THE DETERMINATION OF THE RELATIONSHIP BETWEEN THE PYRETHRINS AND THE YELLOW PIGMENTATION IN PYRETHRUM FLOWERS.

BY

CHARLES ANTHONY ANG'EDU

A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE OF THE UNIVERSITY OF NAIROBI.

JANUARY, 1994

DECLARATION

This is my own original work and has not been presented in any other University.

CHARLES ANTHONY ANG'EDU
CHEMISTRY DEPARTMENT
UNIVERSITY OF NAIROBI
P.O. BOX 30197, NAIROBI.

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

PROF. J.O. MIDIWO
CHEMISTRY DEPARTMENT
UNIVERSITY OF NAIROBI
P.O. BOX 30197, NAIROBI.

DR. W. A. ODINGA
(FORMERLY CHIEF CHEMIST
PYRETHRUM BOARD OF KENYA, NAKURU)
COMMISSION FOR HIGHER EDUCATION
P.O. BOX 44400, NAIROBI.

DEDICATION

To my parents

ACKNOWLEDGEMENT

I express my heartfelt thanks to my supervisors Prof. J.O. Midiwo and Dr. W.A. Odinga for their guidance, encouragement and support throughout this work. I thank the Pyrethrum Board of Kenya, Nakuru for providing the funds and part of the facilities used to carry out this study. I am deeply greatful to the entire staff at the research laboratories of the Board for their kind support to me in one way or the other at all times I was in need while carrying out bits of this study at Nakuru. I also thank the Board's Agronomy department and the Horticultural and Research station, Molo staff for having enabled me to sample the pyrethrum flowers used in this study.

I feel deeply indebted to the 'make shift' Iteso committee led by Mr. William Ikileng' for raising my tuition fees for the master of science degree course. Special thanks to Messrs, Mathew Emukule and Boaz Ikongo who were the guests of honour at the public fundraising that enabled me to continue with this course for their generous donations, advice and encouragement. Indeed I have no enough words to thank everybody who participated in this fundraising for the moral and financial support, big or small. Thanks to all of you.

Thanks to my parents, Mr. Jeremiah Ekisa and Mrs. Blandina Namukuru Ekisa for their patience, support and encouragement during the difficult times. My sincere thanks also go to my brothers and sisters for wishing me well in this undertaking. To my relatives and friends for their contribution in one way or the other, I say I am grateful to all of you. My word of thanks is also due to the Physics department, University of Nairobi for allowing me to use their computers for preparing this thesis. I am grateful to the chairman and the entire staff of the chemistry department University of Nairobi for their support in the completion of my Msc. course work and thesis. Finally I am greatful to my employer Kenya Medical Research Institute (KEMRI) for granting me study leave and for all the support during the difficult time. Indeed to all those not mentioned here who contributed towards the successful completion of this project, I say thank you.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

GLOSSARY

PBK	-	Pyrethrum Board of Kenya
AOAC	-	The official method of analysis of Pyrethrins of the Association of Agricultural Chemists (1984)
Tlc	-	Thin layer chromatography
Ptlc	-	Preparative thin layer chromatography
n-hexane (AF)	-	Aromatic free n-hexane

Hplc	-	High performance liquid chromatography
R_f	-	Relative to solvent front value
$A^{1\%}_{1cm}$	-	Specific absorbance
${}^{1,2,3}A^{1\%}_{1cm}$	-	Specific absorbance in various electromagnetic regions : 1 - (265-300nm), 2 - (300-400nm) and 3 - (400 - 470nm)
BHT	-	Butylated hydroxytoluene
PBO	-	Piperonylbutoxide
P1	-	pyrethrin 1
P2	-	pyrethrin 2
C1	-	cinerin 1
C2	-	cinerin 2
J1	-	jasmolin 1
J2	-	jasmolin 2
PI	-	Pyrethrins I
PII	-	Pyrethrins II
PI/PII	-	PyrethrinsI / Pyrethrins II ratio.
PHP	-	Potassium hydrogen thallate
THF	-	Tetrahydrofuran
WSPE	-	World standard pyrethrum extract
M	-	Linear correlation slope
C	-	Linear correlation constant

ABSTRACT

Pyrethrum (*Chrysanthemum cinerariaefolium* Vis) is the source of the colourless six closely related insecticidal ester compounds collectively referred to as "Pyrethrins". The pyrethrum flower is yellow at its central part and consequently the pyrethrum extract is yellow in colour. The intensity of the yellowness in extracts, mainly attributed to the presence of the carotenoidal and flavonoidal pigments found in pyrethrum flowers has been visually observed to relate closely to the Pyrethrins content but no analytical verification of this observation has been done before.

Twenty different clones and varieties of pyrethrum flowers were sampled from the National Pyrethrum and Horticulture Research Centre, Molo and used in this investigation. The Pyrethrins content for each sample was determined using spectrophotometric, Association of Official Analytical Chemists (AOAC) 8th edition and High Performance Liquid Chromatography (HPLC) analytical techniques. A 250 - 700nm scan of n-hexane extracts showed various absorption peaks in the UV and visible regions at which 1% (w/v) n-hexane (aromatic free) extract absorbances were read using a double beam spectrophotometer.

Statistical correlations between the total Pyrethrins, Pyrethrins I, Pyrethrins II, individual ester concentrations, and Pyrethrins I / Pyrethrins II ratios with Specific absorbances ($A^{1\%}_{1cm}$) calculated from mean absorbances in different electromagnetic regions : 265 - 300nm (${}^{1}A^{1\%}_{1cm}$) ,

300-400nm ($^{2}A_{1cm}^{1\%}$) and 400 - 470nm ($^{3}A_{1cm}^{1\%}$) were done using a computer.

The results depict a negative linear correlation between the Pyrethrins and specific absorbances in all the three cited electromagnetic regions. The individual esters also show a significant inverse correlation with the specific absorbances. PI / PII ratios showed no significant correlation with the specific absorbances. From the correlation coefficients, the pigments absorbing at 265 - 300nm show a better correlation with the Pyrethrins than those absorbing at 400 - 470nm. The pigments at the former region were identified as flavonoids and the latter as carotenoids from spectrophotometric scans and tlc screening of column chromatographic fractions.

The yellow colour in the commercial refined pyrethrum extract is undesirable and it is possible to develop the correlation observed here into a quality control parameter and also as an important factor in future pyrethrum crop improvement programs to achieve desired flowers of low yellow pigmentation but high Pyrethrins content either genetically engineered or agronomically cloned.

CHAPTER 1

1.0 INTRODUCTION

The pyrethrum plant, *Chrysanthemum cinerariaefolium* (vis), a member of the Compositae family in the tribe Anthemidae has been extensively studied in respect to its commercial importance as a source of insecticidal components collectively known as Pyrethrins (Casida,1973). Other plants in the Compositae family have also been screened for insecticidal activity but no results of commercial significance have been obtained (Gladinger, 1936 and 1945; Pushpa *et al*, 1979).

1.1 History of Pyrethrum

Commercial use of pyrethrum flowers is thought to have probably originated from Persia. A story holds that a German woman in Dalmatia (present Yugoslavia) picked the pyrethrum flowers for their beauty. After several weeks, the withered flowers she threw to a corner of her house were found surrounded by dead insects. This observation led her to associate the death of the insects with insecticidal property of the plant (Casida, 1973).

The pyrethrum seed was first introduced in Kenya in 1928 from Yugoslavia (formerly, Persia) by Captain Gilbert Walker who established a commercial farm around Nakuru. In 1929 a Mr. Anderson who was the senior entomologist at the then Scott Agricultural Laboratories (now National Agricultural Laboratories) obtained some *Chrysanthemum cinerariaefolium* seeds from the Ministry of agriculture, Harpenden, England (Leppelley,

1973). The crop became popular among the Kenyan farmers in the highland areas to an extent that by 1945, Kenya was and still is the leading world producer of pyrethrum.

The realization, commercial utilization and initial research into pyrethrum dates back to 1915 (Staudinger *et al*, 1924). To date pyrethrum is regarded as a modern insecticide whose composition, methods of analysis, biochemistry and toxicology are well documented (Head, 1973; Whitehead, 1981; Elliot and Janes, 1973; Brown *et al*, 1956; Otieno, 1983; Rose and Haller, 1937; Glennie and Harbone, 1972). The application of the pyrethrum plant extends beyond the insecticidal property. *Chrysanthemum cinerariaefolium* Vis (Compositae / Asteraceae) in addition to being of high economic importance and export potential, these plants have also been cited to be medicinal (Sofowora, 1984; Pyrethrum bureau, 1987). The pyrethrum marc which is the grist residue after extraction of Pyrethrins is formulated into animal feeds as a de-wormer. Success in research efforts above have contributed to a great deal of the advancement in the pyrethrum industry. To date pyrethrum flowers are grown in many diverse locations especially in East Africa.

1.2 Pyrethrum Production

Kenya is the world's leading producer of pyrethrum (fig. 1b). On average Kenya has been producing well over 60% of the total world production of dry flowers. The total annual world production of dry pyrethrum flowers ranges from 12,000 to 23,000 tones (Casida, 1973; Pandita, 1990; KNA, 1993).

A survey of pyrethrum production in Kenya over the years

(Table 1) shows a great fluctuation but there is a steady rise from 1989 to date. The projection of 14,000 tones for 1992/93 and 16,500 tones for 1993/94 is encouraging (KNA, 1993).

Table 1 : Recent and Future Pyrethrum Production Projection in Kenya

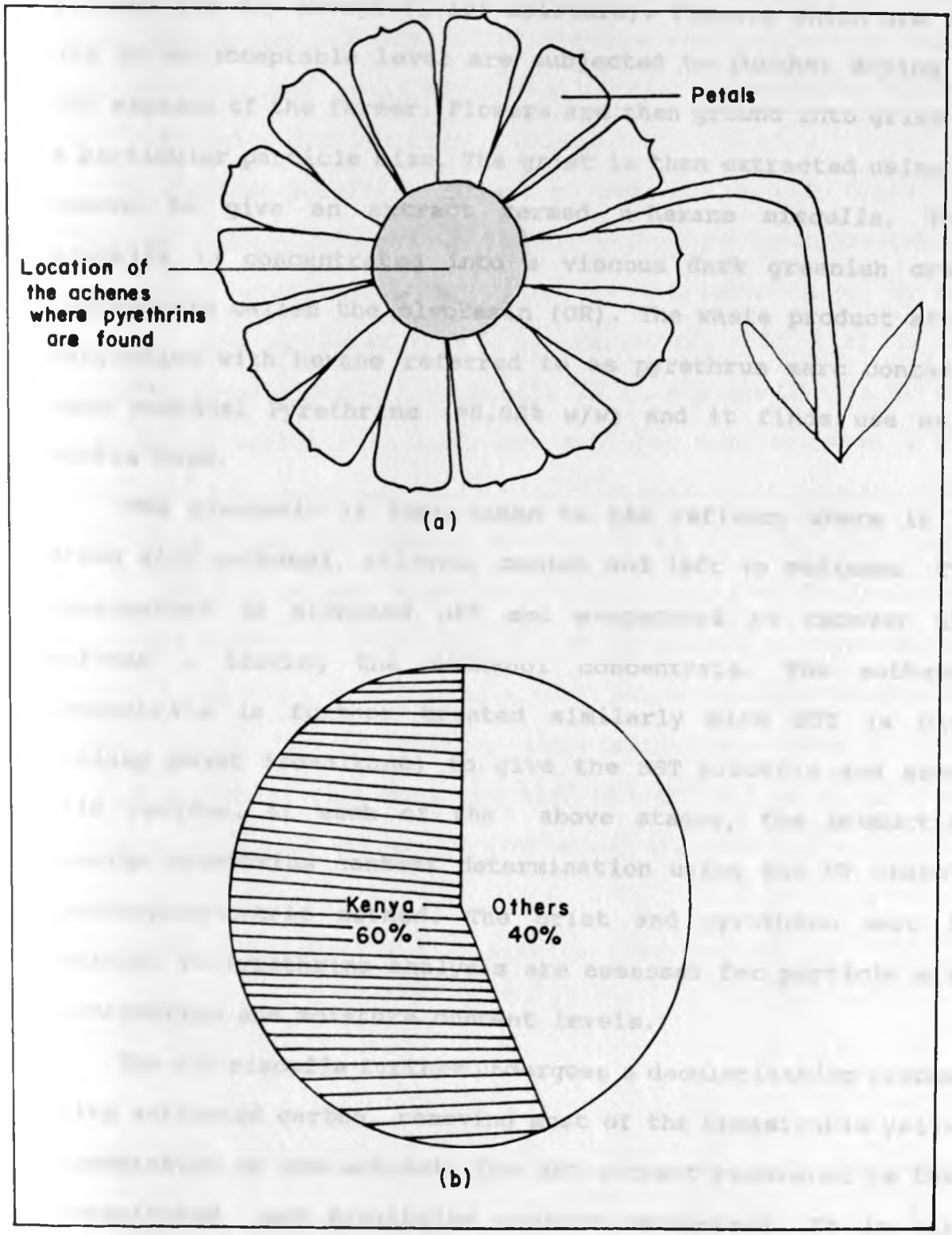
YEAR	PRODUCTION (TONES)	YEAR	PRODUCTION (TONES)
1967/68	11,000	1989/90*	9,000
1968/69	7,000	1990/91*	12,000
1969/70	6,000	1992/93*	14,000 (Projection)
1970/71	10,000	1993/94*	16,500 (Projection)
1971/72	14,000		

* source: (KNA, 1993)

East African countries namely Kenya, Tanzania and Rwanda are the major commercial producers of pyrethrum worldwide. A significant amount of pyrethrum is also grown in Papua New Guinea and Australia (Tasmania). The United States is the major consumer of pyrethrum. In India, Ecuador, Brazil, China and Indonesia pyrethrum is commercially produced mainly for local consumption.

FIG. 1 : (a) A diagram of a mature pyrethrum flower

(b) Pyrethrum production worldwide (1990/91)



1.3 Pyrethrum Processing

At the pyrethrum processing factory flowers are received from farmers and for each delivery it is ascertained that the flowers are dry enough (≤ 10% moisture). Flowers which are not dry to an acceptable level are subjected to further drying at the expense of the farmer. Flowers are then ground into grist of a particular particle size. The grist is then extracted using n-hexane to give an extract termed n-hexane *miscella*. This *miscella* is concentrated into a viscous dark greenish crude concentrate called the oleoresin (OR). The waste product after extraction with hexane referred to as pyrethrum marc contains some residual Pyrethrins (≈0.08% w/w) and it finds use as a cattle feed.

The oleoresin is then taken to the refinery where it is mixed with methanol, stirred, cooled and left to sediment. The supernatant is siphoned off and evaporated to recover the solvent , leaving the methanol concentrate. The methanol concentrate is further treated similarly with SST (a high boiling point iso-alkane) to give the SST *miscella* and green oils residue. At each of the above stages, the product(s) undergo Pyrethrins content determination using the UV visible spectrophotometric method. The grist and pyrethrum marc in addition to Pyrethrins analysis are assessed for particle size distribution and moisture content levels.

The SST *miscella* further undergoes a decolorization process using activated carbon, removing most of the undesirable yellow pigmentation in the extract. The SST extract recovered is then concentrated and Pyrethrins content determined. It is also important to determine the turbidity level of this extract after

which the concentration of this refined pyrethrum extract is standardized to 25% or 50% Pyrethrins using another iso-alkane solvent. The Pyrethrins content determination of the refined extract is done both by the spectrophotometric and AOAC methods. Other quality control determinations are carried out on this final product, for example, specific color absorbance, flash point, viscosity and specific gravity.

The refined pyrethrum extract of commerce usually contains butylated hydroxy toluene (BHT) and piperonyl butoxide (PBO) among other expicients. BHT (**20**) is an antioxidant which prevents loss of Pyrethrins by oxidation. PBO (**19**) is a synergist. Synergists are chemicals which possess little or no Insecticidal activity of their own but when used together with the Pyrethrins, increase their effectiveness by augmenting the insecticidal activity (Shashida *et al*, 1983; Sawicki 1962a and 1962b). Other prominent examples of synergists are tropital and n-octylsulfoxide. Various mechanisms of action of synergists have been put forward including alteration of the cell membranes of the target insects thereby increasing the uptake of the Pyrethrins. These compounds are also thought to play a role in the inactivation of enzymes in the target organism (Izuru, 1973; Price, 1960)

1.4 Pyrethrum flowers and Pyrethrins distribution

1.4.1 The Pyrethrum flower

The flower is daisy like in appearance and is the harvested part of the pyrethrum plant. The flower head drawn above (fig. 1a) is typical of the Compositae family. The flower head is made

up of many small florets sitting on the receptacle. The central part of the inflorescence is yellow whereas the outer (petals) part is white in colour.

Pyrethrum farmers in Kenya, at various localities, grow different clones and varieties of pyrethrum flowers. A few of these are shown in table 2 (Wanjala, 1990; Ottaro, 1978; Tuikong, 1979; Casida, 1973).

Table 2 : Some of the Clones and varieties of *Chrysanthemum cinerariaefolium* flowers grown in Kenya.

LOCAL CLONE	CLONE	VARIETY
Bal 1 Congo C47 Ebiosi Marwanga k7 Nyamasibi Kenya Gekoma Nyankoba	Ma/62/57, 2146, 1408, 2525, Kr/74/122, Ma/62/456, 1708, Mo/70/1124, 194, Sb/66/107[2], Ma/62/246, Ma/62/241, 4331*, Mo/75/4, Ma/62/215, Mo/71/423, L/75/477, Ks/75/336, Ma/62/428, Ma/62/238, Mo/74/473, Ks/71/6, Ks/70/64, Mo/70/223, 1058, 4729, L/75/487, 214 3093, 4098, 4026, 1105, 3863, 1708, 3800, 194	P4[1] K235

Most of the local clones indicated in this table are from Kisii

* Referred to as " standard clone " (Wanjala, 1990)

1 Ottaro (1978) reported of high incidence of naturally occurring triploids in variety P4 in Kenya. Triploids are indicated to be high yielding clones.

2 Is an example of the best recommended triploid clones (Tuikong, 1979)

Clones are plants obtained from continuous vegetative propagation of a single plant of good agronomic traits, for

example, good flower yields, normal flower height, proper lodging and disease resistance. Varieties are obtained from seeds. Subsequent vegetative propagation of seedlings or varieties is restricted because of observed inverse correlation of Pyrethrins and vigour. This may be one of the reasons evident in table 2 above why the Pyrethrum Board of Kenya regards clone production in nurseries preferentially and variety (seed) production only complimentarily.

It is thought that the great variety of pyrethrum clones and different growing conditions contribute to variations in composition of both the Pyrethrins and non-insecticidal components (Parlevliet, 1975). However, within one growing region, there is always some conformity in the composition of the six insecticidal ester homologues.

1.4.2 Pyrethrins distribution in the pyrethrum plant.

The distribution of the Pyrethrins in the pyrethrum plant has been studied (Gladinger, 1936; Head, 1966b). Pyrethrins are found in all parts of the pyrethrum plant but the greatest concentration is in the flower head. It has been found out that over 90% of the Pyrethrins in the flower head are located in the achenes located in the receptacle of the flower head (Chandler, 1951). Head (1936a) indicated that Pyrethrins content in the flower head varies with the maturity of the flower. The concentration of the Pyrethrins is optimal at the stage when the petals are horizontal as shown in fig.1a and it is at this stage when harvesting is recommended (Ikahu and Ngugi, 1990). At harvest, the flowers contain about 80% water and < 10% moisture when properly dried. Drying is usually done in the shade, sun or

by use of mechanical driers. For optimal Pyrethrins content,sun drying at below 40°c is highly recommended (Ngugi and Ikahu, 1991; Munro, 1961; Githinji, 1973). For the samples used in this project, the moisture levels in the fresh and dry flowers compared quite well with the literature figures (Table 3 and 4).

Table 3 : Pyrethrum clone and variety samples from Molo

SAMPLE	FRESH WEIGHT (g)	DRY WEIGHT (g)	MOISTURE LOSS (g)	(%) MOISTURE LOSS	WEIGHT AFTER GRINDING (g)	WEIGHT LOSS AFTER GRINDING (g)	(%) GRIST RECOVERY
C47	912.70	149.60	763.70	83.7	145.00	4.60	96.9
MARWANGA	575.50	95.80	479.70	83.4	88.50	7.30	92.4
NYAMASIBI	596.30	94.40	501.90	84.2	90.60	3.80	96.0
P4	861.10	152.40	708.70	82.3	148.30	4.50	97.3
4331	666.50	103.80	562.70	84.4	99.00	4.80	95.4
Mo/71/423	519.40	82.40	437.00	84.1	79.50	2.90	96.5
Ks/75/316	799.50	119.40	680.10	85.1	114.40	5.00	95.8
Ks/75/313	790.80	144.30	646.50	81.8	139.90	4.40	96.9
K235	636.90	110.00	526.90	82.7	106.40	3.60	96.7
Kr/74/122	897.00	168.80	728.20	81.2	163.20	5.60	96.7
Mo/70/1124	650.30	108.50	541.80	83.3	104.30	4.20	96.1
Sb/66/107	814.10	151.50	662.60	81.4	144.00	7.50	95.0
Ma/70/1013	545.70	107.40	438.30	80.3	103.90	3.50	96.7
Ma/75/4	576.90	89.70	487.20	84.5	87.70	2.00	97.8
L/75/477	777.10	145.10	632.00	81.3	137.00	8.10	94.4
Mo/74/443	617.70	120.30	487.40	80.5	117.90	2.40	98.0
Ks/71/6	308.90	61.80	247.10	80.0	58.40	3.40	94.5
Ks/70/64	598.00	113.50	484.50	81.0	108.50	5.00	95.6
L/75/487	612.40	108.10	504.30	82.3	103.50	4.60	95.7
Mo/70/223	552.50	98.30	454.20	82.2	93.70	4.60	95.3

Table 4 : Water content levels of the dry research samples as determined using Dean and Stark method

SAMPLE	C47	MARWANGA	NYAMASIBI	P4
WATER CONTENT %(v/w)	8.6	5.9	6.8	5.1
SAMPLE	4331	Mo/75/423	Ks/75/336	Ks/75/313
WATER CONTENT % (v/w)	6.9	5.6	6.3	5.6
SAMPLE	K235	Kr/74/122	Mo/70/1124	Sb/66/107
WATER CONTENT %(v/w)	6.6	7.6	7.3	4.8
SAMPLE	Ma/70/1031	Ma/75/4	L/75/477	Mo/74/443
WATER CONTENT %(v/w)	4.6	8.1	6.1	5.2
SAMPLE	Ks/71/6	Ks/70/64	L/75/487	Mo/70/223
WATER CONTENT % (v/w)	8.6	7.0	5.9	5.3

Mean water content = 6.4%

1.5 The Pyrethrins

The widely accepted nomenclature of Pyrethrins adopted in this thesis is as follows: The capital "P" and Roman numerals either "I" or "II" and plural denotation describes the groups Pyrethrins I (PI) and Pyrethrins II (PII) while small "p" and arabic numerals "1" or "2" with singular denotation describes individual esters as jasmolin 1 (J1), cinerin 1 (C1), pyrethrin 1 (P1) and jasmolin 2 (J2), cinerin 2 (C2) and pyrethrin 2 (P2). The abbreviations of the two groups and the individual esters are shown in bracket. Reference to the esters collectively bears capital "P" and plural denotation, "Pyrethrins".

1.5.1 The Chemistry of Pyrethrins

The Pyrethrins (fig. 2) are six closely related esters derived from two acids and three alcohols. The two acid moieties are *chrysanthemic acid (1) and pyrethric acid (2). The alcohol moieties are *jasmolone (3), cinerolone (4) and pyrethrolone (5). The chrysanthemates *jasmolin 1 (3a), cinerin 1 (4a) and pyrethrin 1 (5a) make up Pyrethrins I. The esters of pyrethric acid,*jasmolin 2 (3b), cinerin 2 (4b) and pyrethrin 2 (5b) are referred to as Pyrethrins II. The Pyrethrins have been isolated and fully characterised by various scientists into the above categories and chemical structures (Elliot and Janes, 1973; Pattenden, 1970 ; Bramwel *et al*, 1969).

* *Refer to appendix III for chemical nomenclature.*

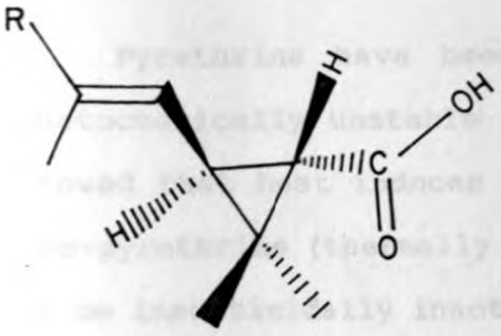
1.5.2 Desirable properties of Natural Pyrethrins

It suffices at this point to cite the desired properties of the pyrethrum extract which contains the Pyrethrins. These properties make pyrethrum a superior insecticide to other insecticides like the synthetic pyrethroids. Pyrethroids are synthetic Pyrethrins, for example, allethrin, tetramethrin and dimethrin. Pyrethrum possesses a strong "kill" and "knockdown" effects to a wide range of pests (Pyrethrum Bureau, 1987). The minimum dose required to 'knockdown' a housefly and a mosquito are 3.32×10^{-9}g and 1.4×10^{-12}g per Kg body weight respectively. It also exhibits a repellency and flushing power that makes insects to quickly get out of their hiding places (Weaving and Sylvester, 1966). Most of all, pyrethrum is biodegradable in the environment and in mammalian tissues. This makes pyrethrum non-persistent in the environment and is also fairly safe to

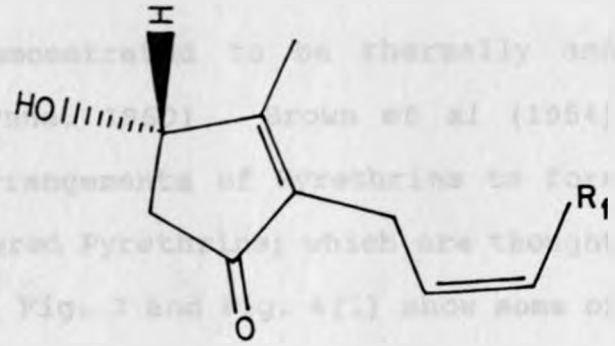
mammals (Griffin, 1973). It has been demonstrated that the Pyrethrins being esters, are rapidly hydrolysed into harmless products in the gut of animals (mammals in particular) which are subsequently excreted (Ware *et al*, 1983). The test of time has also confirmed that pyrethrum is relatively free from potentiality to cause the development of resistance or immunity to many insects unlike the synthetic insecticides. This phenomenon is also explained by its non-persistence in the environment.

