6	0.07	± 0.0041	5.9
	sepals	1.07	± 0.0200	1.8	0.43	± 0.0075	1.7
423	Ray florets	3.53	± 0.1109	3.1	2.20	± 0.0430	2.0
	Petals	3.38	± 0.1051	0.4	0.11	± 0.0120	10.9
	Sepals	2.04	± 0.0100	0.5	0.72	± 0.0146	2.0
122	Ray florets	3.17	± 0.4256	13.4	1.99	± 0.1335	6.7
	Petals	2.88	± 0.9624	33.4	0.13	± 0.0049	3.8
	Sepals	2.83	± 0.7111	25.1	0.92	± 0.0372	4.0

n = 3

Sampling in this case was carried out the same number of times as in the case for the different clones studied.

Figure 25 below is a bar chart of the pyrethrins and pyrethrosin concentration in the different parts of the flower for the clones studied.

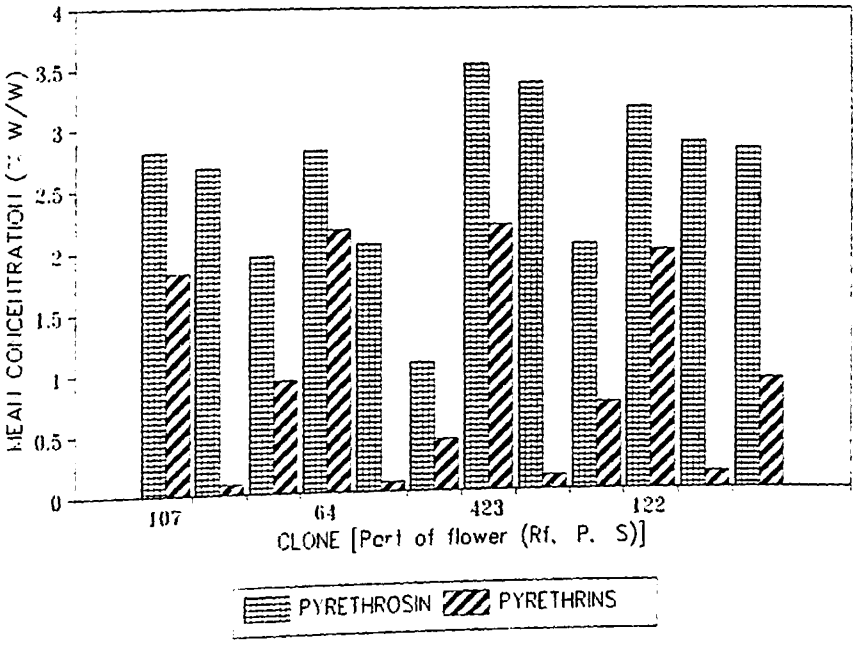


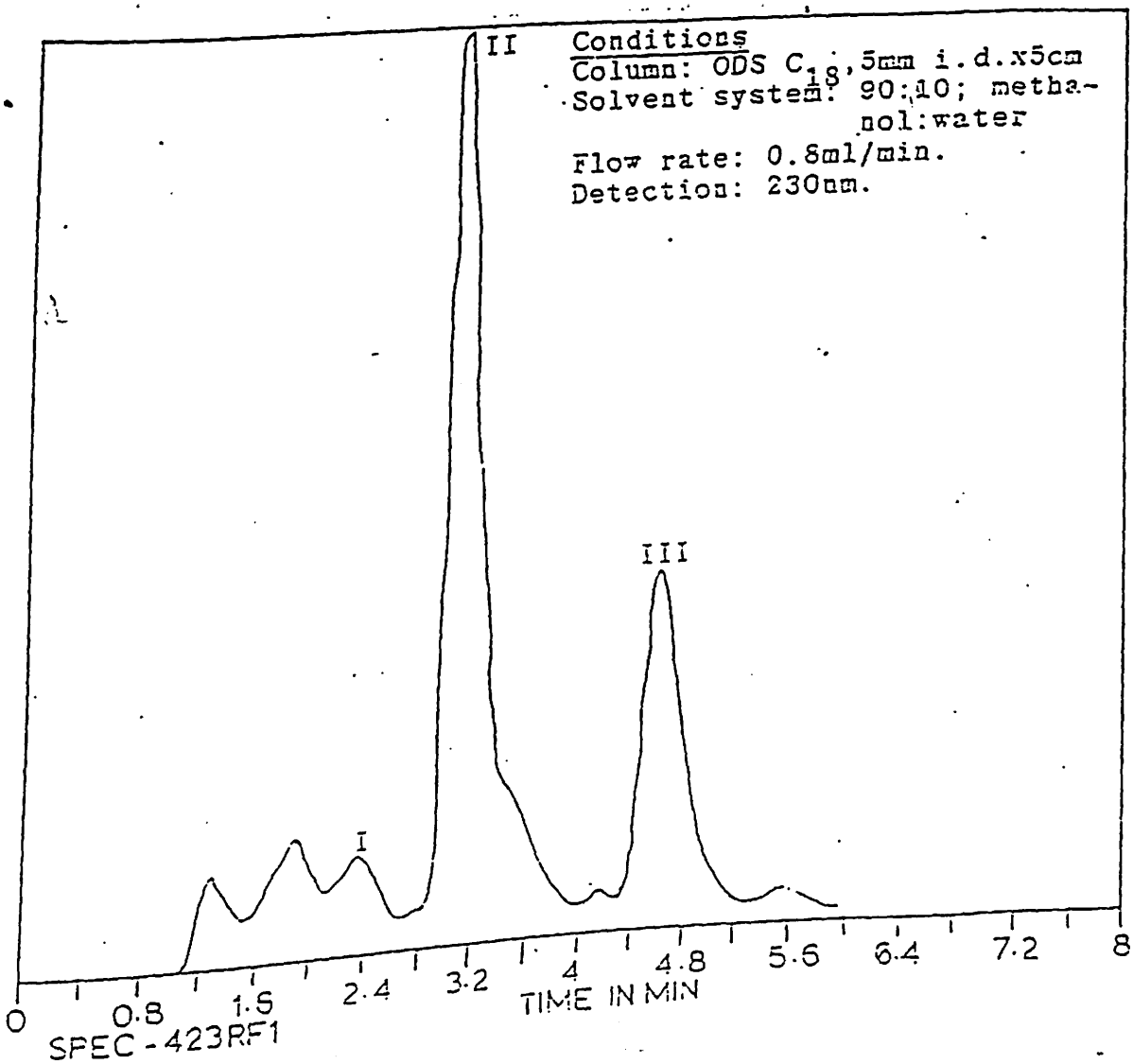
Figure 25: Bar Chart of Pyrethrosin and Pyrethrins Concentration in Different Parts of the Flower (Rf=Ray florets, P=Petals, S=Sepals).

Figure 26, 27 and 28 are chromatograms obtained from the analysis of extracts of ray florets, petals and sepals of clone 423 resepctively. The same computer printing scale has been used so that results can be compared. The pyrethrosin content of the three different parts is not significantly different statistically except in the case of sepals of clones 107 and 64 where this concentration is about half that in the ray florets.

As for the pyrethrins content, statistical analysis shows that there is no significant difference in pyrethrins content for the ray florets in the four clones studied. It also reveals that pyrethrins content is highest in