The commercial survival of the pyrethrum industry is attributed to the natural Pyrethrins' low mammalian toxicity, environmental safety and rapid paralysis of a wide range of flying insects. The pyrethrum extract also exhibits a repellency effect which has not been clearly shown to be caused by the Pyrethrins (Casida, 1973). The competition from the pyrethroids and biotechnological substitutes may be inferior owing to these advantages. All the above and other desirable aspects of Pyrethrins have led to the extensively documented use of these compounds in various formulations for household pest control and storage of farm grains (Cottered, 1950; Desmarchelier *et al*, 1979; Greening, 1979). Chandler (1950) and Dobson (1958) have also documented on the use of Pyrethrins in formulations for public health and related fields without any reported serious health hazards. The advancement of agricultural production of pyrethrum is attributed to the successful application of pyrethrum extract in field and horticultural crops insect pest control (Glynne, 1956; Hagley, 1959, Whitehead and Bowers, 1981;). The next section explains the photochemical properties of Pyrethrins.

FIG. 2: CHEMICAL STRUCTURES OF THE PYRETHRINS

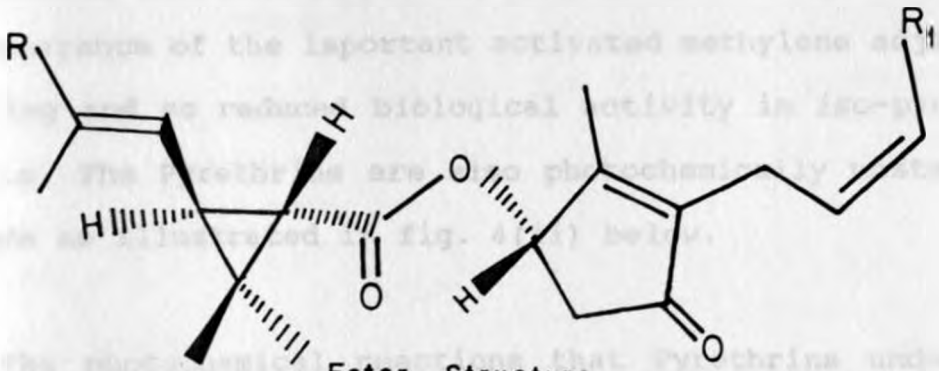


Acid moiety structure



Alcohol moiety structure

Acids				Alcohols		
	R	Acid			R_1	Alcohol
1	CH_3	Chrysanthemic		3	$-CH_2CH_3$	Jasmolone
2	CO_2CH_3	Pyrethric		4	$-CH_3$	Cinerolone
				5	$-CH=CH_2$	Pyrethrolone



Ester Structure

	R	R_1	Ester	Group
3a	$-CH_3$	$-CH_2CH_3$	Jasmolin 1 (J1)	**Chrysanthemates** (Pyrethrins I (PI))
4a	$-CH_3$	$-CH_3$	Cinerin 1 (C1)	
5a	$-CH_3$	$-CH=CH_2$	Pyrethrin 1 (P1)	
3b	$-CO_2CH_3$	$-CH_2CH_3$	Jasmolin 2 (J2)	**Pyrethrates** (Pyrethrins II (PII))
4b	$-CO_2CH_3$	$-CH_3$	Cinerin 2 (C2)	
5b	$-CO_2CH_3$	$-CH=CH_2$	Pyrethrin 2 (P2)	

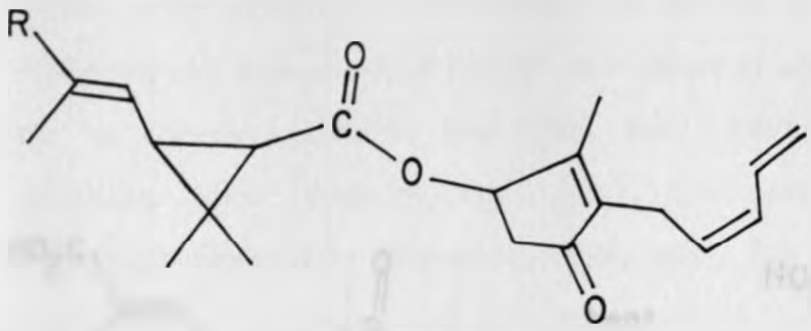
1.5.3 Thermo and Photochemistry of Pyrethrins

Pyrethrins have been demonstrated to be thermally and photochemically unstable (Glynne, 1960). Brown *et al* (1954) showed that heat induces rearrangements of Pyrethrins to form iso-pyrethrins (thermally altered Pyrethrins) which are thought to be insecticidally inactive. Fig. 3 and Fig. 4(i) show some of the products of thermal transformations of the natural Pyrethrin esters (Crombie *et al*, 1971b) and the dicarboxylic acid moiety into *iso*-pyrethrins and acidic products respectively (Otieno and Pattenden, 1979).

It is possible that the resonance conjugation of the unsaturated side chain with the cyclopropane ring involves the disappearance of the important activated methylene adjacent to the ring and so reduced biological activity in *iso*-pyrethrins results. The Pyrethrins are also photochemically unstable and degrade as illustrated in fig. 4(ii) below.

The photochemical reactions that Pyrethrins undergo are mainly photochemical isomerization (Z → E) changes (Fig. 4(ii)). It is indicated that the stereochemical change of chrysanthemum dicarboxylic acid mainly occurs in the cinerins and jasmolins (Kawano *et al*, 1980; Holmstead and Soderland, 1978). Knowing these properties of Pyrethrins, it is then befitting to look at the analytical techniques used to quantify these esters.

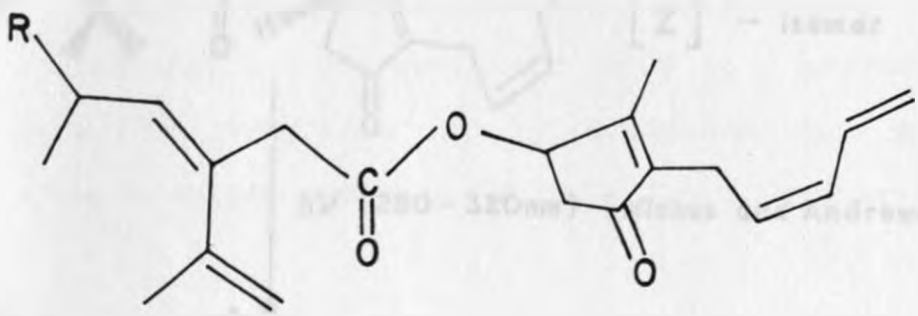
Fig 3 : Some thermal reactions of the natural Pyrethrins



pyrethrin 1 and 2

C_1 to C_2 Bond cleavage with proton transfer

260°C



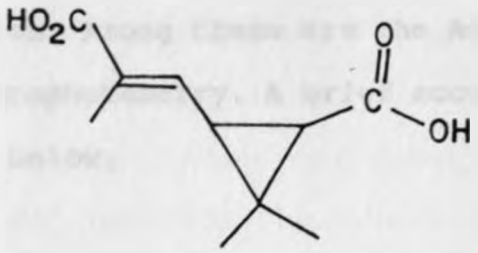
6a Iso - pyrethrin 1 ($R - CH_3$)

6b Iso - pyrethrin 2 ($R - CO_2CH_3$)

Fig.4: (i) Pyrolytic degradation of chrysanthemum dicarboxylic acid.

(ii) Photochemical Z → E transformation of pyrethrin 1 and 2

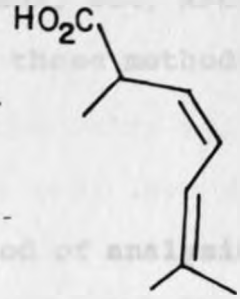
(i)



Chrysanthemum dicarboxylic acid

heat

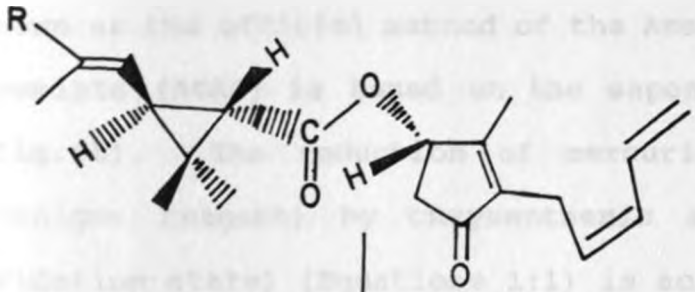
260°C



Acidic produ

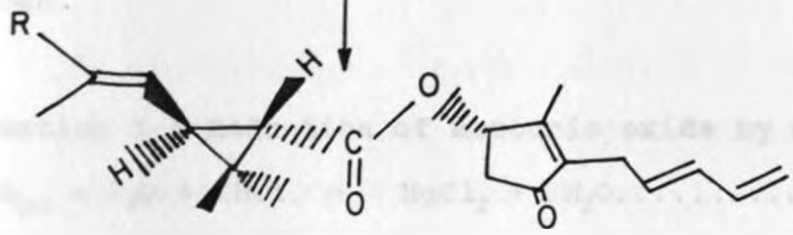
(Major produ

(ii)



[Z] – isomer

hν (290 - 320nm) (Miskus and Andrews, 1972)



[E] – isomer

1.5.4 Analysis of Pyrethrins

There are several methods used for the analysis of Pyrethrins that have been developed over the years arising from the understanding of the chemistry of the Pyrethrins outlined above. Each of these methods has its own limitations and drawbacks ranging from sensitivity, cost, to convenience of application. Among these are the AOAC, PBK, DNP, EDA, GLC, HPLC and Spectrophotometry. A brief account of each of these methods is given below.

1.5.4.1 Mercury reduction method : Official method of analysis of the Association of Agricultural chemists (AOAC)

The most widely used mercury reduction method (appendix I) known as the official method of the Association of Agricultural Chemists (AOAC) is based on the saponification of the esters (fig.5b). The reduction of mercuric sulphate or chloride (Deniges reagent) by chrysanthemic acid to mercurous (+1 oxidation state) (Equations 1:1) is accompanied by a series of characteristic colour changes: Pink → Purple → Blue → Bluish green.

Equation 1 : Reduction of mercuric oxide by chrysanthemic acid

$HgO_{(s)} + H_2O + 2HCl \rightarrow HgCl_2 + 2H_2O$.....(1:1)

$2HgCl_2$ + Chrysanthemic acid $\rightarrow 2HgCl_{(s)} + 2HCl$ .....(1:2)

$2HgCl_{(s)} + 2ICl \rightarrow 2HgCl_{2(aq)} + I_2$ .....(1:3)

$2I_{2(s)} + KIO_{3(aq)} + 6HCl \rightarrow 5ICl + KCl + 3H_2O$ .....(1:4)

Based on the above reactions, Pyrethrins I are estimated by a titrimetric method using iodine monochloride as the indicator base. Similarly Pyrethrins II are determined using the normal acid-base titration using standardized sodium hydroxide as the base. Detailed steps involved in this methodology are given in chapter 2.

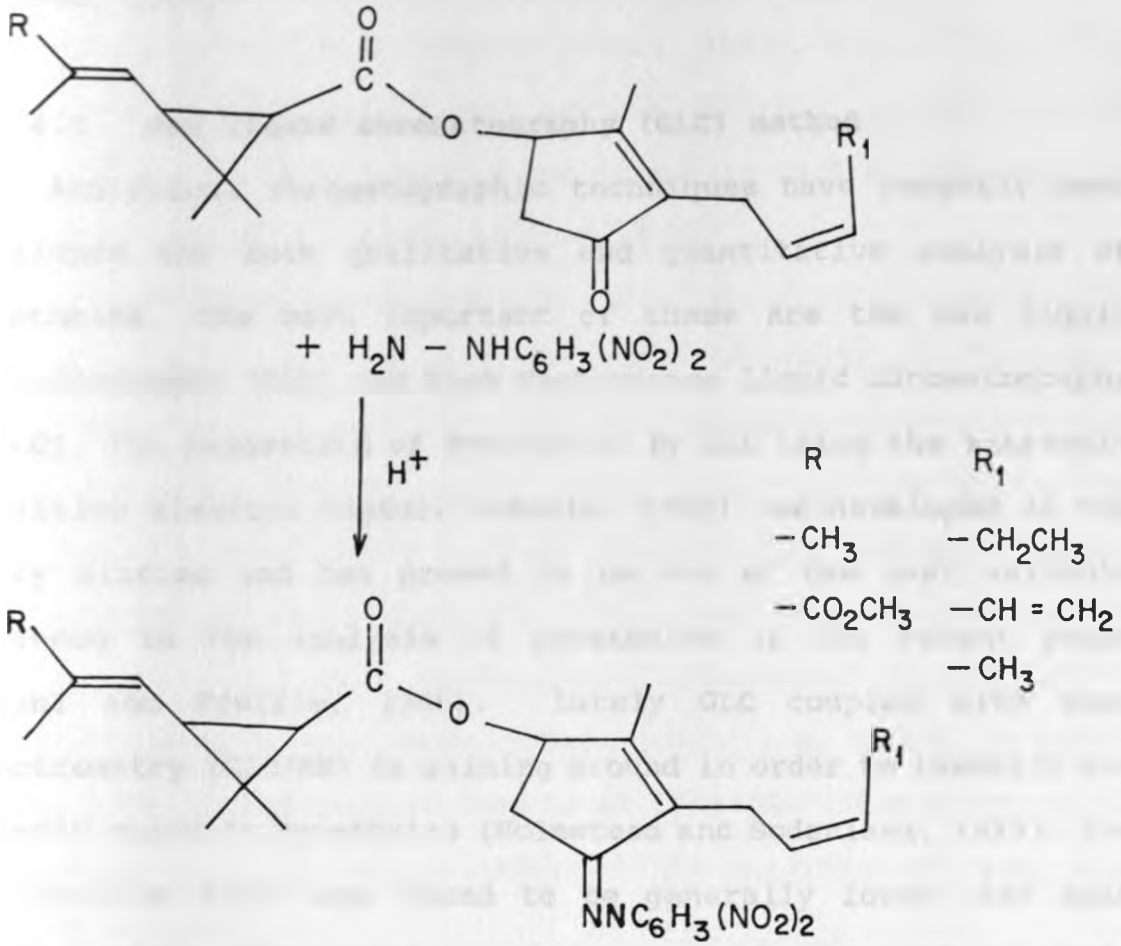
1.5.4.2 **Pyrethrum Board of Kenya (PBK) method.**

The AOAC procedure using sulphuric acid at the fatty acid removal step (section 2.4.5) was shown to exhibit some loss of Chrysanthemic acid during filtration of barium sulphate (Mitchell, 1953). The pyrethrum Board of Kenya (PBK) method (Appendix II), a modification of the conventional AOAC procedure using hydrochloric acid, was developed to accurately estimate Pyrethrins I. PBK results are usually higher (≈ 10%) than the AOAC results. Both AOAC and PBK are relatively expensive and tedious procedures but are so far the only commercialy accepted methods for analysis of Pyrethrins.

1.5.4.3 **2,4-Dinitrophenyl hydrazine (DNP) method.**

The other methods in the analysis of Pyrethrins that are not so prominent currently are the 2,4 - dinitrophenyl hydrazine (DNP) method (Smith, 1959) and the ethylenediamine (EDA) method (Hogsett *et al*, 1953). The former is based on the spectrophotometric estimation of 2,4 - dinitrophenylhydrazones of the Pyrethrins (Equation 2).

Equation 2: Formation of 2,4- dinitrophenylhydrazones of the Pyrethrins



DNP gives lower results than AOAC and it is also capable of giving quantities of individual esters like the HPLC technique only that it is a lot more tedious than the latter.

1.5.4.4 **Ethylenediamine (EDA) method.**

The EDA method employs the reaction under reflux of ethylenediamine with the Pyrethrins liberating pyrethric and chrysanthemic acids. The two acids are then determined by non-aqueous titration with sodium methylate. This method gives very reproducible results in a short time. Initially this method gave only total Pyrethrins content but refinements to it have enabled

the resolution of both Pyrethrins I and Pyrethrins II. EDA like DNP method gives results which are about 10% lower than AOAC (Casida, 1973).

1.5.4.5 **Gas liquid chromatography (GLC) method.**

Analytical chromatographic techniques have recently been developed for both qualitative and quantitative analysis of Pyrethrins. The most important of these are the Gas Liquid Chromatography (GLC) and High Performance Liquid Chromatography (HPLC). The separation of Pyrethrins by GLC using the extremely sensitive electron capture detector (ECD) was developed in the early sixties and has proved to be one of the most valuable advances in the analysis of Pyrethrins in the recent years (Stahl and Pfeifle, 1966). Lately GLC coupled with mass spectrometry (GLC/MS) is gaining ground in order to identify and broadly quantify Pyrethrins (Holmstead and Soderland, 1978). The GLC results have been found to be generally lower that AOAC results may be due to the possible degradation of pyrethrin 2 in the GLC columns. Pyrethrins are usually analyzed using an electron capture detector (ECD), while flame ionization detector (FID) is used for the non-Pyrethrins constituents of pyrethrum extract.

1.5.4.6 **High performance liquid chromatography (HPLC) method.**

High performance liquid chromatography (HPLC) is one of the liquid chromatographic separation techniques which is widely used. It is a fast, high resolving, accurate and highly repetitive and reproducible technique of choice (Otieno *et al*, 1982). HPLC is not limited by thermally labile samples and has

a wider choice of mobile and stationary phases and this makes this technique more versatile than Gas Liquid Chromatography (GLC). In the analysis of Pyrethrins, both normal and reverse-phase HPLC have been attempted (Kamau, 1990). Normal phase HPLC is the most extensively used because it gives better resolved separations for the cinerins and jasmolins that are usually not resolved clearly in reverse-phase HPLC.

Normal phase chromatography (NPC) by definition implies the use of a polar stationery phase and a non-polar mobile phase. The solute elution order in this case is based on the principle that non-polar solutes elute first, polar solutes prefer the stationery phase and elute later. In reverse phase chromatography (RPC) the stationary phase is non-polar and the mobile phase is polar; the solute elution order is the reverse of that observed in normal phase HPLC, i.e. polar compounds elute first and non-polar ones later. Examples of stationary and mobile phase systems applicable in the analysis of Pyrethrins are shown table 5.

The UV absorption detector is the most commonly used in HPLC, being based on the principle of absorption of UV visible light as the effluent from the column is passed through a small flow cell held in the radiation beam (Furniss *et al*, 1989). UV variable wavelength detector has a detection limit of about 1 $ngml^{-1}$ for highly absorbing compounds and since it is a solute property detector, it is relatively insensitive to changes of temperature and flow rate. The complete analytical conditions used for the HPlC determination of individual Pyrethrin ester concentrations are outlined in the chapter 2 section 2.5.2 and 2.5.3

Table 5 : Typical stationary and mobile phases for Normal and Reversed phase HPLC for Pyrethrins analysis.

STATIONARY PHASE	MOBILE PHASE
Normal Phase Oxydipropionitrile	90% hexane : 10% (Acetonitrile:iso-Propanol: Tetrahydrofuran (AIT)) (5:4:1)
Reverse Phase Cyanoethylsilicone	Methanol : H_2O (3:17)

HPLC like the GLC method gives concentrations of individual esters but at ambient temperatures. Normal phase HPLC technique, detection at 230nm offers high resolution without possibilities of Pyrethrins degradation experienced with the GLC technique (Otieno, 1983; Kamau, 1990). A typical HPLC chromatogram of a world standard pyrethrum extract (WSPE 1992) is shown in fig.11a.

1.5.4.7 Spectrophotometry

The spectrophotometric analysis of Pyrethrins is the method of choice for routine work because it is a relatively cheaper and rapid technique. The spectrophotometric methodology is calibrated against the AOAC technique (Beckley, 1950). Briefly, the spectrophotometric determination of the Pyrethrins is done on the basis of the fact that absorption is linearly related to the concentration (percentage (w/w)) Pyrethrins (Details in

chapter 2 section 2.3).

Spectrophotometry gives only total Pyrethrins concentrations unlike the AOAC, GLC and HPLC that are capable of giving Pyrethrins I, Pyrethrins II and individual ester levels. This method is applied to advantage to the analysis of pyrethrum flowers both for payment of pyrethrum farmers and also for individual plant selection in crop improvement programs despite its subjectivity and low accuracy limit due to possible photochemical changes in the alcohol moiety chromophore.

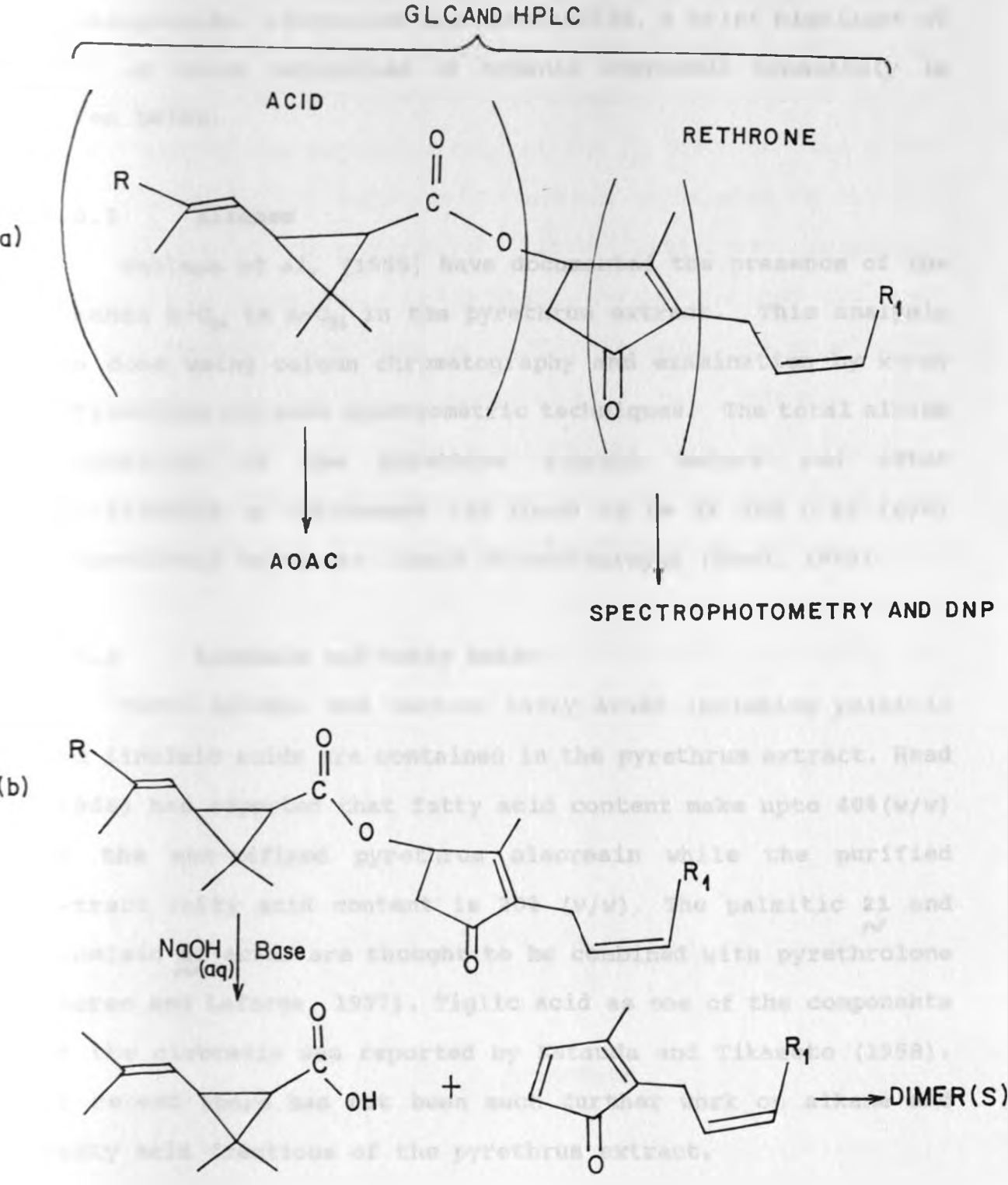
1.5.4.8 **Analytical Basis**

Looking at all the above techniques of Pyrethrins analysis, it is worthwhile to examine the chemical structure of the Pyrethrins once again to notice that these methods are all based on the Pyrethrins' general structure (fig. 5a). AOAC focuses on the acid moieties in the ester molecule while spectrophotometry utilizes the chromophoric group in the alcohol part of the ester structure. DNP is also based on the Ketonic group in the rethrone part of the ester molecule.

The AOAC utilizes the fact that ester molecules can easily be broken down by dilute solutions of aqueous or alcoholic NaOH (Saponification) to give the acid moiety residues which can then be separated and determined. Saponification of Pyrethrins also gives the rethrone residue (fig. 5b) which is capable of polymerization. The GLC and HPLC techniques are based on the entire molecule of the ester.

Fig. 5 : (a) Pyrethrins general structure : The analytical basis

(b) Action of aqueous base on the natural Pyrethrin esters.



1.6 Non-Insecticidal Components of pyrethrum flower extract

Along with the Pyrethrins in pyrethrum flowers, non-insecticidal components isolated and fully characterized include, alkanes, fatty acids, sesquiterpenoids, diterpenoids, triterpenoids, flavonoids and carotenoids. A brief highlight of each of these categories of organic compounds separately is given below.

1.6.1 Alkanes

Wanless *et al*, (1955) have documented the presence of the alkanes n-C_{24} to n-C_{36} in the pyrethrum extract. This analysis was done using column chromatography and examination by x-ray diffraction and mass spectrometric techniques. The total alkane composition of the pyrethrum extract before and after purification or refinement was found to be 4% and 0.2% (w/w) respectively using gas liquid chromatography (Head, 1969).

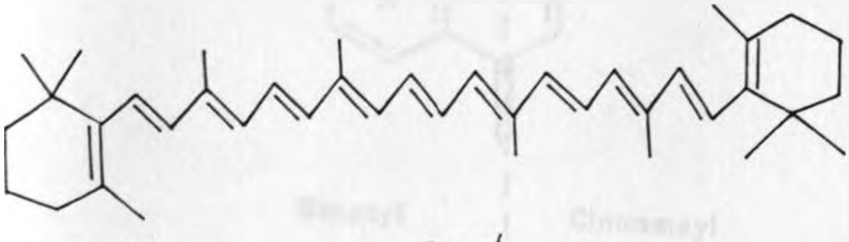
1.6.2 Alcohols and fatty acids

Ceryl alcohol and various fatty acids including palmitic and linoleic acids are contained in the pyrethrum extract. Head (1966) has reported that fatty acid content make upto 40%(w/w) of the non-refined pyrethrum oleoresin while the purified extract fatty acid content is 20% (w/w). The palmitic **21** and linoleic **22** acids are thought to be combined with pyrethrolone (Acree and Laforge, 1937). Tiglic acid as one of the components of the oleoresin was reported by Katsuda and Tikamoto (1958). Of recent there has not been much further work on alkane and fatty acid fractions of the pyrethrum extract.

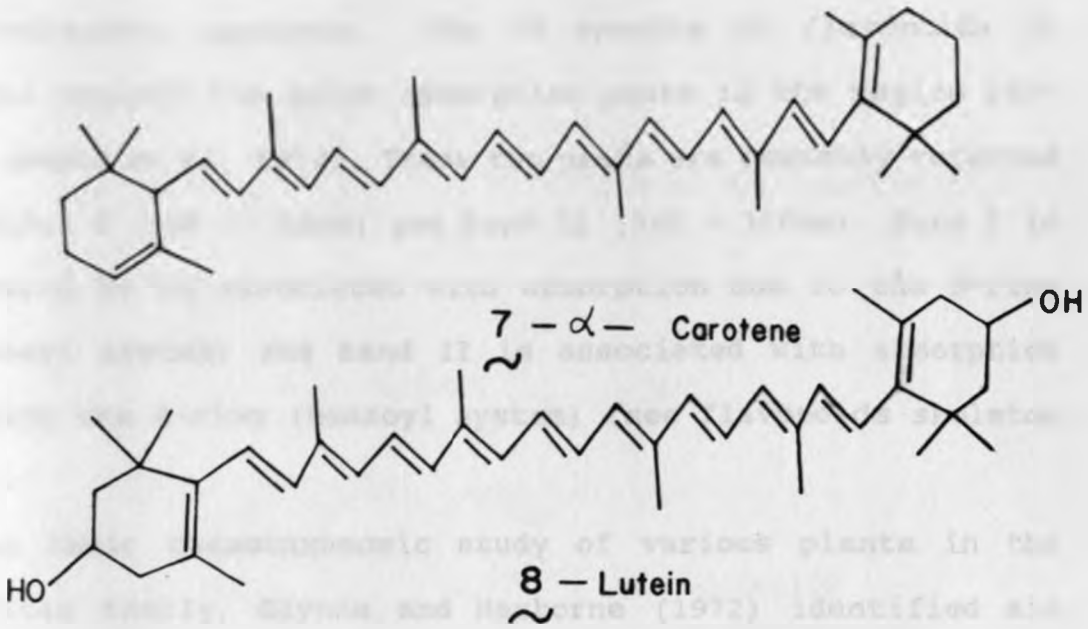
1.6.3 Carotenoids

Carotenoids (fig. 6), ß-carotene (6), α-carotene (7) and lutein (8) have been isolated and identified in the pyrethrum flower head (Head, 1969). Carotenoids are generally yellow pigments which are almost universally distributed in the plant kingdom. Carotenoids represent approximately 0.8 - 0.9% of the composition of the pyrethrum extract and it has also been shown that upto 90% of the carotenoid fraction is related to *cis* and *trans* isomers of the xanthophyll, lutein (Head,1969). Epiphasic carotenoids for example, α-carotene, ß-carotene and lycopene have λmax between 400-470nm in light petroleum. The more polar carotenoids (Hypophasic carotenoids), for example, the xanthophyll lutein and vitamins A1 and A2 have λmax from 280-355nm (Finar, 1975). So the carotenoids absorb both in the UV and visible regions of the electromagnetic spectrum.

Fig. 6 : Chemical structures of carotenoids isolated in pyrethrum flowers.

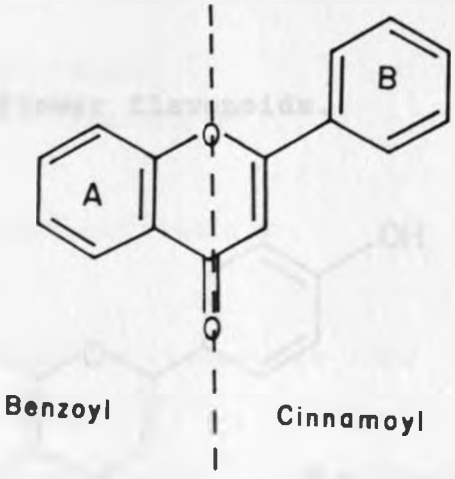


6 — β — Carotene



1.6.4 Flavonoids

The other yellow components of the pyrethrum extract are the flavonoids. Flavonoids are secondary metabolites derived from Shikimic acid (Scheme 1) and these are categorised into five basic groups, flavones, flavonols, aurones, flavanones and chalcones. All flavonoids are similar in their chemical structure based on the flavone skeleton shown below:

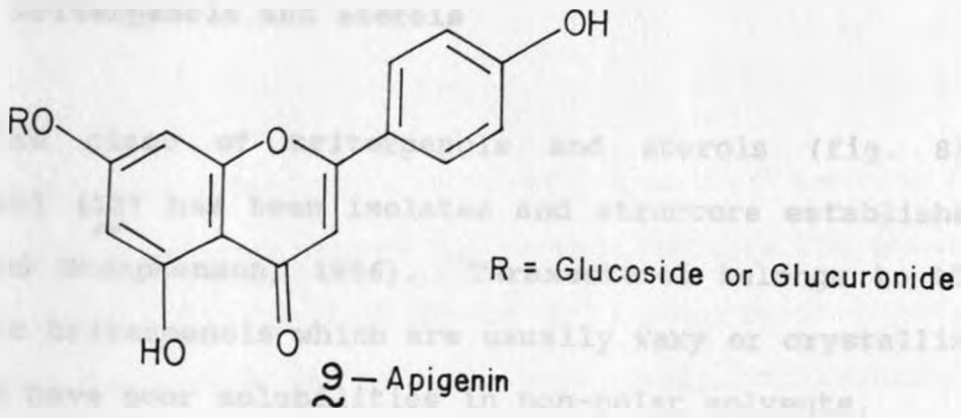


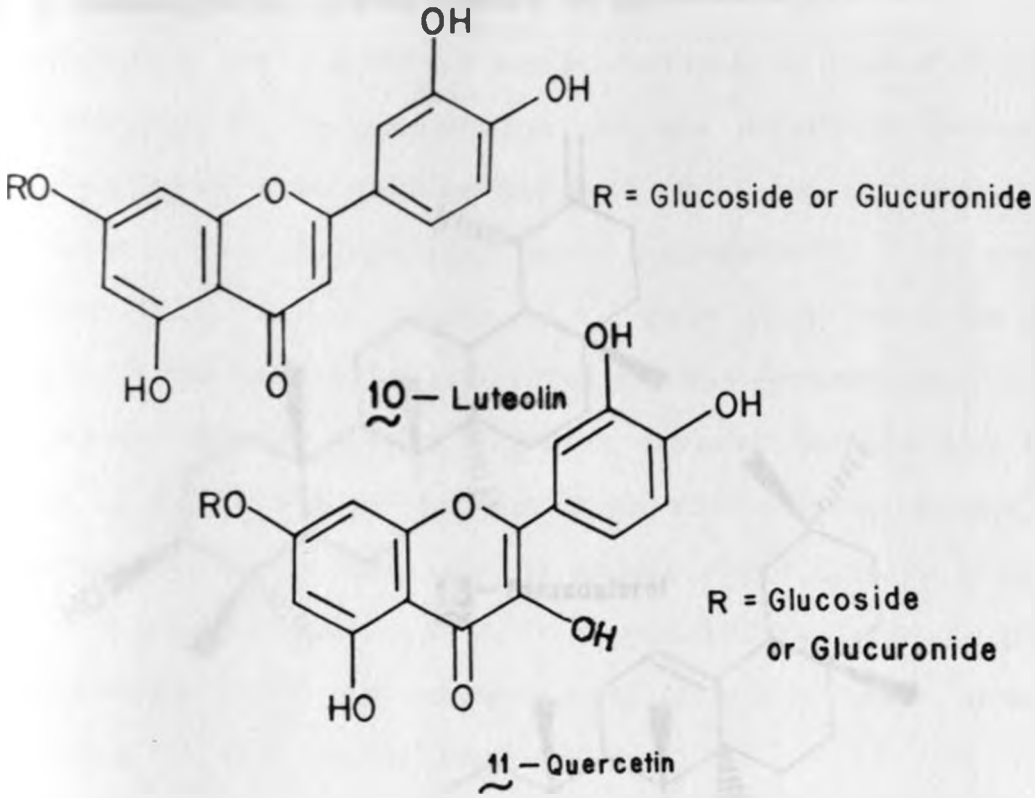
Most flavonoids are yellow compounds which implies that their absorption extends to the visible region of the

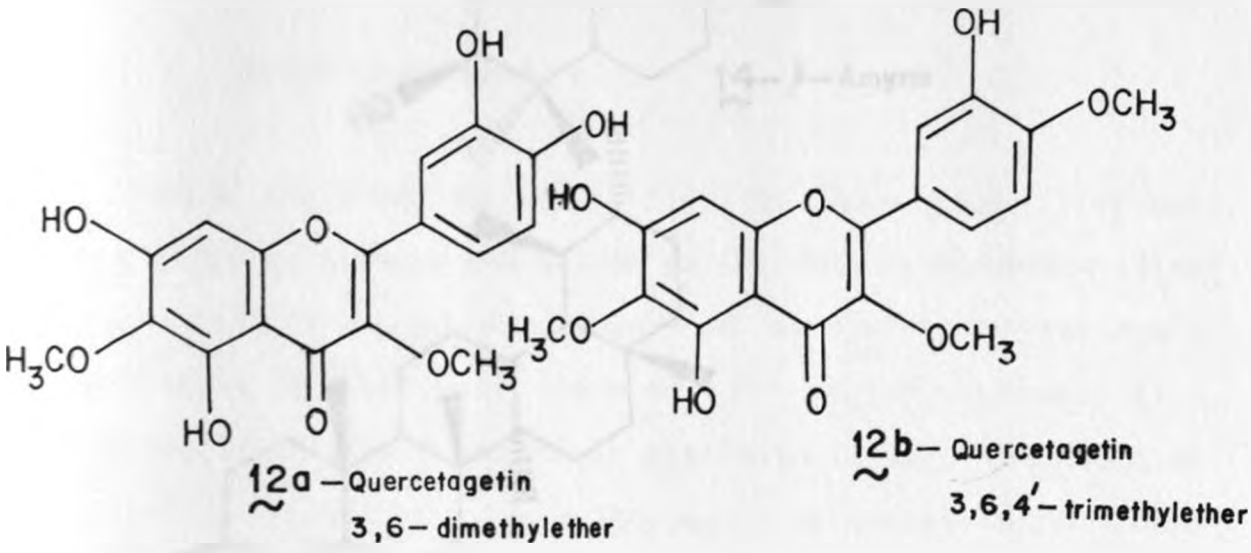
electromagnetic spectrum. The UV spectra of flavonoids in methanol exhibit two major absorption peaks in the region 240-400nm (Mabry *et al*, 1970). These two peaks are commonly referred to as Band I (300 - 380nm) and Band II (240 - 380nm). Band I is considered to be associated with absorption due to the B-ring (cinnamoyl system) and Band II is associated with absorption involving the A-ring (Benzoyl system) (see flavonoids skeleton above).

In their chemotaxonomic study of various plants in the Compositae family, Glynne and Harborne (1972) identified six flavonoid glycosides and methyl ethers of apigenin, luteolin and quercetagetin in pyrethrum flowers from Kenya (fig. 7). These are 7-glucosides and 7 glucuronides of apigenin **(9)**, luteolin **(10)** and quercetin **(11)**. The methyl ethers of quercetagetin are 3,6 - dimethyl ether **(12a)** and 3,6,4′ - trimethyl ether **(12b)**. The quercetagetin ethers are relatively less polar compounds than the glycosides and this may be the reason why the two are the only ones that have been detected in low concentration in the n-hexane pyrethrum extract (Casida, 1973).

Fig. 7 : Pyrethrum flower flavonoids.



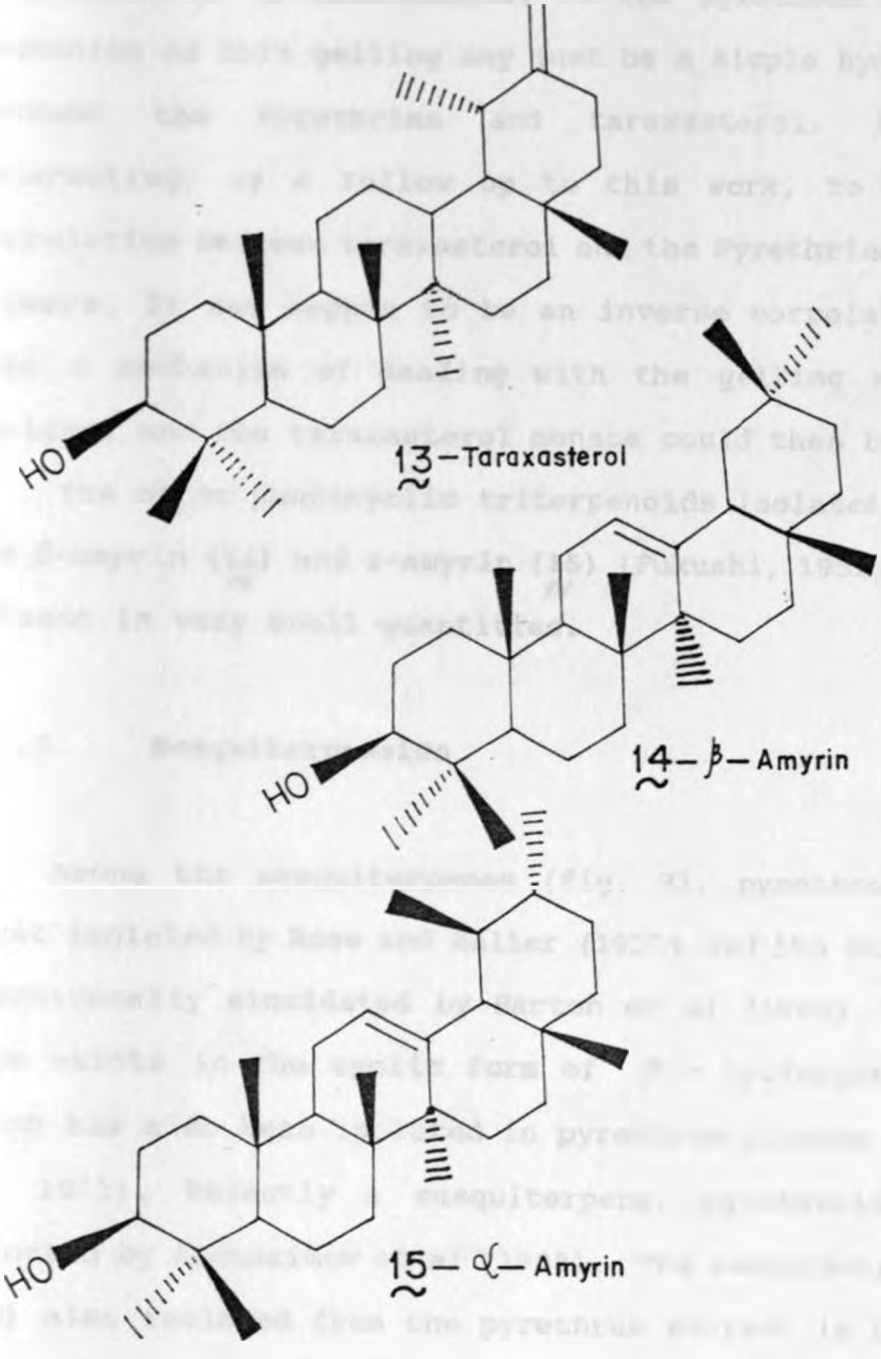




1.6.5 **Triterpenols and sterols**

In the class of triterpenols and sterols (fig. 8), taraxasterol **(13)** has been isolated and structure established (Elliot and Stenphenson, 1966). Taraxasterol belongs to the pentacyclic triterpenols which are usually waxy or crystalline solids and have poor solubilities in non-polar solvents.

Fig. 8 : **Pentacyclic triterpenols in pyrethrum flowers.**



Taraxasterol is undesirable in pyrethrum extract because of the gelling of "pale" that occurs in certain batches and mainly in certain seasons.This is thought to be a phenomenon closely associated to the presence of this pentacyclic triterpenol.

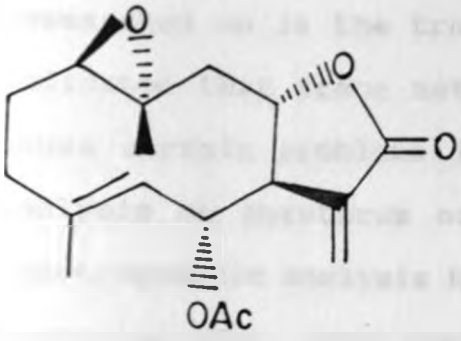
Though the gelling effect can be reversed by agitation and other techniques it is uneconomical to the pyrethrum industry. The mechanism of this gelling may just be a simple hydrogen bonding between the Pyrethrins and taraxasterol. It would be interesting, as a follow up to this work, to find out the correlation between taraxasterol and the Pyrethrins in pyrethrum flowers. It may happen to be an inverse correlation in which case a mechanism of dealing with the gelling effect can be designed and the taraxasterol menace could then be overcome.

The other pentacyclic triterpenoids isolated in pyrethrum are β-amyrin **(14)** and α-amyrin **(15)** (Fukushi, 1952). α-amyrin is present in very small quantities.

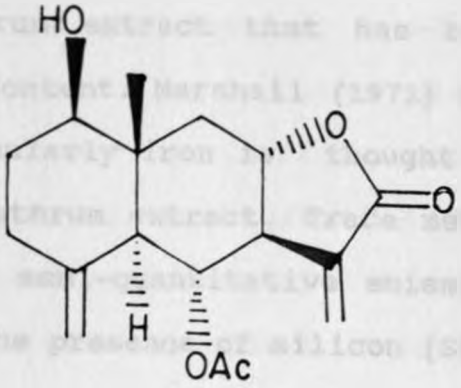
1.6.6 Sesquiterpenoids

Among the sesquiterpenes (fig. 9), pyrethrosin **(16)** was first isolated by Rose and Haller (1937) and its structure later unequivocally elucidated by Barton *et al* (1960). Pyrethrosin also exists in the cyclic form of β - cyclopyrethrosin **(17)** which has also been isolated in pyrethrum flowers (Doskotch *et al*, 1971). Recently a sesquiterpene, pyrethroidin has been reported by Abduazimov *et al* (1985). The sesquiterpene, sesamin **(18)** also isolated from the pyrethrum extract is thought to be synergistic. However, in commercial extracts, piperonyl butoxide **(19)** and butylated hydroxy toluene (BHT) **(20)** are intentionally added to augment the insecticidal activity and stabilize Pyrethrins respectively.

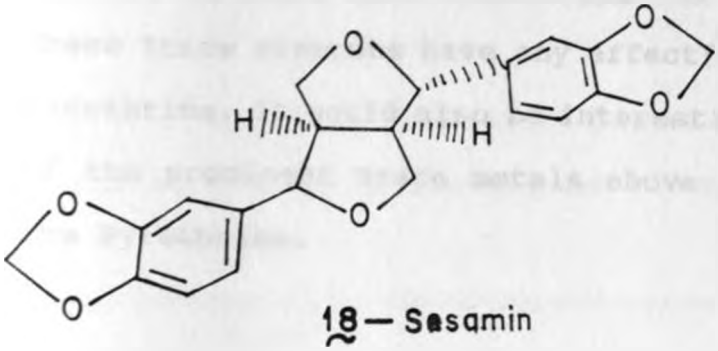
Fig. 9 : Chemical structure of other constituents and additives in pyrethrum extract.



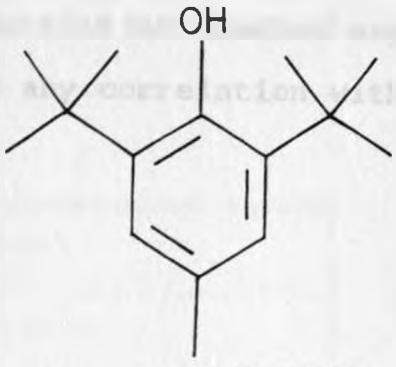
16 — Pyrethrosin



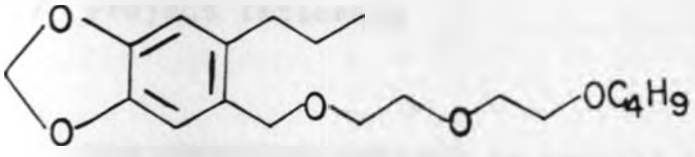
17-β — Cyclopyrethrosin



18 — Sesamin



20 — BHT



19 — Piperonylbutoxide

21 — Palmitic acid

22 — Linoleic acid

1.6.7 Trace metals

Another component of pyrethrum extract that has been researched on is the trace metal content. Marshall (1971) has indicated that trace metals particularly iron is thought to cause certain problems in the pyrethrum extract. Trace metal analysis on pyrethrum extracts by semi-quantitative emission spectrographic analysis has shown the presence of silicon (Si), magnesium (Mg), Iron (Fe) and aluminium (Al) at less than 0.1 ppm levels. Calcium (Ca) and Copper (Cu) were found to be present at less than 0.0001 ppm. It is not confirmed whether these trace elements have any effect(s) on the stability of the Pyrethrins. It would also be interesting to find out whether any of the prominent trace metals above have any correlation with the Pyrethrins.

1.7 Project rationale

The pyrethrum extract is usually yellow and this yellowness differs in intensity from one extract to another. In certain cases, it has been observed that light yellow appearing extracts exhibit high Pyrethrins content and vice-versa. An explanation for this observation is given in the results and discussion section. Compounds that absorb light of wavelength between 400-800nm (visible light) appear coloured to the human eye, the precise colour being a complicated function of wavelengths that compounds subtract from white light. Many compounds, flavonoids

in particular, have strong ultra-violet absorption bands, the shoulders of which tail into the visible region. Subtraction of violet light from white light leaves the complimentary colours which appear yellow / orange to the human eye, and for these reasons, yellow, orange to red in many cases are the most common colours among organic compounds (Kemp, 1991). The flavonoids and carotenoids are naturally yellow pigments and these are the main attributes to the yellow colour of the pyrethrum extract (Odinga, 1992). There are other plants in which carotenoid and flavonoid pigments have been associated with the colours of extracts as shown in table 6 (Goodwin, 1976).

Table 6 : Other plants to which yellow colours in them have been attributed to the carotenoid and flavonoid pigments.

PLANT	Pigment
Rose species	Carotenoids
Primula vulgaris	Flavonols
Antiruliunum majis	Aurones
Lotus coniculatus and *Ulex europeaus*	Carotenoids, Flavonols and Chalcones

The spectrophotometric technique was found most suitable in this work on the basis of its versatility and in-expensiveness. Both the Pyrethrins and the yellow pigments were determined using the same instrument. Despite its subjectivity in terms of

accuracy, no other alternative technique (s), for example, Colorimetric methods were as suitable, results of which reflect the contribution of the carotenoid and flavonoid pigments separately to the yellowness of the pyrethrum extract. The AOAC chemical analysis technique is a widely accepted technique in the quantification of the Pyrethrins despite its high cost. The HPLC adequately compliments this mercury reduction methodology giving the individual ester concentrations from which the distribution of the individual esters the jasmolins, cinerins and pyrethrins in various clones and varieties relative to the non-insecticidal components , the yellow pigments in particular could be evaluated.

The specific objectives in this study were as follows:

(i) To extract and scan clonal and varietal pyrethrum flower samples in order to identify absorption peaks and read absorbances in the UV and visible regions of the electromagnetic spectrum for the Pyrethrins and the non-insecticidal compounds (yellow pigments in particular) using a UV-visible spectrophotometer.

(ii) To quantify the Pyrethrins content in the various pyrethrum samples from different localities using various analytical techniques.

(iii) To determine the Pyrethrins I, Pyrethrins II and Pyrethrins I / Pyrethrins II ratios using the AOAC chemical assay method.

(iv) To assess the distributions of the six individual esters (collectively known as Pyrethrins) using High performance liquid chromatography (HPLC) technique in all the research samples relative to the non-insecticidal components in pyrethrum flowers.

(v) To undertake phytochemical screening of the pyrethrum extracts in order to identify some of the yellow pigments which would give correlative information with the spectrophotometric, AOAC and HPLC results.

(vi) To correlate all the phytochemical, spectrophotometric, chemical assay and HPLC results in order to establish the relationship between the Pyrethrins and the yellow pigments in pyrethrum flowers.

This thesis describes for the first time the intimate relationship between the Pyrethrins and the yellow pigmentation in pyrethrum flowers. Chapter 2 illustrates how this investigation was carried out.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 General:

All the chemical and instrumental analyses were done in replicates of two or four to ensure reproducibility to enhance the accuracy of the final result. Any questionable result(s) obtained from analytical instruments were subjected to acceptability statistical Q-Test (Jefferey *et al*, 1989). All the extractions were made using aromatic free n-hexane (absorbance at λ225nm, air reference, 10mm matched quartz cells $\leq$ 0.003).