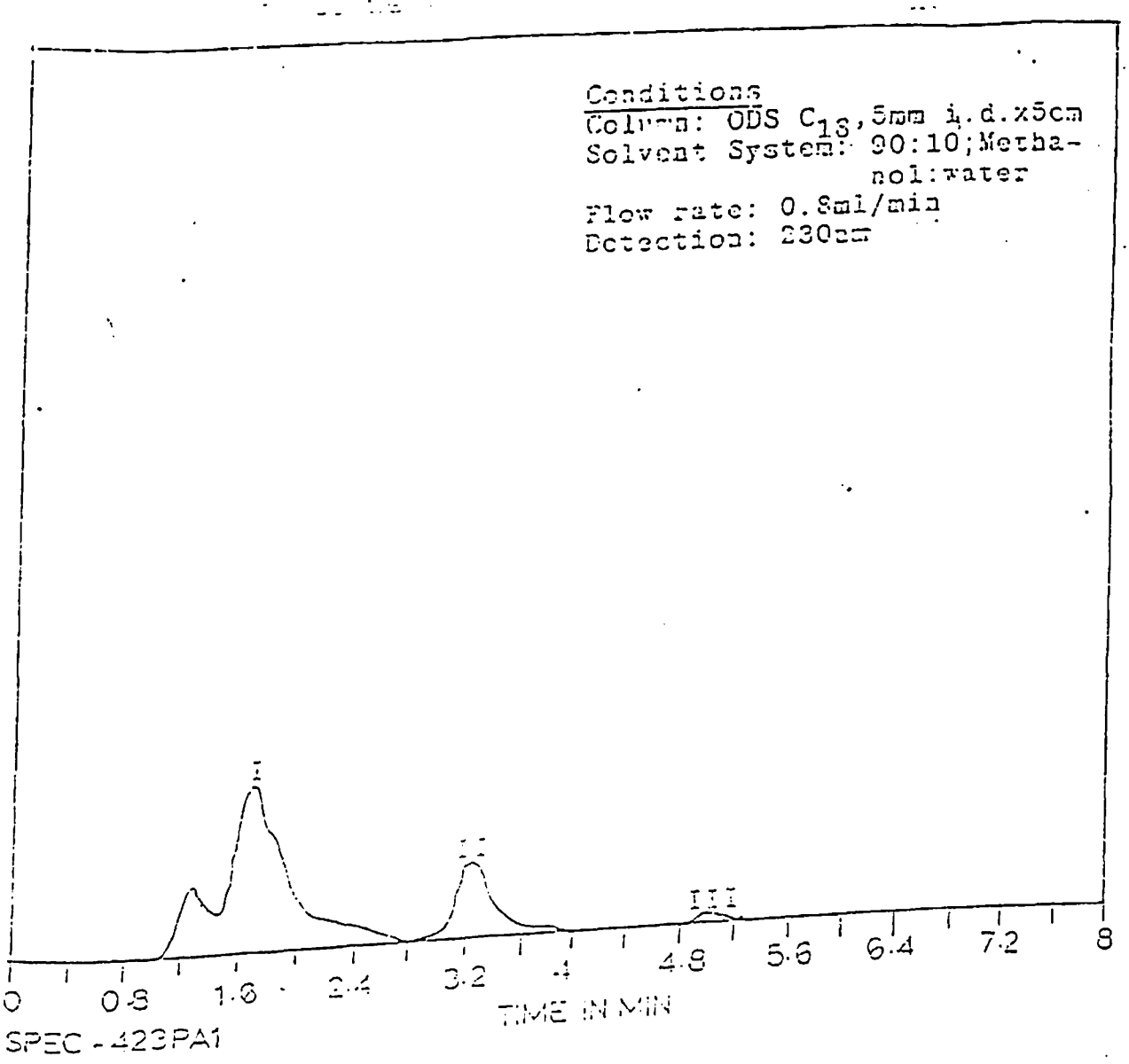
ray florets and lowest in petals. It is clear from bar chart presentation (figure 25) that petals have an insignificant amount of pyrethrins. This has also been clearly shown the chromatograms shown (figures 26, 27 and 28). Statistical analysis shows that in respect to pyrethrins content, the three parts are all different from each other.