De-aromatization of n-hexane was necessary to make the solvent transparent to UV and visible light over the wavelengths of interest cited in this work thereby enhancing accuracy of absorbance measurements made. This clean up of n-hexane was done as follows : double distillation of reagent grade n-hexane, left to cool and gravity run through a 30cm long by 4cm diameter glass column packed with silica gel Grade 1 (activated at 120°c for one hour) and collecting the eluate drop wise into a clean, dry and securely capped winchester bottle. The adsorbent was always replaced after every 1.5 litre eluate continuous collection. For every fresh clean up of n-hexane, the first 15-20cm^3 was discarded. This de-aromatization procedure removes peroxides and aromatic hydrocarbons which would otherwise interfere with absorbance measurements at, for example, λ225nm for the Pyrethrins. The necessity of this procedure can be clearly seen

from a few of the actual sample results shown in table 7. De-aromatized n-hexane sufficiently transparent at λ_{225nm} is termed herein as n-hexane aromatic free {n-hexane (AF)}

Table 7: **n-Hexane de-aromatization absorbances at λ225nm; 1cm silica cells; air reference.**

Solvent	Absorbance	Mean absorbance
n-Hexane (GPR)	0.135,0.242,0.219	0.196 ± 0.046
Distilled n-Hexane	0.066,0.049,0.031,0.069	0.054 ± 0.018
Double Distilled n-Hexane	0.012,0.019,0.020,0.011	0.016 ± 0.005
De-aromatized n-Hexane	0.003,0.002,0.001,0.003	0.002 ± 0.001
PBK* n-Hexane (AF)	0.002,0.003,0.003,0.001	0.002 ± 0.001

* n-Hexane (AF) from Pyrethrum Board of Kenya (PBK)

All other solvents used were distilled prior to use. Distilled water was used for the preparation of various reagents in the wet chemical analyses. All cold extractions of the ground pyrethrum flowers (grist) were carried out for one hour aided by moderate mechanical agitation. Hot extractions were achieved using the conventional soxhlet extractor for at least seven (7) hours.

2.1.1 Optical Cells

The cuvettes used for the spectrophotometric determinations were 10mm quartz cells regarded sufficiently transparent in the UV and visible regions of the electromagnetic spectrum. The cells were kept clean by avoiding contamination as a result of evaporation of solvents from solutions and also avoiding grease film formation on the transparent surfaces by handling the cells only by the etched surfaces.

After use the cuvettes were always emptied and rinsed with n-hexane (AF) then cleaned with distilled water. Finally the cells were rinsed with n-hexane (AF) again and left to dry.

On use, the cells were always rinsed first with n-hexane (AF) and then with the prepared extract before being finally filled for reading using the double beam spectrophotometer. For the purpose of consistency, accuracy and precision only one pair of matched cells were used throughout the analysis. The cells were always placed in the instrument such that the incident radiation enters via the same optical surface every time. One of the cells was specifically marked for the sample and the other as reference cell. Whenever wiping of the cuvette surface(s) was necessary only high quality soft tissue paper was used.

2.2 Materials

Twenty different clones and varieties of pyrethrum flowers originating from various localities, Kisii, Molo, Subukia, Limuru, Keroka and Marindas were sampled in two lots from the

National Pyrethrum and Horticultural Research Centre, Molo nurseries. These were dried in the shade (in an open space inside a laboratory) and ground using a Wiley mill to pass 1mm screen. Precaution was taken during storage of the grist to avoid exposure of the grist to extreme heat or direct sunlight which otherwise would affect the Pyrethrins. Water content levels for the various samples were determined using the Dean and Stark apparatus.

2.2.1 Flower Sampling

The samples required in this work were samples of lowest to highest Pyrethrins content. It was only from different clones and varieties of pyrethrum flowers that this requirement could be satisfied. The samples had to be sampled from one locality to keep other variables like climatic conditions, soil nature, altitude and many other factors constant. Samples were randomly sampled from the nurseries in cultivar plots that were ready for picking at that time. All the plots from which the samples were taken had been subjected to uniform breeding treatment as advised by the plant breeders.

2.2.2 Flower Picking

The samples were hand picked into polythene bags and were immediately weighed to assess their fresh weights (Table 3). Picking early in the morning was found most suitable because at this time it was easier to assess the flowers ready for picking.

These were flowers whose petals were perfectly horizontal early in the morning in the absence of external factors such as heat from the sun. Molo is an area prone to afternoon rain showers which would otherwise interfere with afternoon picking. Due to unavoidable circumstances, picking of pyrethrum flowers in the afternoons and even after raining is at times done within Molo area by the farmers, but this case was not applicable in this project.

The samples were then transported to Nakuru for drying. In the drying laboratory at the chemistry laboratories of the Pyrethrum Board of Kenya, Nakuru, the flowers were spread sparsely on large polythene sheets. The flowers were on daily basis re-spread over and over again for the whole two weeks drying period in order to avoid fermentation of the flowers and also to accelerate the rate of drying. The room was properly ventilated with additional air breeze from the air conditioning fans fitted in the laboratory.

2.2.3 Sample Size

The variational limits for Pyrethrins content in Kenya pyrethrum flowers has been documented to be 0.8 - 2.0% (Casida, 1973). It was suitable to ascertain the number of samples to be taken in this investigation by adopting a relevant statistical approach. Taking into consideration the bulk amount of analyses to be carried out, time factor and expense in terms of cost of analyses in this study, a small sample size was targeted.

A three year study related to this investigation which had been carried out on the performance of ten (10) low attitude, unimproved pyrethrum clones grown in Kisii (Wanjala, 1990) showed that the mean and standard deviation of Pyrethrins content in the various pyrethrum flowers grown in this region were 1.225% and ± 0.133% respectively.

Table 8 : A three year performance study of low altitude unimproved pyrethrum clones grown in Kisii (Wanjala, 1990).

CLONE	1987 (%)	1988 (%)	1989 (%)
K7	1.16	1.20	1.15
Kenya	1.23	1.25	1.26
Marwanga	1.28	1.30	1.03
Gekoma	1.31	1.29	1.17
Nyamasibi	1.34	1.31	1.07
Nyankoba	1.30	1.20	1.15
C47	1.13	1.06	0.95
Ebiosi	1.19	1.17	1.21
Congo	1.20	1.13	1.18
4331	1.61	1.45	1.47

The above study was done in one locality and it involved some of the clones used in the present study. The results showed variability in the Pyrethrins content for the three years as shown in the summarized table 8. This variability was regarded as a sampling error and its mean for the ten samples was found to be 0.135%. Statistically, when a small number of observations is made, the value of standard deviation does not by itself give a measure of how close the sample mean $\bar{x}$ might be to the true

mean μ.

Calculations of confidence interval (limit) is given by

$$u = \bar{x} \pm \frac{ts}{\sqrt{n}} \quad \ldots \quad Equation\ 2{:}1$$

Where t - is the normal student's t - value that is a parameter that depends upon the number of degrees of freedom and the confidence level required. This formula above can be adapted for samples of variable composition and it becomes

$$u = \bar{x} \pm \frac{ts_s}{\sqrt{n}} \quad \ldots \quad Equation\ 2{:}2$$

Where S_s is the standard deviation of individual samples, x is the mean of analytical results, μ is the true mean and n is the number of samples taken.

Equation (2:2) can be re-organized to reflect the number of samples to be taken as:

$$n = (\frac{ts_s}{u-\bar{x}})^2 \quad \ldots \quad Equation\ 2{:}3$$

In equation (2:3), the sampling error is actually represented by

$$(u-\bar{x})$$

i.e. the difference between the sample mean and the actual value μ. From the student's t-tables, the value of t (n-1), 9 degrees of freedom at 95% confidence level is 2.262.

To calculate n (Number of samples), now that t = 2.262, S_s = ± 0.133% and μ-x = 0.135% requires application of equation (2:3) as follows:

$$n = (\frac{2.262\ x\ 0.133}{0.135})^2 \approx 5\ samples$$

At 99% confidence level t = 3.250

$$n = \left(\frac{3.250 \times 0.133}{0.135}\right)^2 \approx 10 \ samples$$

Hence from this test at least 10 samples were required to give results acceptable at 99% confidence level. But 20 samples were confidently handled in this study as shown in the table 9.

Table 9 : Research samples code table

SAMPLE	CODE	SAMPLE	CODE	SAMPLE	CODE	SAMPLE	CODE
C47(c)	1	Ks/75/313	6	L/75/487	11	Ks/70/64	16
Nyamasibi (c)	2	Ma/75/4	7	K235 (v)	12	Ma/70/1013	17
Marwanga (c)	3	Mo/70/223	8	Ks/71/6	13	Mo/70/1124	18
P4 (v)	4	Ks/75/336	9	Sb/66/107	14	Ma/71/423	19
4331	5	L/75/477	10	Mo/74/443	15	Kr/74/122	20

Key: c - indicates local clone; v - indicates variety samples
The samples bearing neither c nor v are hybrid clones
Ks - originating from Kisii; L - originating from Limuru;
Mo - originating from Molo; Kr - originating from Keroka;
Ma - originating from Marindas; Sb - originating from Subukia;
Marwanga, Nyamasibi and C47 are local clones from Kisii.

NOTE: Throughout this thesis unless otherwise stated, the research sample serialisation is as shown in table 9.

2.3 Spectrophotometry:

Spectrophotometric determination of Pyrethrins is based on Beckley's (1950) method. A double beam Pye Unicam SP8-150 spectrophotometer was used. Prior to use, the instrument was always first calibrated using the world standard pyrethrum extract (Odinga, 1992). Absorbances of extracts at λ_{225nm} were done on 0.5% (w/v) n-hexane (AF) cold extracts left to settle overnight and diluted appropriately before reading. The calibration data of WSPE is shown in table 10 and fig.10 below.

The calibration standard stock solution was prepared every time it was necessary by weighing an exact weight of 2g of WSPE into a 200ml volumetric flask and dissolved in n-hexane(AF) filling up to the mark. A dilution of 9ml into 100ml was necessary for the Pye-Unicam spectrophotometer. To make the calibration standards,1,2,3,4,5,6,7 and 8 ml each at a time were pippeted into 100ml volumetric flasks numbered 1,2,3,4,5,6,7 and 8 as shown in table 10. A blank of n-hexane(AF) was also prepared similarly. These standard solutions were always used to check the linearity of response of the spectrophotometer before analysis of Pyrethrins.

Table. 10 : WSPE (1992) Calibration standards absorbance summary.

STANDARD	1	2	3	4	5	6	7	8
	1.0cm^3	2.0cm^3	3.0cm^3	4.0cm^3	5.0cm^3	6.0cm^3	7.0cm^3	8.0cm^3
8.7.92	0.194	0.409	0.604	0.807	1.104	1.217	1.413	1.628
8.7.92	0.198	0.393	0.598	0.782	0.978	1.189	1.394	1.589
9.7.92	0.184	0.388	0.585	0.784	1.003	1.188	1.390	1.591
14.7.92	0.197	0.386	0.6020	0.787	0.981	1.194	1.398	1.594
15.7.92	0.196	0.396	0.590	0.803	0.968	1.187	1.392	1.585
21.7.92	0.191	0.390	0.580	0.783	0.984	1.192	1.393	1.596
12.8.92	0.187	0.390	0.592	0.784	0.978	1.191	1.389	1.599
13.8.92	0.198	0.408	0.609	0.808	1.006	1.206	1.408	1.611
17.8.92	0.188	0.389	0.587	0.795	0.987	1.196	1.410	1.600
20.8.92	0.199	0.391	0.598	0.808	1.008	1.213	1.408	1.606
10.9.92	0.201	0.410	0.613	0.805	0.995	1.212	1.420	1.615
8.12.92	0.194	0.387	0.591	0.806	1.002	1.215	1.413	1.616
AVERAGE	0.194	0.395	0.596	0.796	1.000	1.200	1.402	1.603

Stock Solution : 1.0116g in 100ml n-hexane (AF) in 100ml volumetric flask. A dilution of 9ml made upto the mark in a 100ml flask was made. The standards all in 100ml volumetric flasks were prepared as in row 2 and absorbances of each were recorded as shown in table 10.

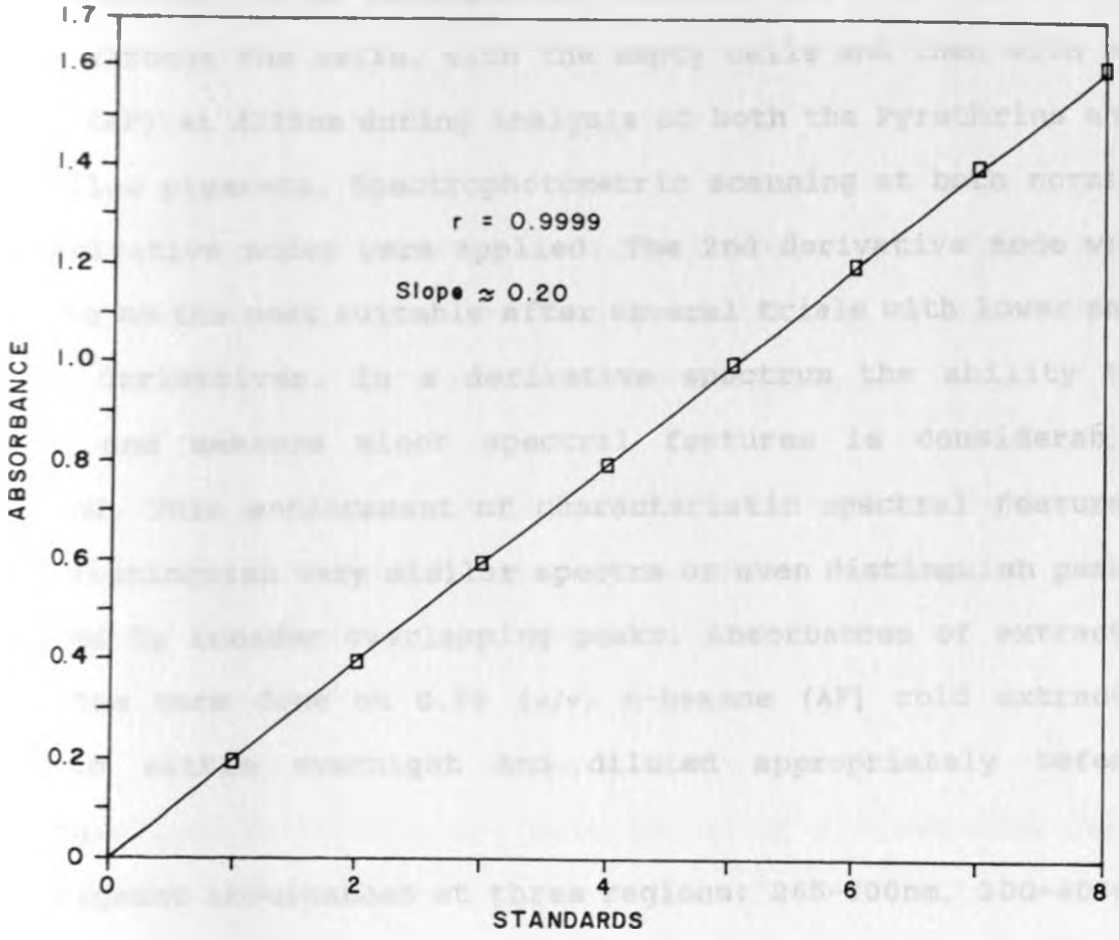


Fig.10: Pye Unicam SP8 - 150 instrument calibration graph of WSPE (1992).

A correlation coefficient of ≥ 0.996 between the absorbances of the standards with the progressive standard solution concentrations indicated linearity of response of the instrument and therefore its suitability for the day's sample analysis (Fig.10). When the standard solutions gave no acceptable readings the instrument was suspected to be faulty and a check using the previous days' sample(s) was made. If the absorbance(s) of the previous day's samples(s) was reproduced, then fresh standard solutions were prepared.

The instrument was at every time of start up put on and left to stabilize for at least 15 minutes. All the operational

settings were adjusted appropriately. The read out was at every time required to be consistently stable. The instrument was zeroed without the cells, with the empty cells and then with n-Hexane (AF) at λ225nm during analysis of both the Pyrethrins and the yellow pigments. Spectrophotometric scanning at both normal and derivative modes were applied. The 2nd derivative mode was found to be the most suitable after several trials with lower and higher derivatives. In a derivative spectrum the ability to detect and measure minor spectral features is considerably enhanced. This enhancement of characteristic spectral features could distinguish very similar spectra or even distinguish peaks obscured by broader overlapping peaks. Absorbances of extracts at λ225nm were done on 0.5% (w/v) n-hexane (AF) cold extracts left to settle overnight and diluted appropriately before reading.

Pigment absorbances at three regions: 265-300nm, 300-400nm and 400-470nm in 1% (w/v)n-hexane(AF) extracts were read without any dilutions. A typical scan, using the 2nd derivative mode from 250-700nm for the pyrethrum extract showed absorption peaks at the following wavelengths : 670, 470, 440, 420, 410, 370, 350, 330, 294, 285, 275 and 265nm. The peaks at 410 and 420nm were noted to vary in position from sample to sample. In some samples the peak at 420nm was missing completely. It was not established what the reason for this occurrence could be, either a solvent effect or molecular chromophoric variation in the compounds absorbing at this wavelengths.

2.3.1 **Spectrophotometric calculation of total Pyrethrins content were done as follows:**

% Pyrethrins = Absorbance at λ_{225nm} x M + C

= A_{225nm} x M + C

where M and C are the linear correlation slope and constant respectively between the Pyrethrins content by AOAC (AOAC, 1984) method with mean absorbances at λ_{225nm}.

Spectrophotometric determination of Pyrethrins content is calibrated against the AOAC mercury reduction assay and it gives good linear regression lines (Casida, 1973).

2.3.2 **Determination of Slope(M) and Constant(C)**

The general formula for calculation of % Pyrethrins using the spectrophotometric method above (Section 2.3.1) is based on the determination of Pyrethrins content of selected (preferably an even number of lowest to highest) Pyrethrins content samples by AOAC and measurement of absorbances of 0.5g in n-hexane (AF) extracts at λ_{225nm}. This UV spectrophotometric method is widely used for rapid routine assay of Pyrethrins. This method is applied to advantage to the analysis of pyrethrum flowers both for payment to the farmers and also for individual plant selection in crop improvement programs at the Pyrethrum Board of Kenya, Nakuru. Application of the two equations 2:4 and 2:5 below based on Karl Pearson's linear regression gives the slope and constant respectively.

$$M = \frac{N\Sigma PA - \Sigma P\,\Sigma A}{N\Sigma A^2 - (\Sigma A)^2} \quad \ldots \text{ Equation 2:4}$$

$$C = \overline{P} - M\overline{A} \quad \ldots \text{ Equation 2:5}$$

Where M = Slope

A = Absorbance intensity at λ225nm

P = % Pyrethrins by AOAC

N = Number of Samples

$\overline{P}$ = Mean % Pyrethrins by AOAC

$\overline{A}$ = Mean absorbance intensity at

λ225nm.

The slope and constant in this work were determined using the Pye Unicam SP8-150 instrument and the twenty research samples. The values of M and C were found to be **1.54** and **0.19** respectively. The Pyrethrum Board of Kenya (Nakuru) slope and constant are 1.44 and 0.29 respectively on a Varian DMS 80 Spectrophotometer fitted with an in-flow cell system suitable for analyzing a large number of samples daily.

2.3.3: **Beer-Lambert's Law**

All the spectrophotometric methodology used in this work is based on the fundamental Beer-Lambert's law which states that the proportion of light absorbed by a solute in a transparent solvent is independent of the intensity of the incident light and is

proportional to the number of absorbing molecules in the light path (Equation 2:6).

$$\log_{10}\left(\frac{I_0}{I}\right) = A = \xi cl \quad \ldots Equation\ 2{:}6$$

where I_0 = intensity of incident light (radiation)

I = Intensity of transmitted light

ζ = Molar absorptivity or Molar extinction coefficient.

c = Concentration of solute in moles/litre

l = Cell path length (cm)

A = absorbance or optical density.

Beer's law is a limiting law and it is strictly valid only at low concentrations (equivalent to absorbance readings upto 1.700). The important point to realise from Beer-Lambert's law is that absorbance (A) is directly proportional to the concentration of the absorbing species.

2.3.4 Specific absorbance

Specific absorbance denoted by $A_{1cm}^{1\%}$ is defined in this work as the projected absorbance of 1% (w/v) n-Hexane pyrethrum extract sample of unit Pyrethrins concentration. This is a proportionality expression that reflects the relative composition of the non-insecticidal components (yellow pigmentation in this case) to the Pyrethrins in the various pyrethrum flowers. The 400 - 470 nm region indicated to be mainly λmax of carotenoid

absorption, ${}^3A_{1cm}^{1\%}$ reflects the carotenoid specific colour absorbance.

The formula expressing the specific absorbance is given by

$$A_{1cm}^{1\%} = \frac{\bar{A} \times 100}{\bar{P} \times w} \quad \ldots \textit{ Equation } 2:7$$

Where $A_{1cm}^{1\%}$ = Specific absorbance

$\bar{A}$ = Mean absorbance for 1% (w/v) n-hexane extract using 1cm cells at a particular region:265 - 300nm (^1A), 300-400nm (^2A) and 400-470 nm (^3A).

$\bar{P}$ = Mean Pyrethrins content by AOAC

W = Exact weight (g) of sample used to obtain a 1% (w/v) n-hexane extract.

2.4 Mercury reduction method : The official method of analysis of the Association of Agricultural Chemists (abbreviated AOAC).

The official methodology of analysis (AOAC, 1984) was used in this work (Appendix I). The actual detailed steps followed are indicated below.

2.4.1 Step 1 : Extraction

Duplicate samples (8.0 g and 9.0g) of grist were weighed accurately into 3.0 cm diameter x 8.0 cm length Whatman cellulose extraction thimbles, plugged with cotton wool and extracted using

soxhlet apparatus for at least seven (7) hours using 100ml n-hexane (AF) in 250ml Erlenmeyer quick fit conical flasks containing a few anti-bumping chips. After complete extraction, the n-hexane extracts were concentrated *in vacuo* to reduce volume to about 50ml.

2.4.2 Step 2 : Dewaxing

The flasks containing the extracts were then carefully stoppered and kept in a refrigerator at 0-5°c overnight. The next morning the extracts were filtered cold through cotton wool into another set of 250ml conical flasks carefully rinsing the cotton wool. This cold filtration was meant to remove the waxes in the extracts. The extract were then evaporated to dryness *in vacuo*.

2.4.3 Step 3 : Saponification

20ml of alcoholic 0.5N NaOH was then added to the dry extracts in a 250ml conical flask containing a few anti- bumping chips and refluxed for 1.5 hours. This process hydrolyses the esters. Because this is an alkali base hydrolysis, it is referred to as saponification.

2.4.4 Step 4 : Evaporation

After saponification, the samples were transferred into 800ml Erlenmeyer beakers and added distilled water to make the volume up to 200ml. The solutions were then evaporated on a hot plate (taking care of the excessive foaming at this stage) to a

volume of 150ml to remove the alcohol. This solutions were then cooled to room temperature with tap water.

2.4.5 Step 5 : Fatty acid removal

The cool solutions were then carefully transferred into 250ml volumetric flasks and added 1.0g of filter-celite each. Celite is a filtering aid medium. 10 ml of 10% $BaCl_2$ solution was then added to each of the volumetric flasks and filled to the mark with distilled water. This mixtures were then shaken vigorously for exhaustive removal of the fatty acids by $BaCl_2$. Yellowish-orange Barium fatty acid salt precipitate was formed. 200ml of extracts were filtered off into a 200 ml volumetric flasks and added two to three drops of phenolphthalein indicator. These filtrates are basic and hence they were neutralised with 20% H_2SO_4. It is necessary to acidify the solutions by adding 1ml of H_2SO_4 acid in excess to precipitate the excess $BaCl_2$ if any. A white precipitate of $BaSO_4$ which formed was then filtered off through Whatman filter paper No.1 coated lightly with a suspension of filter-celite on a butchner funnel aspirator. It was necessary that the precipitate was washed several times with distilled water.

2.4.6 Step 6 : Separation of Chrysanthemic and Pyrethric acids.

The filtrates were then transferred to 500ml separating funnels and extracted twice with two 50ml portions of n-hexane (AF). The n-hexane layers were transferred to 250ml separating funnels and washed twice with 5ml portions of distilled water to

remove traces of aqueous layer. These aqueous washings were not discarded. The resultant portions of the aqueous layers were emptied into 800ml beakers used in step 4. The chrysanthemic acid is a less polar acid than pyrethric acid and therefore in principle the Pyrethrins I are contained in the n-hexane layer while the aqueous layer contains Pyrethrins II. What was achieved here was separation of the two acids subsequently used in quantifying the Pyrethrins I and Pyrethrins II. At this point the aqueous layer was set to evaporate for about 1hr as in step 4 down to 50mls. This was then cooled to room temperature (25°C) with tap water.

2.4.7 Step 7(a) : Determination of Pyrethrins I

The hexane layers were extracted twice with 5ml portions of 0.1N NaOH solution. At each of these partitioning steps careful washing and transfers were undertaken to avoid losses of the analyte. Chrysanthemic acid has a high solubility in dilute basic aqueous solutions and so 0.1N NaOH was suitably used here. The basic extract was then placed in a 100ml beaker and the n-hexane layer was discarded.

2.4.8 Step 8 : Mercury reduction

10ml of Deniges reagent was added to the basic extract. The 100ml beaker and its contents were then kept for one hour in a dark water-bath at 25°c. The extract on adding Deniges reagent changed colour to pink and then gradually to purple → light blue → deep blue-green colour. These colour changes are characteristic of chrysanthemic acid. The intensity of these colours depend on

the concentration of the acid. The mixture was then removed from the dark water bath and immediately added 3mls of saturated NaCl solution followed by 20ml amyl alcohol. The saturated NaCl precipitates Mercurous Chloride (HgCl) (Refer to equation 1 :1 - 1:4). The precipitate was briefly boiled and filtered through a small filter paper carefully transferring all the precipitate to the filter paper. A further 10ml amyl alcohol was added to the empty beaker and boiled briefly again to precipitate HgCl. The white precipitate of HgCl was filtered through the same filter paper. The filter paper was then washed twice with 10ml of distilled chloroform using the same 100ml beaker above. This helps remove all traces of alcohol from the filter paper. The filter paper was then placed in a 200ml conical flask. The beaker was washed with 50ml of 60% HCl aqueous solution and the sides of the beaker wiped thoroughly with cotton wool on a small glass rod before emptying both the HCl solution and the piece of cotton wool into the 200ml conical flask-containing the filter paper and $HgCl_{(s)}$. 20ml of $CHCl_3$ was then put into the same 100ml beaker above and added to the 200ml conical flask contents. 1ml of iodine mono chloride (ICl) indicator was added and titration with 0.01MKIO_3 solution carried out. The titration was done with constant shaking until the pink colour in the chloroform phase just disappeared.