Fig. 26 Chromatogram of the extract of Ray Florets of Clone 423



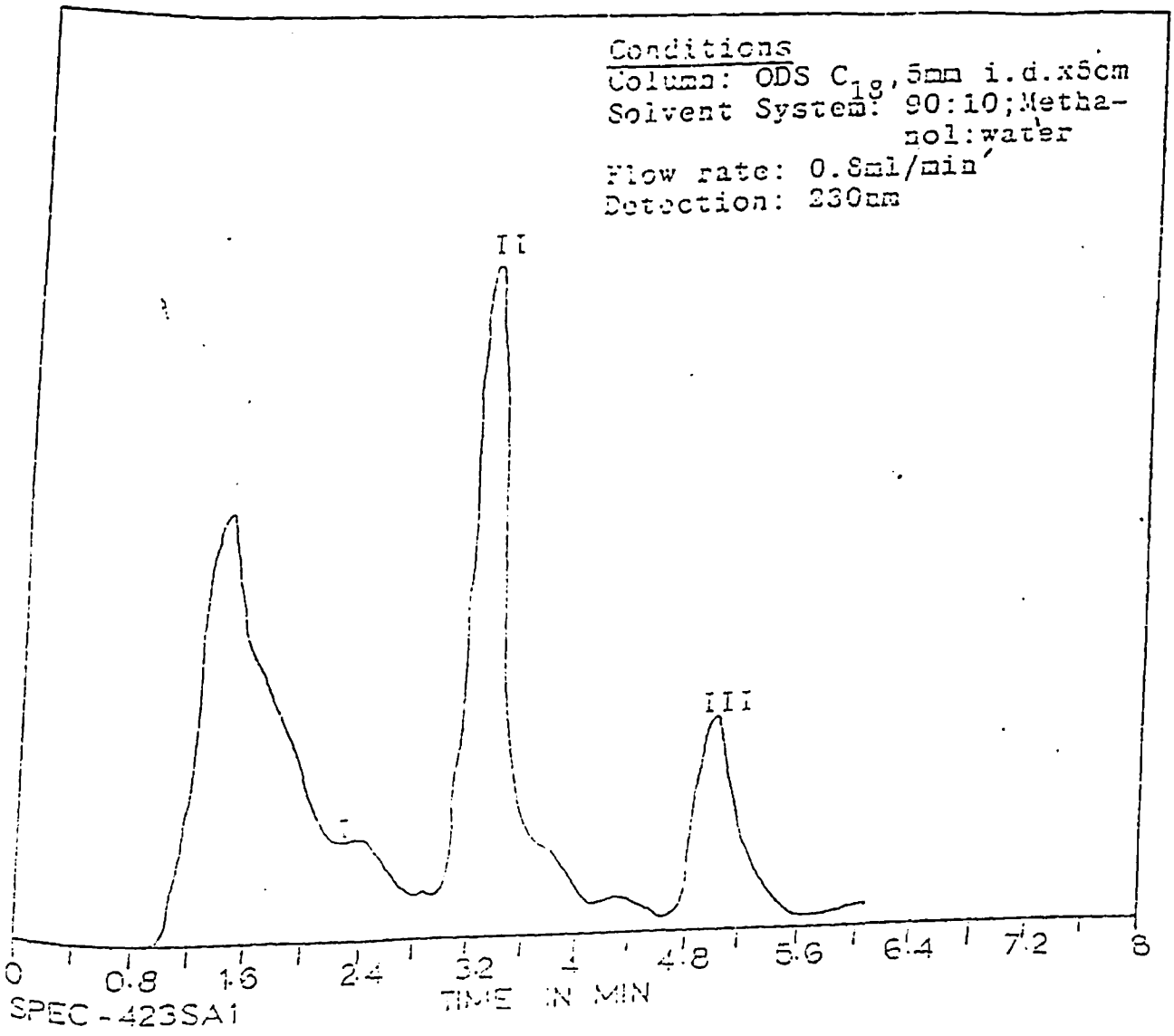
Identification of peaks: I pyrethrosin
II pyrethrins II
III pyrethrins I

Fig. 27 Chromatogram of the extract of Petals of Clone 423



Identification of peaks:
I pyrethrosin
II pyrethrins II
III pyrethrins I

Fig. 28 Chromatogram of the extract of Sepals of Clone 423



Identification of peaks:

I	pyrethrosin
II	pyrethrins II
III	pyrethrins I

The ratio for pyrethrins in the different parts was obtained as follows:-

Part of flower	Mean pyrethrins concentration	Ratio
Ray florets	2.0417	20.9836
Petals	0.0973	1.0
Sepals	0.7479	7.6865

$$\frac{Rayflorets}{Petals} = 20.9836 \approx 21.0$$

$$\frac{Sepals}{Petals} = 7.6865 \approx 8.0$$

$$\frac{Rayflorets}{Sepals} = 2.7299 \approx 3.0$$

To test these ratios, the pyrethrins concentration in the ray florets is divided by 21 and that for sepals by 8 and then the means compared statistically using the f-test. The level of significance taken $\alpha = 0.05$. Analysis of the data thus obtained shows that the variance ratio lies outside the critical region showing that the mean pyrethrins concentration in the different parts is not significantly different hence the ratios for ray florets to petals is 21, that for ray florets to sepals is 3 whereas that for sepals to petals is 8.

Inference

All the samples analysed for both pyrethrins and pyrethrosin have quite low values of standard deviations. Of all the samples analysed for pyrethrosin 83% have coefficient of variation ranging between 0.4% and 13.5%; whereas 92% of the samples analysed for pyrethrins had coefficients of variation ranging between 0.8% and 10.9%. Only one sample had coefficient of variation of 16.5% which is not also very high. This shows that the concentrations of pyrethrosin and pyrethrins do not vary with different seasons.

It has been observed that the pyrethrosin concentration in the three different parts of the flower is not significantly different; whereas pyrethrins content in the petals is insignificant in relation to the amount of pyrethrins in the sepals and ray florets. Consequently, if petals are removed before extraction of the flowers for pyrethrins, there would be a significant reduction in pyrethrosin in the extract without there being a significant reduction in the concentration of pyrethrins in the extract.