2.4.9 Calculation of Pyrethrins I concentration

The % (w/w) Prethrins I was calculated as follows:

$$\% \text{ Pyrethrins I} = 0.7125 \times \frac{V_I}{W_I}$$

Where V_1 = Titre volume (volume of 0.01M KIO_3 used) (cm^3)

W_1 = Weight of sample (g)

0.7125 = Stoichiometric factor for Pyrethrins I.

2.4.10 Derivation of the Pyrethrins I Stoichiometric factor

Experiments have shown that:

1ml of 0.01M KIO_3 ≡ 0.0057g of Pyrethrins I

Initially in step 5 above, the sample was made up to 250ml out of which only 200mls underwent analysis.

Let the weight of the sample extracted at step1 = W_1 (g)

Let the volume of 0.01M KIO_3 used on titration = V_1 (cm^3)

The amount of Pyrethrins I in the 200mls = (V_1 x 0.0057)g

For the total sample of 250ml the amount of Pyrethrins I

$$= \left(\frac{250}{200} \times \frac{V_I}{W_I} \times 0.0057\right) g$$

$$\%Pyrethrins\ I = \left(\frac{250}{200} \times \frac{V_I}{W_I} \times 0.0057 \times 100\right) \%$$

$$= 0.7125 \times \frac{V_I}{W_I}$$

2.4.11 Illustrative example for % Pyrethrins I calculation

Sample: 4331 (one of the set of results)

Sample weights: A - 8.0673g B - 9.0017g.

Reagent	0.01MKIO$_3$ PI'S		0.02NNaOH PII'S	
	A	B	A	B
Final Volume (cm^3)	11.10	23.55	8. 95	18.75
Initial Volume (cm^3	0.00	11.10	0.00	8.95
Blank (cm^3)	0.05	0.05	0.05	0.05
Titre Volume (cm^3)	11.05	12.40	8.90	9.80
% (w/w)	0.98	0.98	0.54	0.53

$$\%Pyrethrins\ I = \frac{0.7125\ X\ 11.05}{8.0673}\ (A) = \frac{0.7125\ X\ 12.40}{9.0017}\ (B)$$

$$= 0.9759\% \quad (A) = 0.9814\% \quad (B)$$

$$\simeq 0.98\% \quad (A) \simeq 0.98\% \quad (B)$$

Mean % Pyrethrins I = 0.98%

2.4.12 **Step 7(b) : Determination of Pyrethrins II**

After evaporating the aqueous layer containing Pyrethrins II acid moiety (step 6) down to about 50ml and cooling to room

temp (20°c) the solution was transferred into a 50ml separating funnel containing about 20g NaCl and 10ml conc. HCl such that a saturated solution of NaCl was formed. The sodium Chloride solution supersaturates the aqueous layer with NaCl and therefore reduces the solubility of pyrethric acid in the aqueous layer.

The aqueous layer was then extracted three times with diethyl ether washing twice with 10ml portions of saturated NaCl solution to remove traces of HCl. This step is very critical such that any traces of acid left over would lead to inaccuracies in the titre volume determination. The total diethyl ether layer was then filtered (to remove undissolved excess NaCl) through a cotton plug and residue washed with additional 10ml of diethyl ether. The total diethyl ether extract in a 500ml conical flask was then evaporated to dryness in a water-bath designed to recover the solvent. The sample was then put in an oven at 100°c for ten minutes. On removing the sample from the oven, a current of compressed air was blown into the flask to remove HCl fumes. The sample was then dissolved in 2ml of neutral alcohol and added 20 ml of acid free water. Both the neutral alcohol and acid free water were prepared by neutralization of absolute alcohol and de-ionized water using dilute sodium hydroxide to make PH value to 7. Two drops of phenolphthalein indicator were then added to the conical flask containing the sample and titrated with 0.02N NaOH.

2.4.13 **Calculation of Pyrethrins II Concentration**

$$\% \text{ (w/w) Pyrethrins II} = 0.4675 \times \frac{V_{II}}{W_{II}} \times TF$$

V_{II} = Volume of 0.02N NaOH (cm^3)

W_{II} = Weight of Sample extracted (g)

TF = Titration factor due to the hygroscopic alkali base NaOH

0.4675 = Stoichiometric factor for PyrethrinsII

2.4.14 **Derivation of Pyrethrins II Stoichiometric Factor**

Experiments have shown that 1ml of 0.02N NaOH is equivalent to 0.00374g of Pyrethrins II i.e. 1ml 0.02N NaOH ≡ 0.00374g of Pyrethrins II. At step 5 above only 200ml of the 250ml total extract were analyzed. 250/200 factor is to be applied in total Pyrethrins II concentration determination.

Let weight of Sample = W_{II} (g)

Let Volume of 0.02N NaOH used = V_{II} (cm^3)

Therefore amount of Pyrethrins II in 200ml = (V_{II} x 0.00374)g

Total Pyrethrins II in 250ml

$$= \left(\frac{250}{200} \times V_{II} \times 0.00374\right) g$$

$$\% \ Pyrethrins\ II = \left(\frac{250}{200} \times \frac{V_{II}}{W_{II}} \times 0.00374 \times 100\right) \%$$

Incorporating the titration factor (TF) explained below:

$$\% \ Pyrethrins\ II = \left(\frac{250}{200} \times \frac{V_{II}}{W_{II}} \times 0.00374 \times 100 \times TF\right) \%$$

$$\% \ Pyrethrins\ II = \left(0.4675 \times \frac{V_{II}}{W_{II}} \times TF\right) \%$$

2.4.15 **The titration factor (TF)**

Sodium hydroxide and potassium hydroxide are highly hygroscopic bases. A certain amount of alkali carbonate and water are present in these reagents on standing for some time which implies that exact results cannot be obtained in titrimetric determinations when using these bases and hence the necessity to standardize these alkalis just before use. Potassium hydrogen phthalate (PHP) is one of the primary standards used in standardization of Sodium hydroxide.

PHP is a substance of high purity (99.9%) (Jefferey *et al*, 1989). Drying at 120°c for 2 hours before use is recommended. To determine the titration factor using PHP the following steps were followed:

0.8g of PHP (Wp) were weighed exactly in duplicate (A and B) and dissolved in acid free water in 200ml volumetric flasks and volumes made up to the mark.

Taking one of the two samples to explain further, 10ml of the solution was pippeted into a conical flask and titrated with the prepared 0.02N NaOH solution using phenolphthalein indicator.

The calculation of the titration factor was done as follows:

Let the average titre volume 0.02N NaOH = V_p (cm^3)

Let the weight of PHP = Wp (g)

Concentration of Sodium hydroxide = 0.02N

Number of moles of NaOH in Vp (cm^3)

$$= \frac{0.02 \times V_p}{1000} \ moles$$

Number of moles in PHP in

$$10ml = (\frac{0.02 \times Vp}{1000}) \ moles$$

Exact weight of PHP in

$$10ml = (\frac{0.02 \times Vp}{1000} \times 204.22) \ g$$

The expected weight of PHP if the concentration of sodium hydroxide is exactly 0.02N is given by

$$(\frac{Wp}{200} gml^{-1} \times 10ml) = \frac{W_p}{20} \ g$$

$$Titration Factor (TF) = \frac{Expected \ weight \ of \ PHP \ calculated}{Exact \ weight \ PHP \ used}$$

$$TF = \frac{W_p \times 1000}{0.02 \times V_p \times 204.22 \times 20}$$

$$TF = \frac{12.2417\ W_p}{V_p}$$

Where Wp = weight of PHP used (g)

Vp = Volume of 0.02 NaOH used on 10ml PHP (cm^3)

Other recommended primary standards in addition to potassium hydrogen phthalate are Benzoic acid ($C_6H_5CO_2H$; RMM 122.12), Succinic acid $(CH_2CO_2H)_2$; RMM = 118.09), Potassium hydrogen iodate $(KH(IO_3)_2$; RMM = 118.09) and sulphamic acid (NH_2SO_3H ; RMM = 97.09) (Jefferey *et al*, 1989). The titration factor explained above is an expression of change of basicity of sodium hydroxide with respect to potassium hydrogen phthalate.

2.4.16 Illustrative example for % Pyrethrins II calculation

(Refer to data for sample 4331 above)

TF = 1.0430

A B

$$\%\,PyrethrinsII = \frac{0.4675\ X\ 8.90\ X\ 1.0430}{8.0673} = \frac{0.4675\ X\ 9.80\ X\ 1.0430}{9.0017}$$

= 0.5379% = 0.5308%

Mean % Pyrethrins II = 0.5343%

≈ 0.53%

2.5 High performance liquid chromatography (HPLC)

2.5.1 Samples

Exact weights of 1g of the grist samples each time in duplicate were cold extracted for one hour aided by moderate mechanical agitation using 40ml n-Hexane (AF) as the solvent in 100ml volumetric flasks. Volumes were made up to the mark with the same solvent. The extract was then left to settle for at least two (2) hours before injection into the instrument. This procedure was repeated on the following day for the same sample using the same instrument thus checking for reproducibility.

2.5.2 Instrument:

A varian model 5000 liquid chromatograph equipped with a varian UV-100 detector, flow cell 4.5μl and a varian integrator model 4400 was used.

2.5.3 Operational Conditions:

Sample injection system: Manual loop injector with a sample load capacity of 10 μl

Detector Settings: λmax 230nm; Absorbance range (sensitivity) = 0.5 Auf.

High Pressure pump: Multi-head reciprocating type capable of minimizing pulsation;

Flow rate = 0.8 ml/minute.

Integrator settings: Chart speed = 1cm/min ; Attenuation = 8; Peak threshold (PT) = 6

Column: A varian micropak CN-5 nitrile, 30cm x 4 mm id and ambient column temperature.

Pressure: 14-16 atmospheres

Mobile phase: Reservoir A:(Acetonitrile : Isopropanol:Tetrahydrofuran) (5:4:1)
Reservoir B: n-Hexane (AF)
Combination: 10%A and 90%B

Syringe: 10µl (Wedge tipped) Hamilton syringe

Glassware: 100ml and 10ml volumetric flasks cleaned to dry and further rinsed with acetone (Distilled)

Reagents and Solvents: Acetonitrile - HPLC grade
Isopropanol - HPLC grade
Tetrahydrofuran (THF)-HPLC grade
n-Hexane - Aromatic free

Acetone - Analar grade

WSPE - 1992 {provided by Pyrethrum Board of Kenya ; Purity - 19.42 % (10.8% PI ; 8.62 % PII)}

WSPE is World Standard Pyrethrum Extract.

Before acceptable readings were taken various dilutions of the standard and at times test samples were made. Peak height readings were preferred to peak areas due to convenience of use of peak heights which are less bulky. Actual HPLC chromatograms of the WSPE and pyrethrum flower extracts are shown in fig.11

2.5.4 **Pyrethrins content calculations were undertaken as follows:**

$$\% \ Pyrethrins \ (w/w) = \frac{H_s \ X \ C_{std}}{H_{std} \ X \ C_s} \ X \ \% \ Purity \ of \ WSPE$$

where H_s = Average peak height of sample

H_{std} = Average peak height of WSPE

C_s = Concentration of sample (gm/l)

C_{std} = Concentration of WSPE

Fig.11: (a) WSPE (1992) chromatogram

(b) Pyrethrum flower grist extract HPLC chromatogram 1

(c) Pyrethrum flower grist extract HPLC chromatogram 2

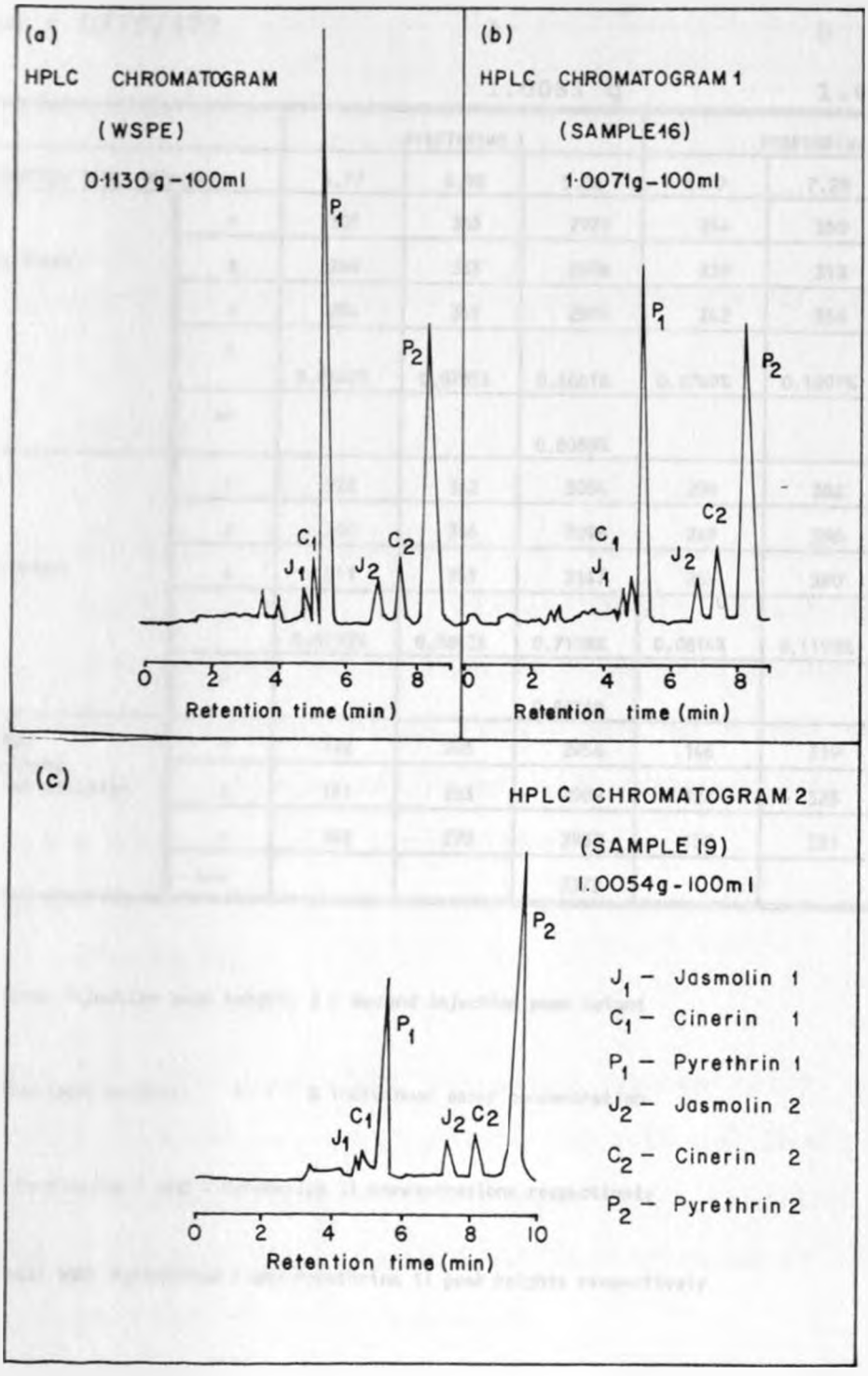


Fig. 11 : (a) WSPE (1992) HPLC Chromatogram .

(b) Pyrethrum flower grist extract HPLC Chromatogram 1

(c) Pyrethrum flower grist extract HPLC Chromatogram 2

2.5.5 Illustrative example of Pyrethrins content determination using HPLC method.

Sample : L/75/477 A 1.0081 g B 1.0081 g

		PYRETHRINS I			PYRETHRINS II		
RETENTION TIME (Minutes)		4.77	4.98	5.34	6.79	7.29	8.07
A	1	305	365	2921	244	359	1593
1.0081g-100ml	2	264	333	2996	239	353	1579
	3	284	349	2959	242	356	1586
	*	0.0642%	0.0785%	0.6661%	0.0740%	0.1091%	0.4854%
	**			0.8088%			0.6685%
B	1	322	342	3084	290	382	1628
	2	300	366	3096	240	396	1683
1.0081g-100ml	3	311	355	3147	265	390	1656
	*	0.0703%	0.0803%	0.7118%	0.0814%	0.1198%	0.5088%
	**			0.8624%			0.7100%
WSPE 1992	1	172	293	2956	148	319	1489
0.1079g-100ml	2	151	263	2909	127	323	1557
6.6ml-10ml Dilution	3	162	278	2933	138	321	1523
	***			3373			1982

Key: 1 - First injection peak height; 2 - Second injection peak height

3 - Mean peak height; * - % individual ester concentration

** - % Pyrethrins I and % Pyrethrins II concentrations respectively

*** - Total WSPE Pyrethrins I and Pyrethrins II peak heights respectively

Summary of the above results

	Pyrethrins I (PI) % w/w	Pyrethrins II (PII) % w/w	Total Pyrethrins % w/w
A	0.81	0.67	1.48
B	0.86	0.71	1.57
Mean	0.84	0.69	1.53

2.6 Phytochemical screening of the pyrethrum flower extract

2.6.1 General

All solvents were distilled prior to use. Analytical thin layer chromatography (t.l.c.) plates (Merck aluminium sheet precoated with silica gel 60 UV-254) were used throughout the phytochemical screening. Merck silica gel 60 G was used for preparative t.l.c. whereas silica gel (230-400 mesh) was used for the gravity elution column chromatography.

2.6.2 Extraction

500g of grist was cold extracted sequentially using n-hexane, dichloromethane, ethylacetate and methanol in a 5.0 litre flat bottomed conical flask with intermittent stirring using a glass rod. Each of the extracts was concentrated *in vacuo* and yields of each are shown in table 11.

Table 11: Phytochemical screening Pyrethrum extracts summary.

	n-Hexane extract	CH_2Cl_2 extract	EtOAc extract	CH_3OH extract
Description	(Thick yellowish - green mass)	(Thick dark brown slurry)	(Thick sticky dark brown mass)	(Thick brown mass)
Weight(g)	12.99	19.96	5.65	68.99

2.6.3 **Preliminary work :**

The pyrethrum extracts above were tested for various classes of organic compounds as follows:

2.6.4 **Test for sterols and triterpenoids**

0.5g of each of the extracts were re-extracted with 25ml dry chloroform. 5ml of the $CHCl_3$ extract was mixed with 0.5ml Ac_2O followed by two drops of conc. H_2SO_4 acid. A greenish blue coloration was observed in all the extracts. In the n-hexane, dichloromethane, and ethylacetate extracts, the greenish blue coloration gradually changed to a pinkish colour. This observation was indicative of the presence of sterols and triterpenoids in the pyrethrum extracts.

2.6.5 **Test for flavonoids**

5ml chloroform extracts above were treated as follows:

(a) added 4ml 1% $AlCl_3$ in methanol. A yellow coloration was observed. This indicated the presence of flavonoids

(b) added 4ml 1% KOH. A yellow colour was noted in the aqueous layer indicating the presence of flavonoids

(c) added 0.5ml conc.HCl plus a few Magnesium turnings. A red coloration was observed. Presence of flavonoids in the pyrethrum extract confirmed.

2.6.6 Thin Layer Chromatography (t.l.c.)

Thin layer chromatography of each of the extracts was performed using precoated analytical plates activated prior to use at 120°C for 10 minutes. Various sports of interest were visualised using a UV lamp (254nm and 366nm), spray reagents and other techniques illustrated below. The screening done for the pyrethrum extracts was biased towards the yellow compounds of relevance to this research project.

2.6.6.1 n-hexane extract

For the n-hexane extract t.l.c on silica gel precoated plates developed in $CHCl_3$: cyclohexane (1:3) gave two yellow spots Rf. 0.50 and 0.81. There was a yellow spot at the origin which did not move. Only the yellow spot at the origin on exposure of the plate to NH_3 vapour intensified in colour. On a second plate spotted and developed similarly, the compound at Rf. 0.50 charred deep blue on spraying with conc. H_2SO_4 after heating at 120°C for 10 minutes. The same compound on another t.l.c. plate developed similarly on spraying with 20% $SbCl_3$ in Chloroform (Carr-Price reagent) changed colour to deep blue after heating the plate briefly at 120°C. This spot at Rf. 0.50 was suspected to be a carotenoid compound while the polar one(s) at the origin could be flavonoid compounds. The spot at Rf. 0.81 was thought to be one of the common chlorophyll pigments.

T.l.c. of n-hexane extract using $CHCl_3$: n-hexane (1:3) gave identical results to the previous solvent system. T.l.c of this extract run alongside an n-hexane carrot extract developed in n-hexane : ethylacetate (3:1) gave identical yellow spots in the two extracts at Rf. values, 0.14, 0.27, 0.89, 0.91. All this four spots showed no noticeable response to NH_3 vapour. Exposure of a similarly developed plate to iodine vapour in an iodine chamber showed this same spots together with other spots thought to be Pyrethrins because these were not visible in daylight but fluorescent blue in both short and long UV light.

2.6.6.2 Dichloromethane and Ethylacetate Extract

The CH_2Cl_2 extract spotted on a t.l.c. plate and developed in n-hexane: chloroform (3:1) solvent system gave two yellow spots at Rf. 0.40 and 0.66. Both these spots were fluorescent in UV light and these colours intensified on exposure to NH_3 vapour. The EtOAc extract in the same solvent system showed two yellow spots at Rf. 0.14 and 0.63 which both responded to NH_3 vapour by intensification of the yellow coloured spots.

2.6.6.3 Methanol Extract

The Methanol extract spotted and developed in n-hexane : EtOAc (1:3) solvent system showed three yellow spots at Rf. 0.14, 0.26 and 0.80 which all responded to NH_3 vapour including a polar compound(s) at the origin. All these spots were fluorescent in both short and long UV wavelengths.

Both the ethylacetate and methanol extracts when co-chromatographed in n-hexane: EtOAc (1:3) showed many spots on

exposure to iodine vapour.

2.6.6.4 Re-extraction with n-hexane

A little of each of the dichloromethane, ethylacetate and methanol extracts were re-extracted with n-hexane and t.l.c. of resultant n-hexane extract showed interesting results in n-hexane : ethylacetate (1:3) system. The dichloromethane (n-hexane extract) showed two yellow spots at Rf's 0.69 and 0.91 identical to the initial dichloromethane extracts. In addition there was a yellow spot at the origin which did not move but this yellow spot was the only one which intensified on exposure to NH_3 vapour. These spots at 0.69 and 0.91 could be carotenoids in dichloromethane which easily separated into n-hexane layer. The ethylacetate (n-hexane extract) gave no yellow spots at all, so did the methanol (n-hexane extract).

2.6.7 Column Chromatography (n-hexane extract)

A column was run for the n-hexane extract as follows : 6g of n-hexane extract was charged with 12g of silica gel (230-400 mesh). The solvent in the charged sample was evaporated to dryness *in vacuo*. 48g of silicagel (230 -400 mesh) was packed under n-hexane into a 3cm diameter glass column. Normal gravity polarity gradient elution using 500ml n-hexane, 500ml 5% ethylacetate in n-hexane, 500ml 10% ethylacetate in n-hexane, 300ml 12% ethylacetate, 200ml 13% EtOAc in n-hexane and finally 200ml 15% EtOAc in n-hexane was done after sample introduction. 56 eluates were collected and after t.l.c. screening resulted into 15 fractions. No yellow pure compounds were achieved. Four

fractions f_2-f_5 were further combined. This contained six compounds, three of which were visibly yellow and the other three were fluorescent in UV light having been developed in n-hexane : EtOAc (3:1).

Another small column was run similarly for this combined fraction (3g) eluting using 500ml 5% Et_2O in n-hexane. 10% Et_2O in n-hexane and 15% Et_2O in n-hexane. 60g of silica gel (230-400 mesh) for this column was packed under n-hexane: Et_2O (17:3). A fraction f2 of this gave a strong intense yellow compound Rf. 0.42 in n-hexane: CH_2Cl_2 (17:8) solvent system but a fluorescent compound at the solvent front was noted.

2.6.8 Preparative thin layer chromatography (Ptlc)

To isolate a pure compound from the above fraction 2, preparative t.l.c. was attempted. 25 x 25 cm glass plates were coated with silica gel 60G using 1:2 ratio of absorbent to de-ionized water. The slurry was mixed thoroughly for 75 seconds and spread on clean and dry glass plates with a spreading machine. The plates were left to dry overnight and were activated at 120°C for 1/2 hour before streaking the sample (1.2g). The plates were developed in n-hexane: CH_2Cl_2 (17:8) and the yellow band at Rf. 0.38 was scrapped off and regained in CH_2Cl_2 and filtered off using a butchner funnel. The yellow extract was concentrated *in vacuo*. Tlc of this concentrated extract in n-hexane: CH_2Cl_2 (17:8) gave a strongly intense yellow spot at Rf. 0.66. A fluorescent spot at the origin was again noted and this may be the reason why this compound failed to crystallise out adequately. At this point, the compound was dried *in vacuo* and

re-extracted in n-hexane (AF). This extract of n-hexane (AF) was then subjected to a 250-700nm scan using a spectrophotometer. The spectrum showed absorption peaks at 420nm, 440nm and 470nm (fig. 27(b)).

A similar scan of ß-carotene, authentic sample (from city chemical corporation, New York) in n-hexane (AF) also showed absorption peaks at 410nm, 440nm and 470nm (fig. 28). All this information having in mind the pyrethrum sample analysis results (CHAPTER 3) indicated that the peaks at 410, 420, 440 and 470 nm are likely to be carotenoid peaks.

2.6.9 Column Chromatography (Dichloromethane extract)

The dichloromethane extract was also subjected to separation using column chromatography. 10g of the extract was charged with 20g of silica gel (230-400 mesh). 180g of the same absorbent was packed under dichloromethane. Polarity gradient elution using 500ml CH_2Cl_2, 500ml 5% EtOAc in CH_2Cl_2 and 500ml 20% EtOAc in CH_2Cl_2. 14 eluates were collected. The only eluate that showed yellow spots on t.l.c. was eluate 14. In n-hexane: EtOAc (2:3) eluate 14 had a prominent yellow spot at Rf. 0.54. This eluate also showed other two fluorescent spots at Rf 0.31 and 0.77. This prominent yellow spot was also fluorescent under short UV and its colour intensified on exposure to NH_3 vapour indicating the presence of a flavonoid compound.