It should be noted that the results discussed are valid for the samples collected at random from Pyrethrum Board's Molo Research Station. Further studies need to be conducted to test whether similar results would be obtained on analysis of samples collected from other areas where pyrethrum is grown. I would however, not expect too much variation since for example, concentrations of pyrethrins in clone 4331 has been observed as follows in an experiment to determine how pyrethrins vary with picking intervals.

Picking intervals	Percentage pyrethrins content
14 days	1.50
21 days	1.50
28 days	1.46 (ref. 72)

In my case, pyrethrins content for this clone was found to be 1.61% (see table 3).

3.7.0 Analysis of Oleoresin and Pale extract.

These two samples were supplied by pyrethrum Board of Kenya and did not need to be extracted before analysis like all the other samples used in the study.

The product obtained after 'solvent extraction' of ground dry pyrethrum flowers with n-hexane is oleoresin which is dark and viscous containing about 30% by weight of pyrethrins (46). Pale extract is obtained after its clean up and is what is mainly exported by the Pyrethrum Board of Kenya.

Table 5 shows the results obtained after HPLC analysis of the above two samples whereas figure 29 shows a representation of the results obtained in a bar chart form. Two chromatograms (figure 30 and 31 have also been shown. It ought to be noted that peak I in both figures appears clearer when the scale is unaltered and very clear on the chart paper. However, when the scale is not reduced, peak II and III go off the scale of the paper. Also, it has previously been mentioned that the same scale has been used in all the chromatograms with the

exception of the first chromatogram so that comparisons could be made.

Table 5: Percentage Concentration of Pyrethrins and Pyrethrosin in the Pale Extract and Oleoresin

Sample	Mean Pyrethrosin Concentration % (w/w)	s.d	c.v.	Mean pyrethrins concentration % (w/w)	s.d	c.v.
Pale extract	0.50	±0.053	10.6	18.10	±0.110	0.6
Oleoresin	33.93	±0.973	2.9	28.98	±0.110	0.4