2.6.10 Preparative thin layer chromatography (Dichloromethane extract)

Preparative t.l.c. was found appropriate to isolate the

yellow compound above. The sample (0.9001g) was streaked on preparative t.l.c. plates (thickness, 2mm) were developed in EtOAc: n-Hexane (3:2). The yellow isolate regained in the same solvent mixture was 0.0950g. Tlc on an analytical precoated silica gel UV $_{254}$ plate developed in EtOAc: n-Hexane (3:2) showed a yellow spot at Rf. 0.65. The yellow spot strongly and readily intensified on exposure to NH_3 vapour. This solid compound was not appreciably soluble in n-hexane but readily dissolved in methanol. Recrystallisation was not achieved using this set of solvents as expected. Out of curiosity, a little amount of the isolate in n-hexane (AF) showed some faint yellow colour which indicated slight solubility of the compound in n-hexane. A spectrophotometric scan (250-400nm) of n-hexane solution containing this yellow isolate revealed a strong absorption peak at 275nm which coincided perfectly with one of the wavelengths which the pyrethrum research samples showed (fig. 29). This gave an indication that the type of compounds absorbing in the region 265-400 nm include the yellow flavonoid pigments.

2.7 Data analysis

Because these were samples of varying composition and small sample size, the value of standard deviation s does not by itself give a measure of how close the sample mean $\bar{x}$ of the Pyrethrins content in each of the samples might be to the true mean. It is however possible to calculate the confidence interval to estimate the range within which the true mean may be found but the reliability of results was considered most suitable for this work by use of the statistical Q-test (Jeffery *et al*, 1986). The Q-

test is a reliable measure used widely by analysts to arrive to a sensible decision as to whether certain results, particularly instrumental, may be rejected or accepted.

$$Q = \frac{|Questionable\ Value - Nearest\ Value|}{Largest\ Value - Smallest\ Value}$$

The product of this quotient is known as the calculated value of Q. There are critical values of Q with which the calculated Q is compared with at various confidence levels. If the calculated Q value exceeds the critical value then the questionable result is rejected otherwise if the calculated value of Q is less than the critical one, then the questionable result is accepted. The data was subjected to linear regression analysis as indicated below.

2.7.1 Correlation and Regression

The interest in this work was to examine how variations in Pyrethrins content associated with the variations in the non-insecticidal components, yellow pigments in particular, in pyrethrum flowers. For this purpose the best index of association or relationship was the Karl Pearson's product moment coefficient of correlation, r. A coefficient of correlation is a single number that reflects to what extent variations in one variable go with variations with the other.

A computer was used to run linear regression analyses using the INSTAT programme using a BBC computer. Regression graphs were drawn using the LOTUS package using an IBM computer. The INSTAT package was also used to make scatter plots used to ascertain linearity of relationship before regressing the variants.

2.7.2 Spearman's Rank Difference Correlation Coefficient , ρ (rho)

After inspection of results it was noticed that certain samples had the same Pyrethrins content and some had equal values of specific absorbances. Also the distribution of the specific absorbances $^{2}A^{1\%}_{1cm}$ and $^{3}A^{1\%}_{1cm}$ showed some degree of skewness as the scatter plots depict. This occurrence necessitated use of different statistical data treatment other than the determination of the Pearson's product moment correlation coefficient (r) known as Spearman's Rank Difference correlation coefficient denoted by ρ(rho). Normal distribution is not a prerequisite in the calculation of ρ like in r. But it must be clarified here that r is based on both the sizes of the measures (Quantitative)as well as their relative positions in the series of results while ρ takes account of the positions only and this is why r values are emphasized in this thesis despite this limitation of applicability in small sample determinations. ρ values are only given on exploratory basis.

$$\rho = 1 - \frac{6\sum D^2}{N(N^2-1)}$$

where ρ = Spearman's Rank - difference coefficient

D = Difference between ranks

D^2 = Difference between ranks squared

ΣD^2 = Summation of squares of differences between ranks

N = Sample size.

CHAPTER 3

3.0 RESULTS AND DISCUSSION

3.1 RESULTS

Preliminary investigations in this work on various aspects were carried out. The water content levels of the pyrethrum flowers determined from the difference in fresh and dry weights of the flowers were fairly uniform and were found to be 82.5 ± 1.5%. For the dry samples as determined using the Dean and Stark method, the average water content was found to be 6.4 ± 1.2%.

Spectrophotometric scanning of the World Standard Pyrethrum Extract (WSPE) and various pyrethrum flower extracts all in n-hexane (Aromatic Free) in the ultra violet and visible regions of the electromagnetic spectrum showed interesting results. The WSPE n-Hexane 0.5% (w/v) solution spectrum (fig 12) shows a high intensity absorption peak at λ_{225nm}. No noticeable absorbance between 300 - 700nm was observed. The reason for this is that, WSPE is prepared from the refined pyrethrum extract from which even the pigments absorbing at 400 - 470nm may have been removed almost completely. A peak at 285nm is noticeable.

A spectrophotometric scan (230-700nm) on a 5% (w/v) composite pyrethrum grist n-hexane (AF) extract fig.13(a) showed four prominent absorption peaks at 470, 440, 420 and 275nm. There were also peaks noticeable between 300-400nm region. Close to 230nm region the absorbance went off scale due to the absorption of the Pyrethrins around this region.

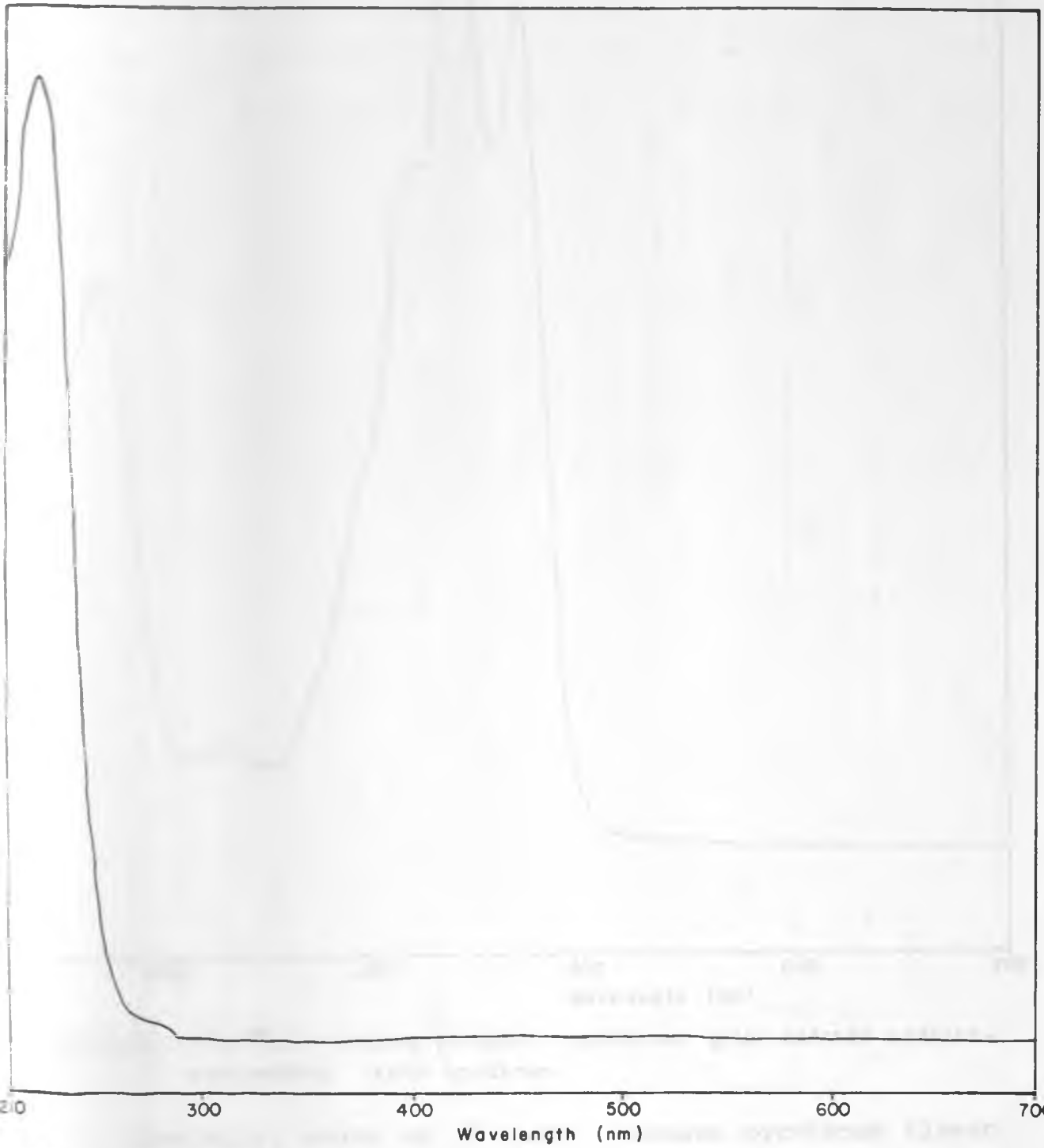


Fig. 12 : WSPE (1992) in n-Hexane (AF) spectrophotometric scan (210 - 700nm)

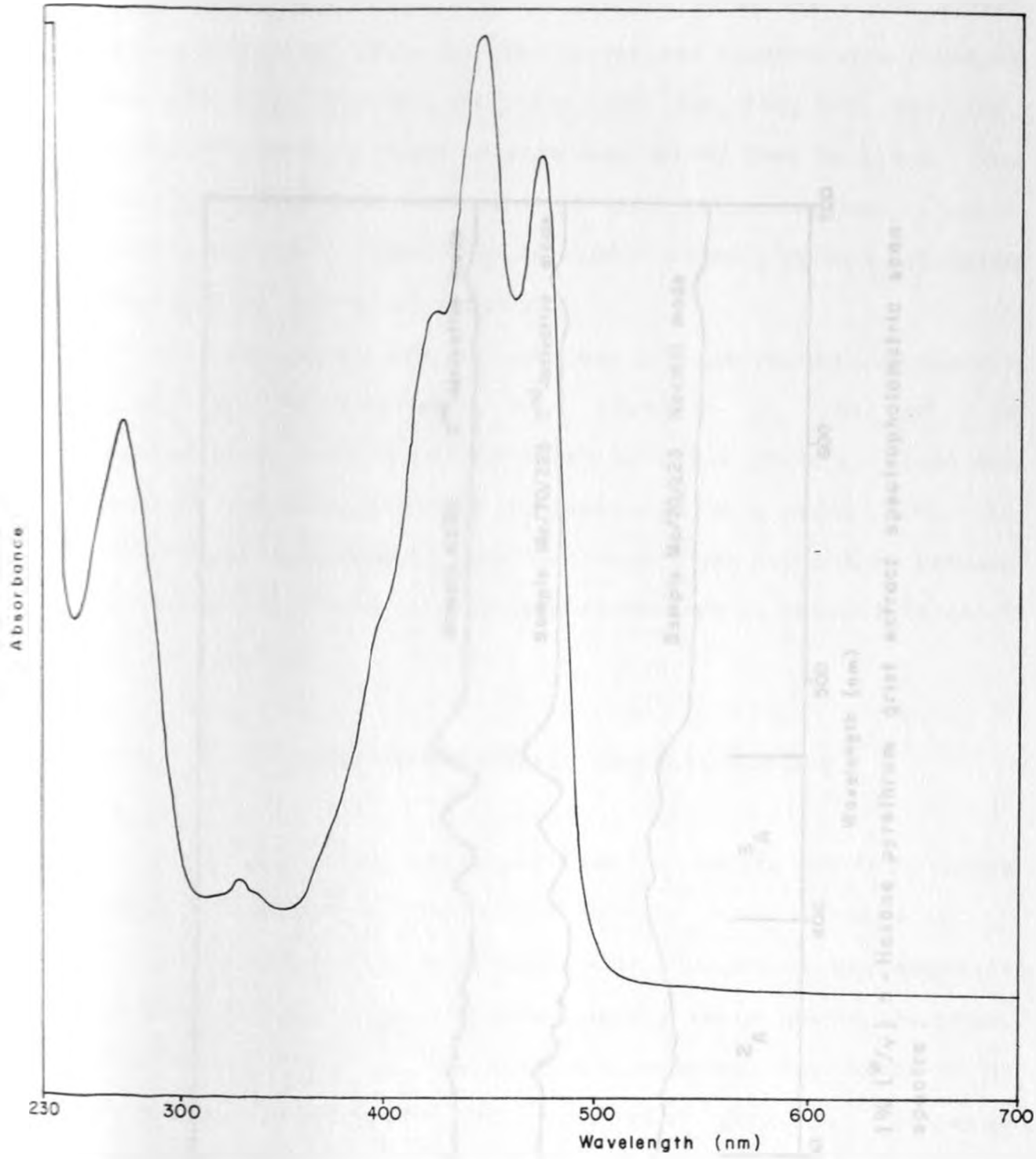


Fig. 13(a): 5% ($^{w}/_{v}$) n-Hexane composite pyrethrum grist extract spectrophotometric scan spectrum.

Similarly, scans on 1% (w/v) n-hexane pyrethrum flower grist samples Mo/70/223 and K235 were done and the spectra are shown in fig. 13(b).

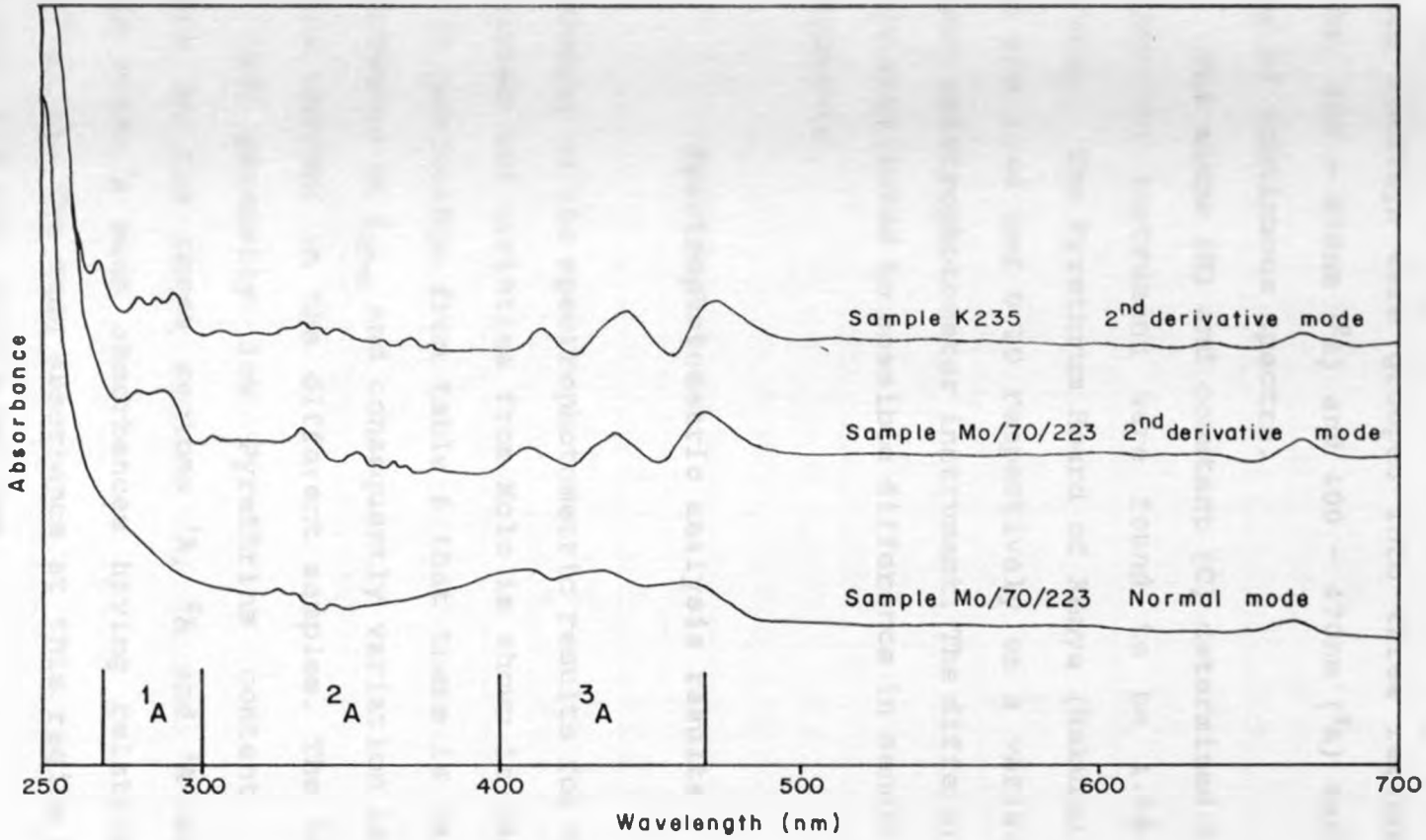


Fig. 13(b): 1% ($^{W}/_{V}$) n-Hexane pyrethrum grist extract spectrophotometric scan spectra.

Second derivative mode spectra are shown above the normal mode spectrum and peaks in the derivatised spectra were noted at 670, 470, 440, 420, 410, 390, 370, 350, 330, 294, 285, 280, 275, 265nm. The peak at 420nm in some samples shifted to 410nm. The absorbance readings were grouped into three regions: 265 - 300nm (^1A), 300 - 400nm (^2A) and 400 - 470nm (^3A) each of which comprised of continuous spectra.

The slope (M) and constant (C) determined for the Pye Unicam SP8-150 instrument were found to be **1.54** and **0.19** respectively. The Pyrethrum Board of Kenya (Nakuru) slope and constant are 1.44 and 0.29 respectively on a varian DMS - 80 double beam spectrophotometer instrument. The difference between the two is attributed to possible difference in sensitivities of two instruments.

3.1.1 Spectrophotometric analysis results

A summary of the spectrophotometric results for the various sample clones and varieties from Molo is shown in table 12.

It is noticeable from table 6 that there is variation in the absorbances at λ_{225nm} and consequently variation in the total Pyrethrins content in the different samples. The local clone samples had generally low Pyrethrins content. The mean absorbance at the three regions ^1A, ^2A and ^3A also showed variation with ^1A mean absorbances having relatively higher values (Fig.14). The mean absorbance at this region were found to be 0.646 $\pm$ 0.035, fairly uniform.

Table 12: Spectrophotometric analysis results for the various sample clones and varieties from Molo.

Slope (M) = 1.54 and constant (C) = 0.19

SAMPLE	A_{225nm}	Total Pys (%)	$^1\overline{A}$	$^2\overline{A}$	$^3\overline{A}$	$^1A^{1\%}_{1cm}$	$^2A^{1\%}_{1cm}$	$^3A^{1\%}_{1cm}$
1	0.445	0.88	0.642	0.176	0.189	72.8	20.2	21.7
2	0.609	1.13	0.646	0.233	0.254	57.7	20.8	22.7
3	0.695	1.26	0.705	0.211	0.191	56.4	16.9	15.3
4	0.806	1.43	0.671	0.212	0.156	46.6	14.7	10.8
5	0.860	1.45	0.689	0.219	0.168	45.6	14.5	11.1
6	0.889	1.56	0.713	0.208	0.164	46.0	13.4	10.6
7	0.890	1.56	0.690	0.188	0.178	43.9	12.0	11.5
8	0.903	1.58	0.667	0.152	0.099	42.2	9.7	6.3
9	0.908	1.59	0.651	0.258	0.243	40.7	16.3	15.4
10	0.928	1.62	0.670	0.203	0.142	41.6	12.7	8.9
11	0.930	1.62	0.637	0.205	0.176	39.3	12.7	10.9
12	0.937	1.63	0.634	0.204	0.248	38.9	12.5	15.2
13	0.936	1.63	0.607	0.193	0.181	37.2	11.3	10.6
14	0.980	1.70	0.600	0.201	0.184	35.1	11.6	10.6
15	0.998	1.73	0.637	0.163	0.106	36.4	9.4	6.1
16	1.020	1.76	0.595	0.204	0.132	33.8	11.6	7.5
17	1.021	1.76	0.607	0.178	0.094	34.1	10.0	5.3
18	1.071	1.84	0.615	0.206	0.191	33.4	11.2	10.4
19	1.109	1.89	0.625	0.218	0.138	30.6	11.7	7.4
20	1.201	2.04	0.628	0.154	0.059	25.0	7.6	2.9

Key:

$^1\overline{A}$ - Mean absorbance in the region 265-300nm

$^2\overline{A}$ - Mean absorbance in the region 300-400nm

$^3\overline{A}$ - Mean absorbance in the region 400-470nm

($^1A^{1\%}_{1cm}$) - Specific absorbance in the region 265-300nm

($^2A^{1\%}_{1cm}$) - Specific absorbance in the region 300-400nm

($^3A^{1\%}_{1cm}$) - Specific absorbance in the region 400-470nm

A_{225nm} - Absorbance at λ_{225nm} , 1-20 - refer to the various sample clones and varieties as coded in table 9.

It was not possible to correlate the % Pyrethrins with the mean absorbances. The mean absorbances at ^2A and ^3A showed similarity in trends. Most of the samples with high mean absorbance at ^2A also had high mean absorbance at ^3A and vice versa (Fig.14).

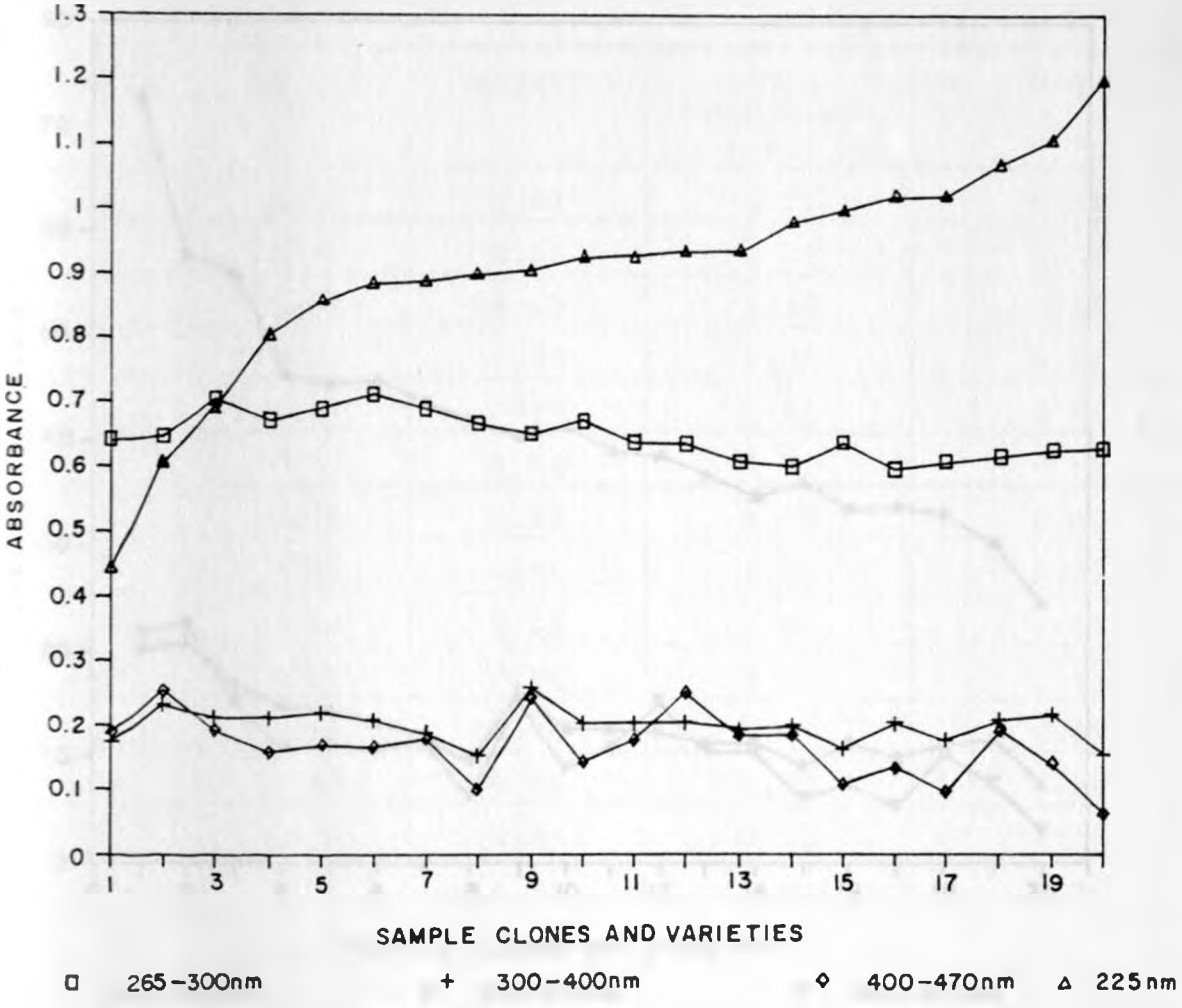


Fig.14: Variation of absorbances at 225nm, 265-300nm, 300-400nm and 400-470nm.

The variation in the calculated specific absorbances shown in Fig.15 depict a general negative trend. The samples with high Pyrethrins content generally show low specific absorbance values in all the three regions.