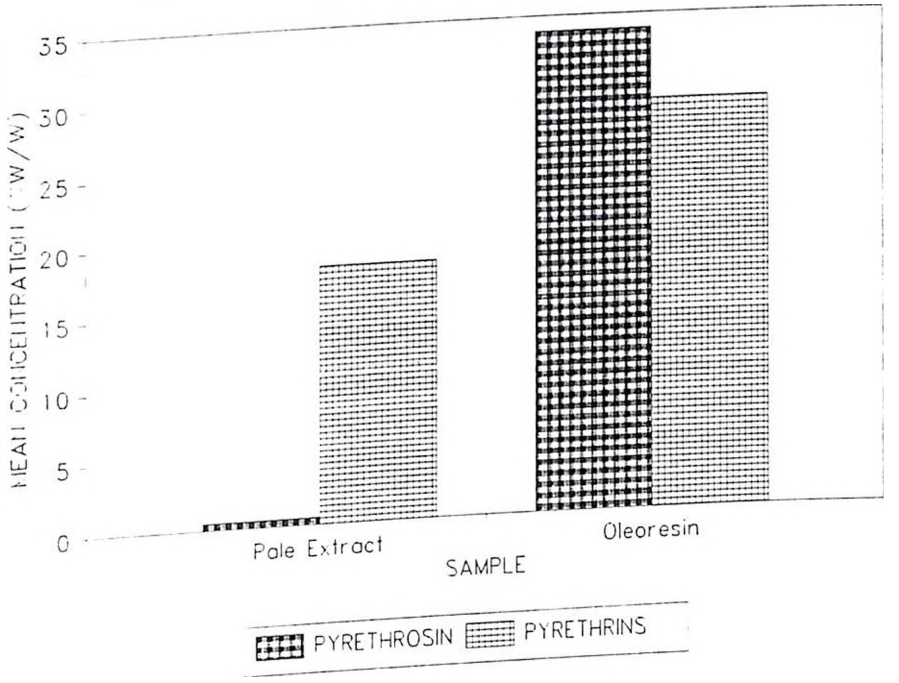
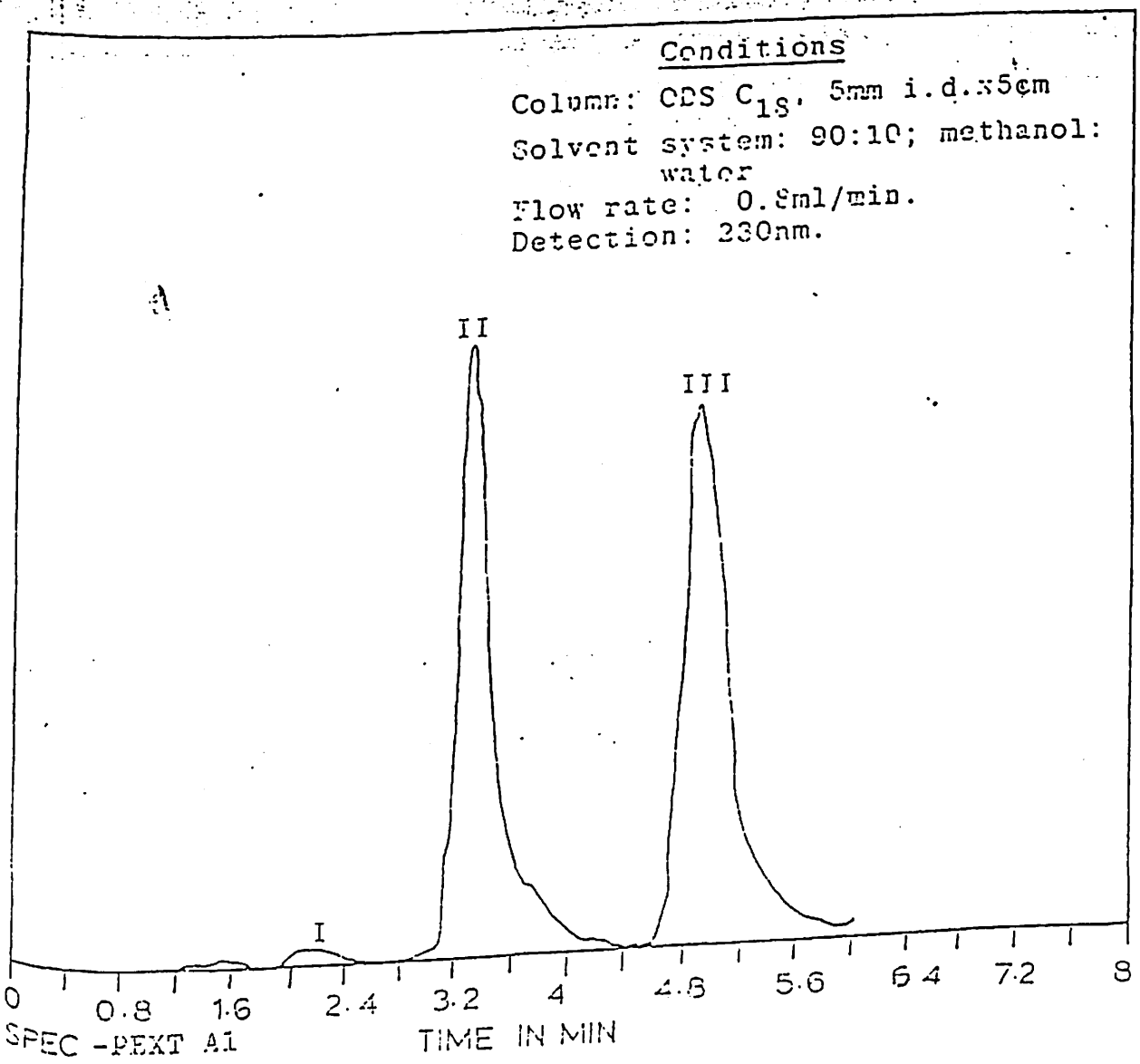


Fig. 29: Bar Chart Showing Percent Concentration of Pyrethrins and Pyrethrosin in Pale Extract and Oleoresin.