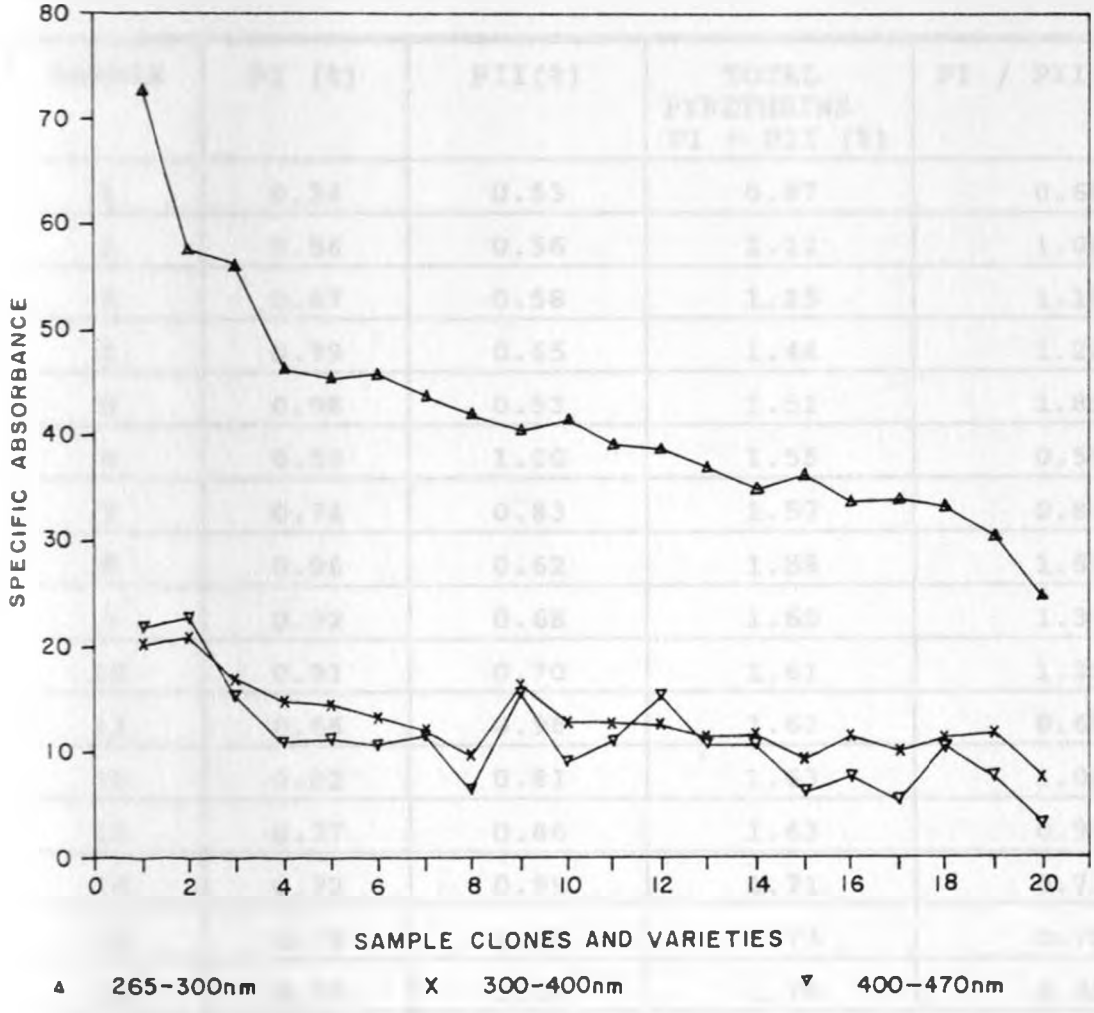


Fig.15: Specific absorbance variation in the three electromagnetic regions.

3.1.2 AOAC Chemical analysis results.

The spectrophotometric results outlined and explained in section 3.1.1 above showed only the total Pyrethrins content in the various samples. Table 13 is more elaborate and it shows the Pyrethrins I and Pyrethrins II contents in the various samples as determined using the AOAC method.

Table 13 : AOAC analysis results for the various sample clones and varieties from Molo.

SAMPLE	PI (%)	PII(%)	TOTAL PYRETHRINS PI + PII (%)	PI / PII RATIO
1	0.34	0.53	0.87	0.64
2	0.56	0.56	1.12	1.00
3	0.67	0.58	1.25	1.16
4	0.79	0.65	1.44	1.22
5	0.98	0.53	1.51	1.85
6	0.55	1.00	1.55	0.55
7	0.74	0.83	1.57	0.89
8	0.96	0.62	1.58	1.55
9	0.92	0.68	1.60	1.35
10	0.91	0.70	1.61	1.30
11	0.66	0.96	1.62	0.69
12	0.82	0.81	1.63	1.01
13	0.77	0.86	1.63	0.90
14	0.72	0.99	1.71	0.73
15	0.73	1.00	1.73	0.73
16	0.70	1.06	1.76	0.66
17	0.76	1.02	1.78	0.75
18	0.95	0.89	1.84	1.07
19	0.57	1.29	1.86	0.44
20	0.86	1.17	2.03	0.74
MEAN	0.75	0.84	1.58	0.96

The mean Pyrethrins I, Pyrethrins II, total Pyrethrins and Pyrethrins /Pyrethrins II ratios were found to be 0.75 ± 0.16%, 0.84 ± 0.22%, 1.58 ± 0.26% and 0.96 ± 0.36 respectively.

A comparison of the variation between the total Pyrethrins content with the Pyrethrins I / Pyrethrins II ratios shows great fluctuation. Certain samples of low total Pyrethrins content have high PI / PII ratio and vice versa. On average 70% of the samples had ≤ 1.0 value of Pyrethrins I / Pyrethrins II ratio (Fig.16).

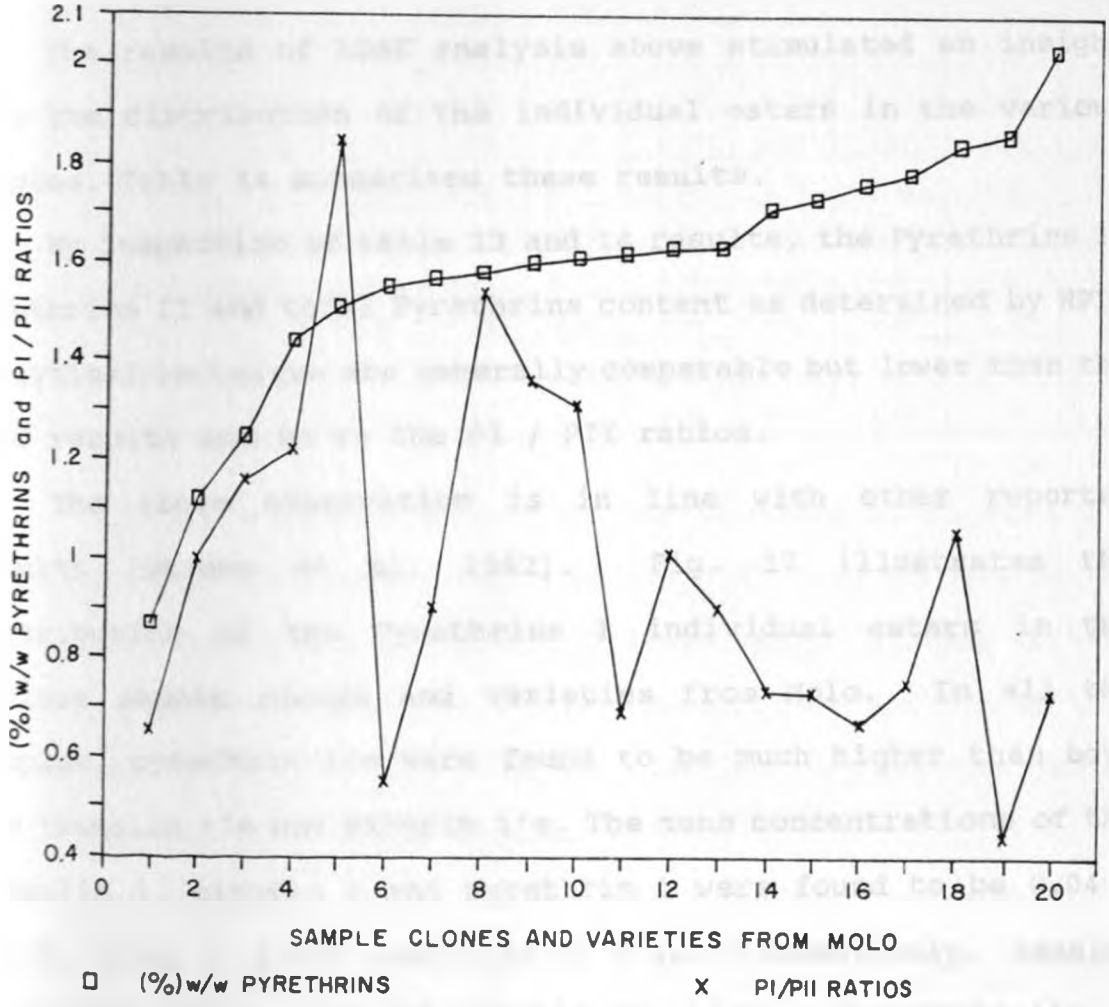


Fig.16: The variation of (%) total Pyrethrins with PI/PII ratios.

The distribution of Pyrethrins I and Pyrethrins II in the various clone and variety samples was assessed as indicated in Fig.19. The various samples showed great fluctuation in Pyrethrins I and Pyrethrins II content. Sample Ma/71/423 had the highest Pyrethrins II content while local clone C47 and clone 4331 had the lowest. Clone 4331 regarded as a standard clone had the highest Pyrethrins I and subsequently the highest PI / PII ratio (Fig. 16).

3.1.3 **HPLC analysis results.**

The results of AOAC analysis above stimulated an insight into the distribution of the individual esters in the various samples. Table 14 summarises these results.

By inspection of table 13 and 14 results, the Pyrethrins I, Pyrethrins II and total Pyrethrins content as determined by HPLC analytical technique are generally comparable but lower than the AOAC results and so to the PI / PII ratios.

The above observation is in line with other reported results (Otieno *et al*, 1982). Fig. 17 illustrates the distribution of the Pyrethrins I individual esters in the various sample clones and varieties from Molo. In all the samples, pyrethrin 1's were found to be much higher than both the jasmolin 1's and cinerin 1's. The mean concentrations of the Jasmolin 1, cinerin 1 and pyrethrin 1 were found to be 0.04 ± 0.02%, 0.06 ± 0.02% and 0.55 ± 0.12% respectively. Samples Mo/70/223, 4331 and Ks/75/336 had the highest pyrethrin 1's.

Table 14: HPLC analysis results for the various sample clones and varieties from Molo.

SAMPLE	J1(%)	C1(%)	P1(%)	PI(%)	J2(%)	C2(%)	P2(%)	PII(%)	PI+PII (%)	PI/PII RATIO
1	0.02	0.02	0.30	0.34	0.02	0.06	0.30	0.38	0.72	0.89
2	0.03	0.05	0.43	0.51	0.03	0.07	0.32	0.42	0.93	1.21
3	0.03	0.08	0.50	0.61	0.05	0.09	0.36	0.52	1.11	1.22
4	0.07	0.05	0.70	0.82	0.07	0.06	0.44	0.57	1.39	1.44
5	0.07	0.05	0.82	0.94	0.05	0.06	0.40	0.51	1.45	1.84
6	0.03	0.05	0.42	0.50	0.07	0.09	0.80	0.96	1.46	0.52
7	0.04	0.06	0.57	0.67	0.06	0.12	0.53	0.71	1.38	0.94
8	0.07	0.07	0.71	0.85	0.06	0.05	0.47	0.58	1.43	1.47
9	0.09	0.06	0.82	0.97	0.06	0.06	0.43	0.55	1.52	1.76
10	0.07	0.08	0.69	0.84	0.08	0.11	0.50	0.69	1.53	1.22
11	0.05	0.05	0.57	0.67	0.10	0.13	0.61	0.84	1.51	0.80
12	0.06	0.07	0.74	0.87	0.06	0.08	0.54	0.68	1.55	1.28
13	0.03	0.08	0.69	0.80	0.06	0.13	0.56	0.75	1.55	1.07
14	0.05	0.06	0.58	0.69	0.07	0.15	0.73	0.95	1.64	0.73
15	0.03	0.03	0.66	0.72	0.07	0.09	0.72	0.88	1.60	0.82
16	0.05	0.07	0.60	0.72	0.10	0.16	0.68	0.94	1.66	0.77
17	0.07	0.08	0.63	0.78	0.09	0.15	0.65	0.89	1.67	0.88
18	0.03	0.14	0.69	0.86	0.06	0.19	0.60	0.85	1.72	1.01
19	0.05	0.05	0.47	0.57	0.10	0.10	0.96	1.16	1.73	0.49
20	0.04	0.10	0.62	0.76	0.11	0.23	0.79	1.13	1.89	0.67

The mean concentrations of the jasmolin 2, cinerin 2 and pyrethrin 2 were found to be 0.06 ± 0.02%, 0.10 ± 0.04% and 0.51 ± 0.15% respectively. Samples Ma/71/423, Ks/75/313 and Kr/74/122 had the highest concentration of pyrethrin 2. The trends in the contents of the jasmolin 2's and cinerin 2's like the jasmolin 1's and cinerin 1's are similar (Fig.18)

As shown in Fig.19, the results of the two methods are comparable but generally the HPLC results are lower than AOAC results and particularly so in the Pyrethrins II. The Pyrethrins I from both methods are of low level as compared to Pyrethrins II except for the sample which had PI / PII ratio higher than

1.0. The PI / PII ratios from HPLC are generally higher than for AOAC indicating the possibility of AOAC over estimating Pyrethrins II.

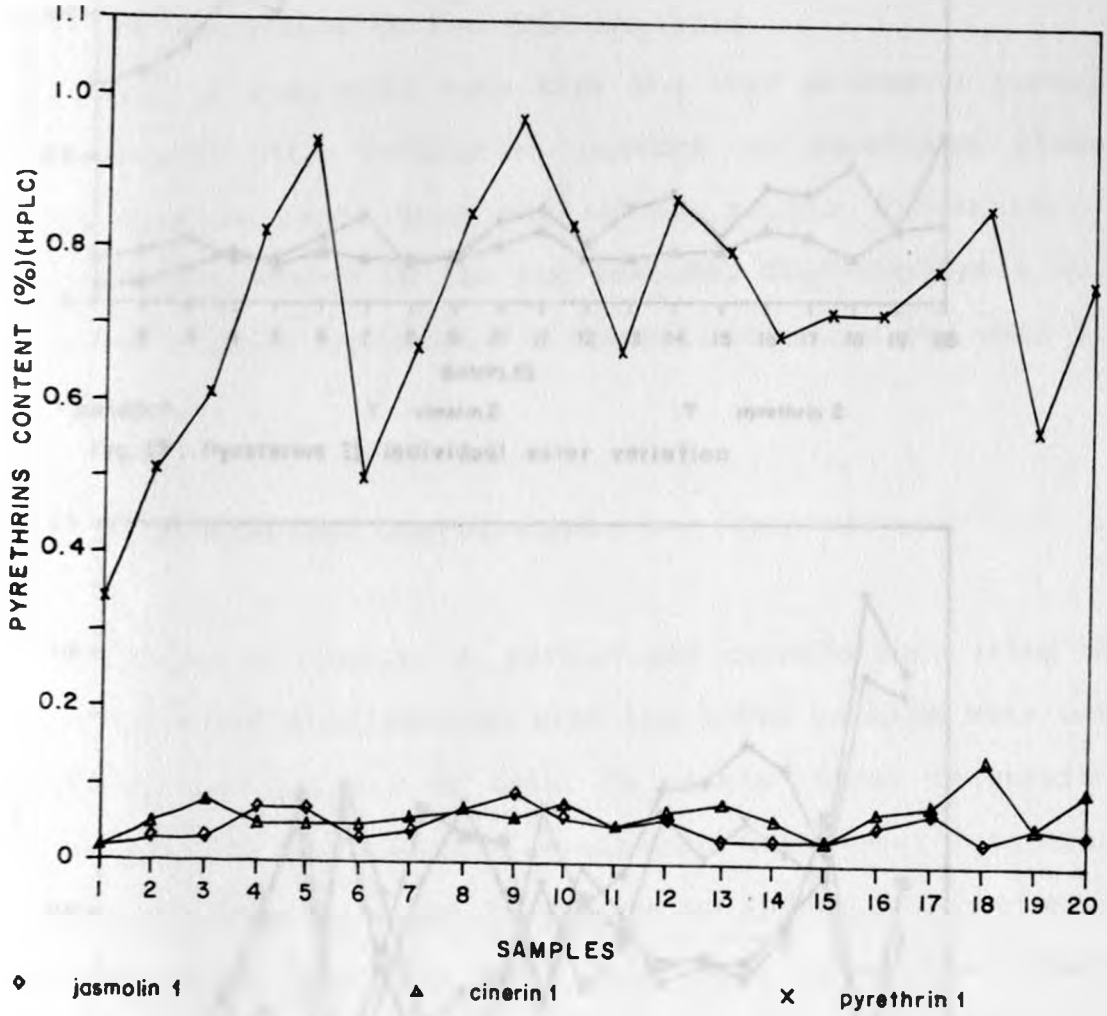


Fig. 17 : Pyrethrins I individual ester variation .

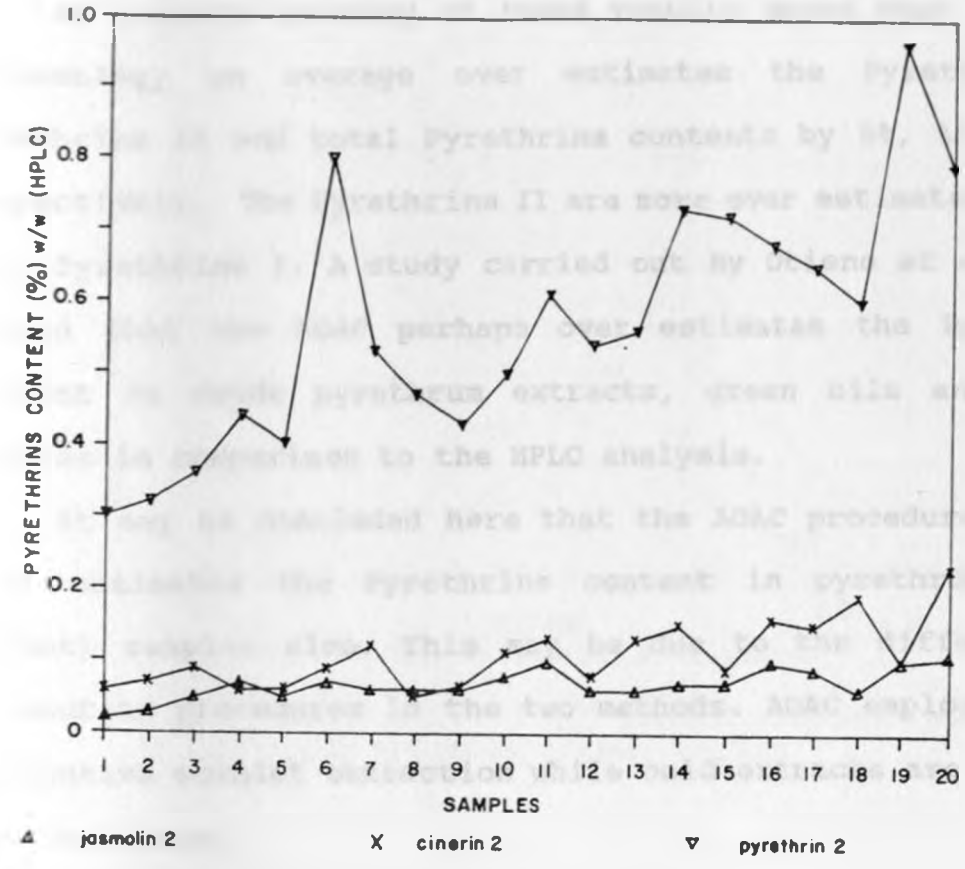


Fig. 18: Pyrethrins II individual ester variation

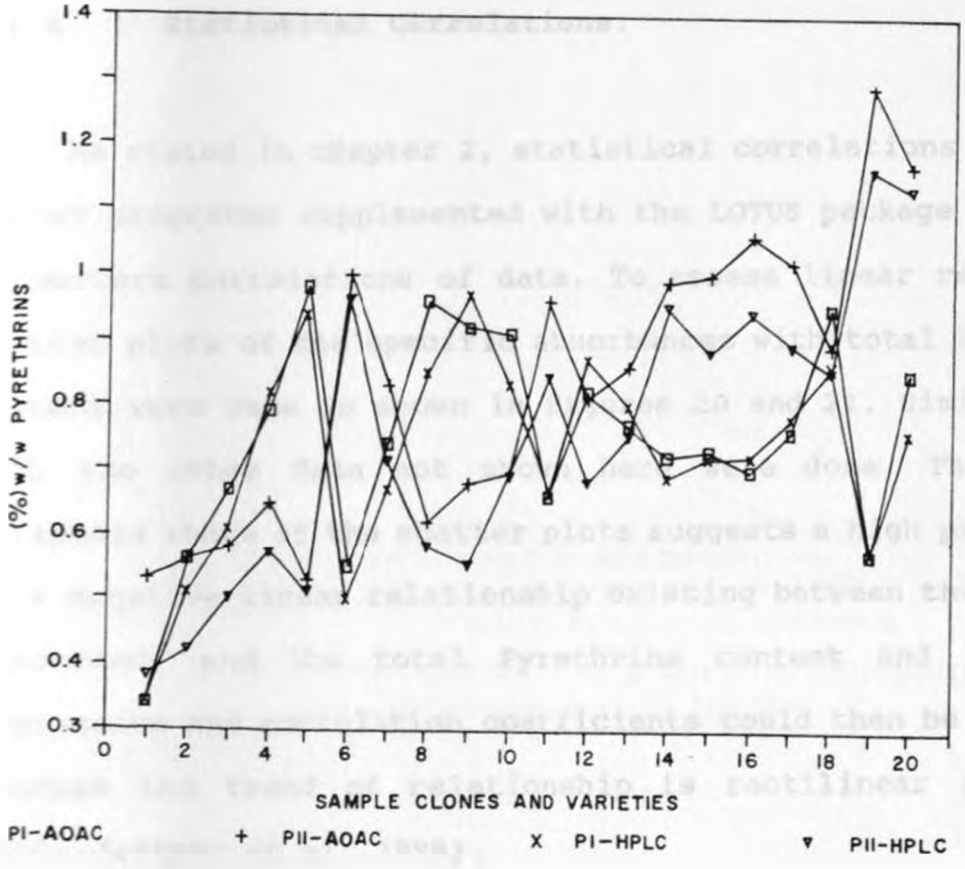


Fig. 19: A comparison of Pyrethrins I and Pyrethrins II contents by AOAC and HPLC analytical techniques.

An indepth scrutiny of these results shows that the AOAC methodology on average over estimates the Pyrethrins I, Pyrethrins II and total Pyrethrins contents by 8%, 11% and 8% respectively. The Pyrethrins II are more over estimated by AOAC than Pyrethrins I. A study carried out by Otieno *et al* (1982) showed that the AOAC perhaps over estimates the Pyrethrins content in crude pyrethrum extracts, green oils and sludge samples in comparison to the HPLC analysis.

It may be concluded here that the AOAC procedure perhaps over estimates the Pyrethrins content in pyrethrum flower (Grist) samples also. This may be due to the difference in extraction procedures in the two methods. AOAC employs a more exhaustive soxhlet extraction while cold extracts are used for HPLC analysis.

3.1.4 **Statistical Correlations.**

As stated in chapter 2, statistical correlations using the INSTAT programme supplemented with the LOTUS package were used to perform correlations of data. To assess linear regression, scatter plots of the specific absorbances with total Pyrethrins content were done as shown in figures 20 and 21. Similar plots with the other data not shown here were done. The general ellipsoid shape of the scatter plots suggests a high probability of a negative linear relationship existing between the specific absorbance and the total Pyrethrins content and so linear regression and correlation coefficients could then be evaluated because the trend of relationship is rectilinear (Aggarwal, 1990; Jeffrey *et al*, 1989).

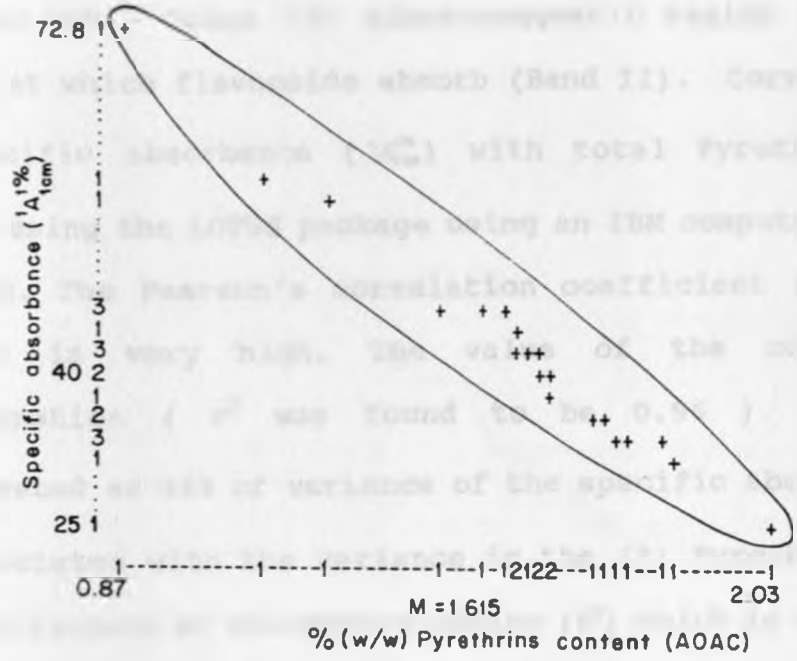


Fig.20 : Scatter plot of ($^1A^{1\%}_{1cm}$) specific absorbance with total Pyrethrins content (AOAC)

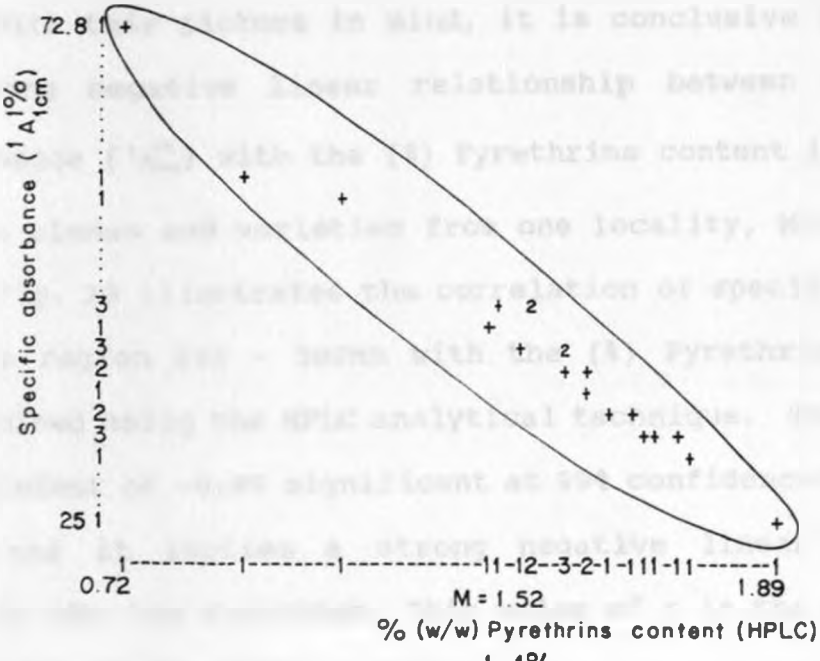


Fig.21: Scatter plot of ($^1A^{1\%}_{1cm}$) specific absorbance with total Pyrethrins content (HPLC)

The 265 - 300nm (^1A) electromagnetic region is within the region at which flavonoids absorb (Band II). Correlation graph of specific absorbance ($^1A^{1\%}_{1cm}$) with total Pyrethrins content (AOAC) using the LOTUS package using an IBM computer is shown in fig. 22. The Pearson's correlation coefficient (r) of -0.98; P(0.01) is very high. The value of the coefficient of determination (r^2 was found to be 0.96). This can be interpreted as 96% of variance of the specific absorbance ($^1A^{1\%}_{1cm}$) is associated with the variance in the (%) Pyrethrins content. The coefficient of non-determination (κ^2) which is defined as the proportion of the variance in specific absorbance not associated with the variance in the Pyrethrins content is only 4%. κ^2, defined as the coefficient of alienation given by $\kappa^2 = 1 - r^2$, reflects the degree of non-relationship.