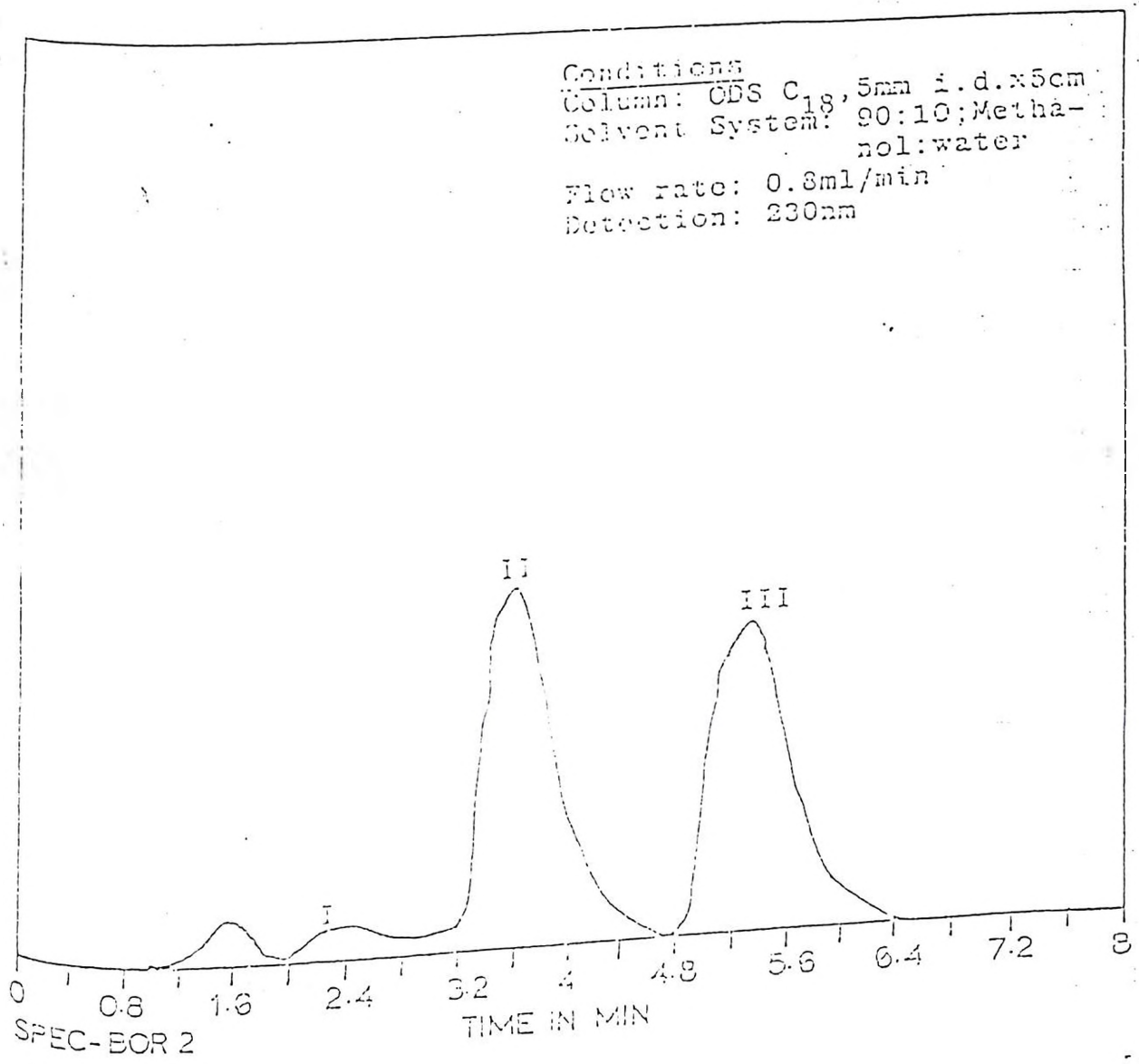
The method of statistical analysis chosen in this case is the student's t-test. The level of significance chosen remains as $\alpha = 0.05$.

Fig. 30 **Chromatogram of Pale Extract (Commercial Pyrethrum extract)**



Identification of peaks:
I pyrethrosin
II pyrethrins II
III pyrethrins I

Fig. 31 Chromatogram of Oleoresin



Identification of peaks: I pyrethrosin
II pyrethrins II
III pyrethrins I

3.7.1 Pyrethrins and Pyrethrosin Content in Oleoresin and Pale Extract.

When the t-test is conducted on the data for pyrethrins obtained for the two samples above, it is observed that 't' lies within the critical region. This means that the pyrethrins content in the pale extract is significantly different from the pyrethrins content in oleoresin. A ratio for this is obtained in the following way:-

$$\frac{\textit{Pyrethrins content in pale extract}}{\textit{Pyrethrins content in oleoresin}} = \frac{18.10}{28.98} = 0.62$$

Thus if the pyrethrins content of oleoresin is multiplied by this factor and then the data for both statistically compared both are found not to differ significantly as the computed value of 't' is found to lie outside the critical region.

As for the pyrethrosin content of both samples, t-test reveals that the two concentrations are significantly different. The ratios for these are obtained as follows:-

$$\frac{\textit{Pyrethrosin content in oleoresin}}{\textit{Pyrethrosin content in pale extract}} = \frac{33.93}{0.5} = 67.86$$

When the pyrethrosin content of pale extract is multiplied by this factor (67.86) and then t-test conducted again, the value of t obtained is found to lie outside the critical region. Hence the pyrethrosin content of oleoresin is 67.86 times more than that for pale extract.

A look at the standard deviations in the table shows good precision and reliability of data obtained.

Inference

It should be noted that though the amount of pyrethrins in the pale extract and oleoresin has been established in the past, the concentrations of pyrethrosin in both substances and indeed in all the samples analysed in this study had not been established prior to this study.

The work has also shown that the clean up procedure adopted by pyrethrum Board of Kenya is quite effective in reducing the amount of pyrethrosin in oleoresin to an insignificant amount when obtaining the pale extract. It should also be noted that further work needs to be done to establish safe levels of pyrethrosin.

3.8.0 Analysis of Mosquito Coils

The aim of this analysis as earlier mentioned was slightly different from that of the other samples taken for the study. The aim was not to compare the amount of pyrethrins/pyrethrosin in the mosquito coils but to establish whether pyrethrosin was present in the mosquito coils. The mosquito coils chosen for the study were Doom mosquito coils and Mos. Kill mosquito coils which were purchased from a leading supermarket chain. The extraction procedure chosen and the method of analysis was similar to that adopted for the pyrethrum dust samples.

Table 6 represents the results obtained after analysis of the two samples and figure 32 is a representation of the results obtained in bar chart form.

Table 6: Percentage Concentration of Pyrethrins and Pyrethrosin in two types of Mosquito Coil Studied

Sample	Mean pyrethrosin concentration % (w/w)	S.D.	c.v.	Mean pyrethrins concentration % (w/w)	S.D.	c.v.
Mos. Kill mosquito coil	0.25	± 0.018	7.2	0.09	± 0.014	15.2
Doom mosquito coil	0.69	± 0.018	2.6	0.97	± 0.167	16.6

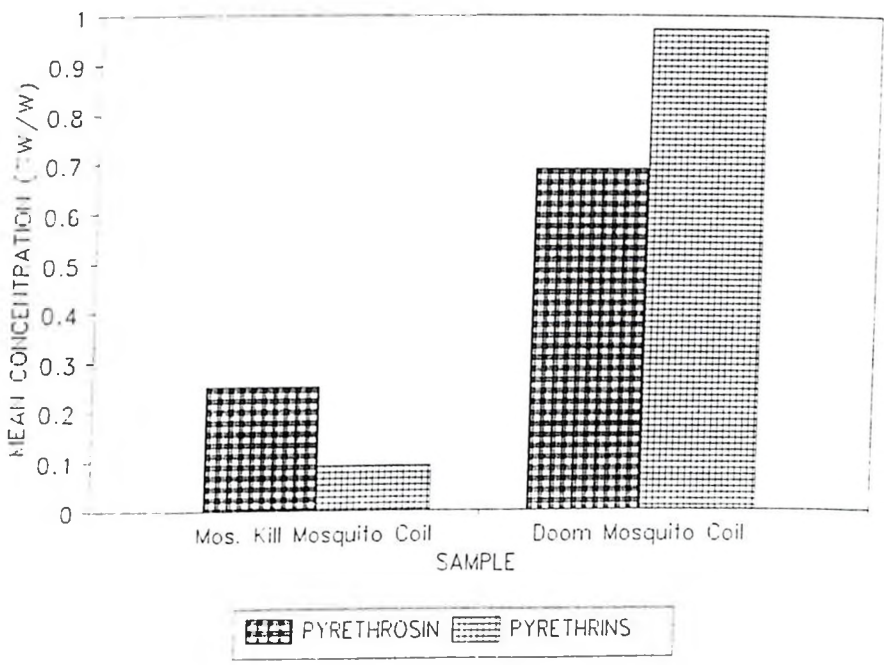


Fig. 32: Bar Chart Showing Percent Concentration of Pyrethrins and Pyrethrosin in two types of Mosquito Coils.

Inference

Both types of mosquito coils have been shown to contain both pyrethrins and pyrethrosin. Since both mosquito coils have been found to contain pyrethrosin. Safe levels of pyrethrosin should be established to know

whether the levels of pyrethrosin in these two types of mosquito coils are high enough to be harmful or not. Studies should, therefore, be conducted to establish the effects of burning a mosquito coil containing pyrethrosin and assess the lethal dose.

The standard deviations and the coefficients of variation are not too high as to dramatically affect the data obtained.

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