With this picture in mind, it is conclusive that there is a strong negative linear relationship between the specific absorbance ($^1A^{1\%}_{1cm}$) with the (%) Pyrethrins content in the various sample clones and varieties from one locality, Molo.

Fig. 23 illustrates the correlation of specific absorbance in the region 265 - 300nm with the (%) Pyrethrins content as determined using the HPLC analytical technique. The correlation coefficient of -0.98 significant at 99% confidence limit is very high and it implies a strong negative linear relationship between the two variables. This value of r is the same as the r value found with AOAC results.

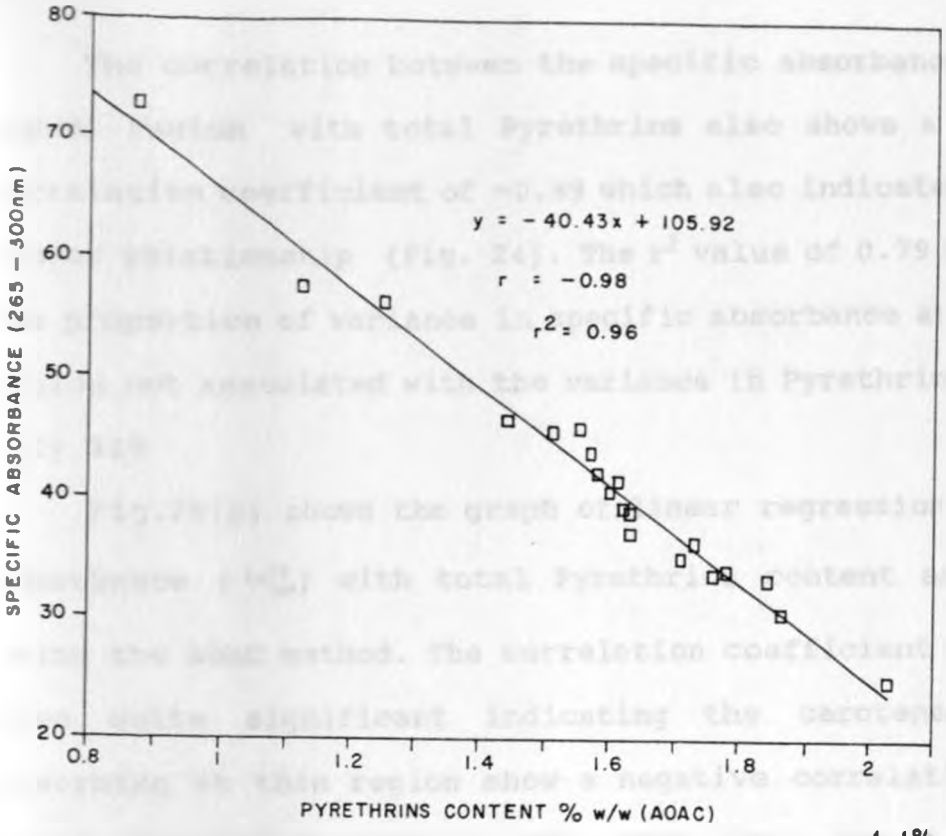


Fig. 22 : Linear regression graph of specific absorbance ($^{1}A^{1\%}_{1cm}$) with total Pyrethrins content (AOAC) .

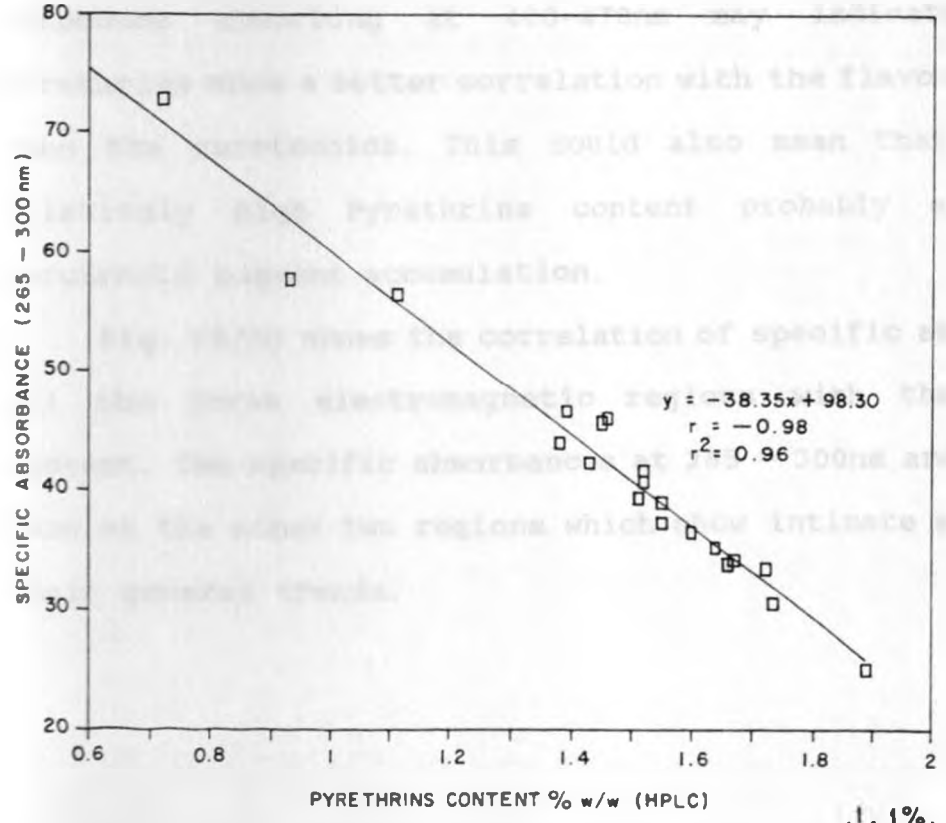


Fig. 23: Linear regression graph of specific absorbance ($^{1}A^{1\%}_{1cm}$) with total Pyrethrins content (HPLC) .

The correlation between the specific absorbance at (300 - 400nm) region with total Pyrethrins also shows a significant correlation coefficient of -0.89 which also indicates a negative linear relationship (Fig. 24). The r^2 value of 0.79 implies that the proportion of variance in specific absorbance at 300 - 400nm region not associated with the variance in Pyrethrins content is only 21%

Fig.25(a) shows the graph of linear regression of specific absorbance ($^{s}A_{1cm}^{1\%}$) with total Pyrethrins content as determined using the AOAC method. The correlation coefficient of - 0.85 is also quite significant indicating the carotenoid pigments absorbing at this region show a negative correlation with the total Pyrethrins content. The fact that the r value with compounds absorbing at 265-300nm region is higher than for the compounds absorbing at 400-470nm may indicate that the Pyrethrins show a better correlation with the flavonoid pigments than the carotenoids. This could also mean that flowers of relatively high Pyrethrins content probably exhibit high carotenoid pigment accumulation.

Fig. 25(b) shows the correlation of specific absorbances in all the three electromagnetic regions with the Pyrethrins content. The specific absorbances at 265 - 300nm are much higher than at the other two regions which show intimate similarity in their general trends.

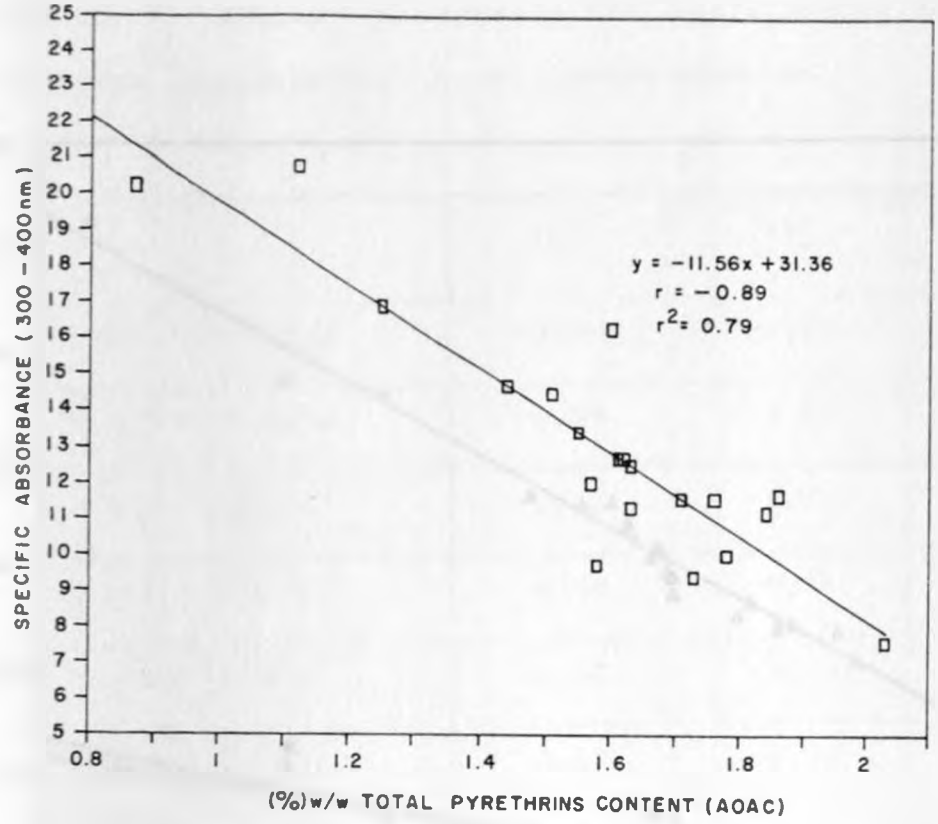


Fig 24 : Graph of linear regression of specific absorbance ($^{2}A^{1\%}_{1cm}$) with total Pyrethrins content (AOAC).

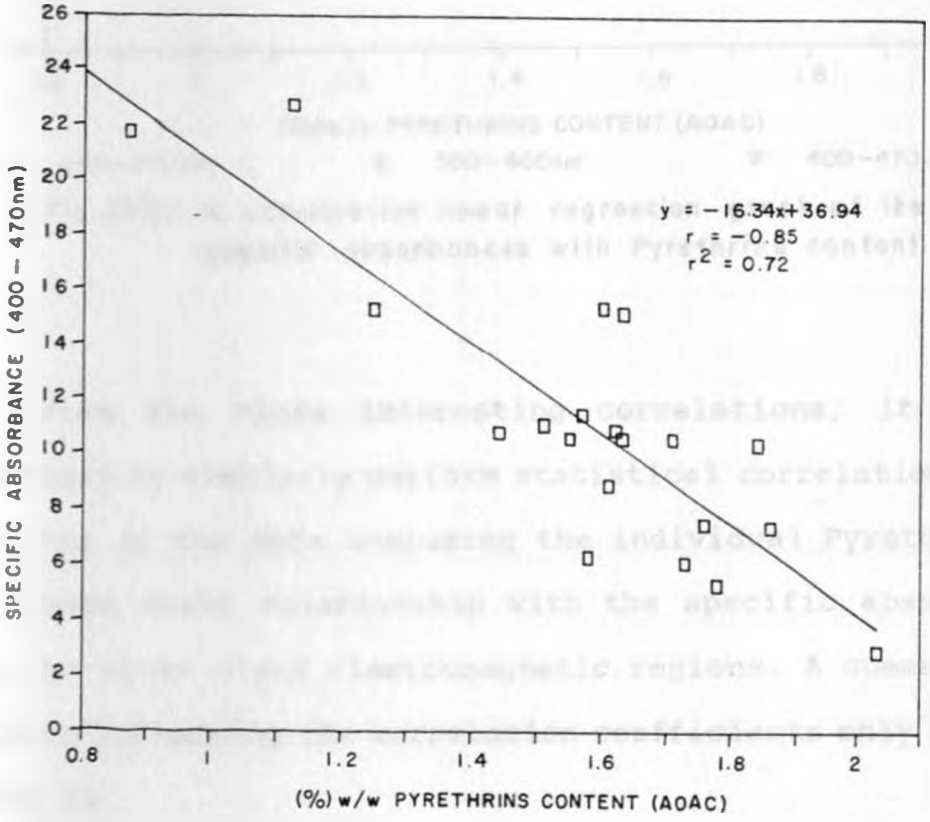


Fig.25(a): Graph of linear regression of specific absorbance ($^{3}A^{1\%}_{1cm}$) with total Pyrethrins content (AOAC).

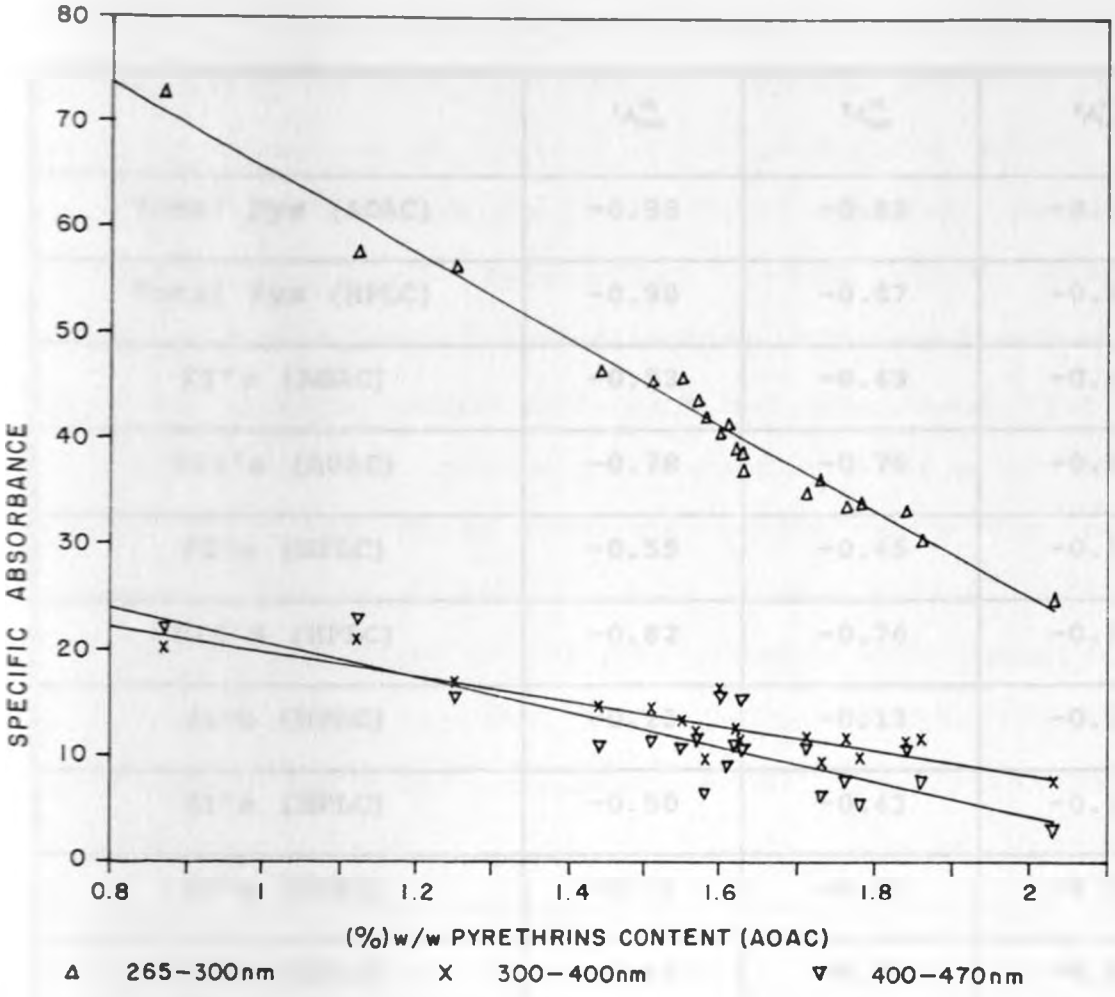


Fig. 25(b): A comparative linear regression graph of the three cited specific absorbances with Pyrethrins content (AOAC)

From the above interesting correlations, it was found necessary to similarly perform statistical correlations with all the rest of the data including the individual Pyrethrin esters to assess their relationship with the specific absorbances at all the three cited electromagnetic regions. A summary of this results indicating the correlation coefficients only is shown in table 15.

Table 15 : Summary of correlation coefficients between the Pyrethrins contents with specific absorbances.

	$^1A_{1cm}^{1\%}$	$^2A_{1cm}^{1\%}$	$^3A_{1cm}^{1\%}$
Total Pys (AOAC)	-0.98	-0.89	-0.85
Total Pys (HPLC)	-0.98	-0.87	-0.84
PI's (AOAC)	-0.53	-0.49	-0.43
PII's (AOAC)	-0.78	-0.70	-0.69
PI's (HPLC)	-0.55	-0.45	-0.39
PII'S (HPLC)	-0.82	-0.76	-0.76
J1's (HPLC)	-0.23	-0.13	-0.23
C1's (HPLC)	-0.50	-0.43	-0.32
P1's (HPLC)	-0.51	-0.43	-0.37
J2's (HPLC)	-0.82	-0.73	-0.83
C2's (HPLC)	-0.65	-0.60	-0.54
P2's (HPLC)	-0.76	-0.71	-0.72
PI / PII (AOAC)	+0.18	+0.17	+0.15
PI / PII (HPLC)	+0.28	+0.34	+0.33

Note: At 99% confidence limit, >± 0.54 correlation coefficient is significant basing on twenty samples (Fisher and Yates, 1963).

Key: Pys - Pyrethrins ; PI's - Pyrethrins I ; PII's - Pyrethrins II

As a matter of confirmation of the results above, five samples selected at certain Pyrethrins content levels as shown in table 16 were each extracted with n-hexane (AF) to give approximately 1% (w/v) Pyrethrins content extracts and re-analysed using the UV visible spectrophotometer again. From table 16 it is clear that the five different samples of approximately equal Pyrethrins content show different absorbances at the various wavelengths. Visually it was noticed that the original sample C47 which had the lowest Pyrethrins content had the most intense yellow colour of the grist extract. Sample Kr/74/122 which was the sample with the highest Pyrethrins content showed the least intense yellow extract.

The yellow colour of the five extracts when viewed against a white background in 25ml vials increased from Kr/74/122 ▸ Mo/70/1124 ▸ Ks/75/313 ▸ Nyamasibi ▸ C47. The vials which were coded arbitrarily as 1,2,3,4 and 5 were arranged in order of increasing yellowness corresponding to the above sample order by three different persons who had no prior idea of the nature of the samples. It is evident from this results that samples of low Pyrethrins content have proportionally high yellow pigmentation levels. An initial attempt to visually array the grist samples in terms of the yellow colour proved futile at the beginning of the project. The mean absorbances of the five samples at the three regions 265-300nm, 300-400nm and 400-470nm decreased steadily with increase in the Pyrethrins content depicting a negative linear correlation (fig. 26).

Table 16: Selected Samples {≈ 1% (w/w) Pyrethrins Content} absorbance readings at various Wavelengths.

	1	2	3	4	5
Sample	C47	NYAMASIBI	Ks/75/313	Mo/70/1124	Kr/74/122
Weight (g) in 100ml n-Hexane (AF)	0.5747	0.4032	0.3268	0.2747	0.2404
Absorbance at λ_{225nm}	0.487	0.504	0.493	0.443	0.495
Pyrethrins Content. (%)	0.94	0.97	0.95	0.87	0.95
Sample\Wavelength (nm)	C47	NYAMASIBI	Ks/75/313	Mo/70/1124	Kr/74/122
265	0.345	0.329	0.252	0.191	0.151
275	0.290	0.251	0.192	0.145	0.112
280	0.270	0.234	0.183	0.135	0.113
285	0.240	0.209	0.165	0.121	0.102
294	0.206	0.158	0.124	0.088	0.078
330	0.098	0.094	0.063	0.039	0.042
350	0.065	0.078	0.046	0.027	0.028
380	0.068	0.090	0.043	0.022	0.024
385	0.074	0.097	0.046	0.022	0.025
410	0.107	0.131	0.058	0.029	0.033
420	0.091	0.107	0.050	0.024	0.026
440	0.076	0.083	0.044	0.020	0.019
470	0.058	0.061	0.032	0.015	0.015
670	0.028	0.039	0.021	0.013	0.017
$^{1}\overline{A}$	0.270	0.236	0.183	0.136	0.112
$^{2}\overline{A}$	0.076	0.090	0.050	0.028	0.030
$^{3}\overline{A}$	0.074	0.110	0.045	0.022	0.023

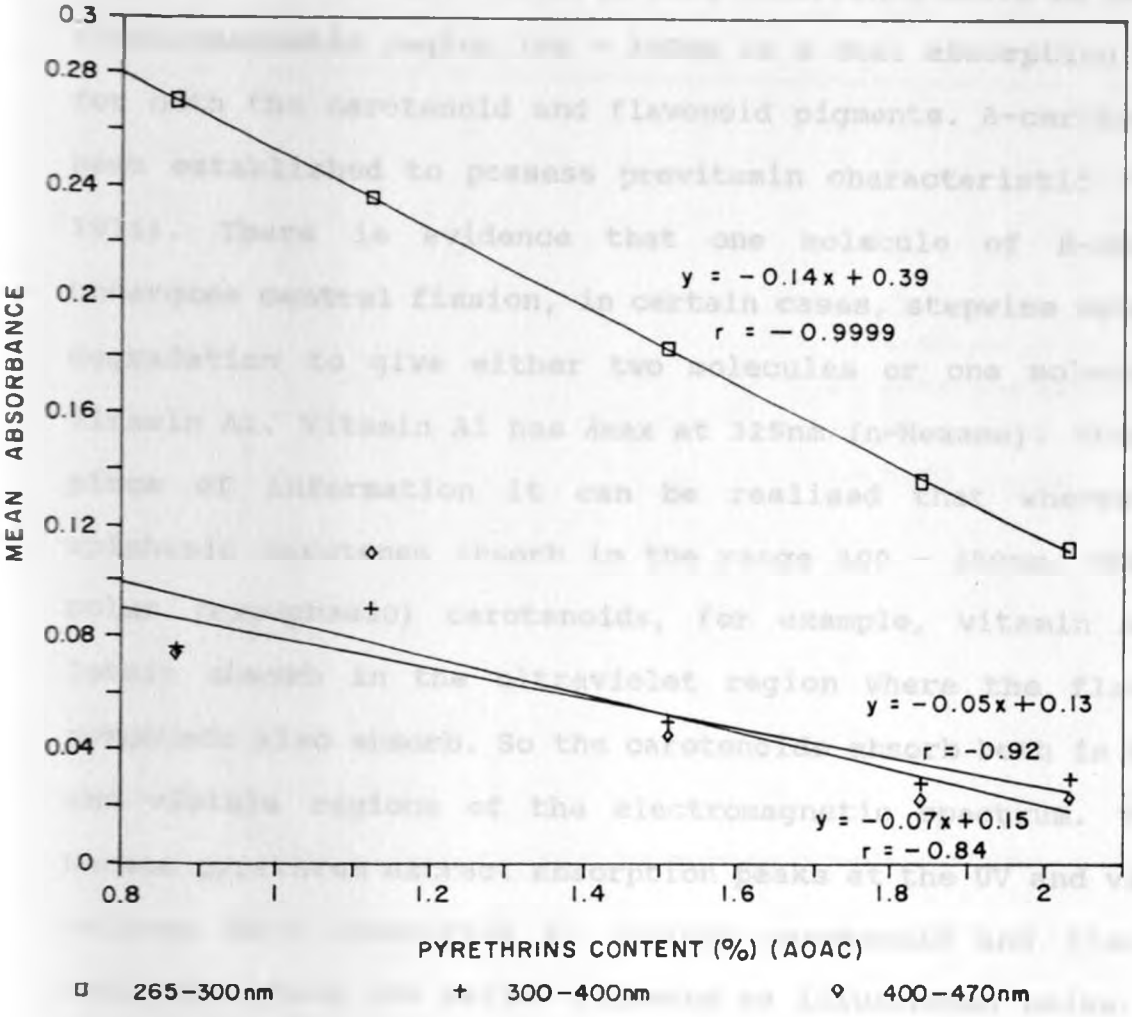


Fig.26: Linear regression of mean absorbances with (%) w/w Pyrethrins content in the five selected sample clones.

The correlation coefficient of -0.99, -0.92 and -0.84 in the three regions ^{1}A , ^{2}A and ^{3}A are close to the ones registered with all the twenty samples when using specific absorbance index. This confirms that proportionally, a strong negative linear relationship exists between the Pyrethrins and the yellow pigmentation in pyrethrum flowers. Similarity in the

correlations of pigments at 300-400nm and 400-470nm with the total Pyrethrins has also been observed here as in fig.25(b).

A possible explanation of this occurrence could be that the electromagnetic region 300 - 400nm is a dual absorption region for both the carotenoid and flavonoid pigments. ß-carotene has been established to possess provitamin characteristic (Finar, 1975). There is evidence that one molecule of ß-carotene undergoes central fission, in certain cases, stepwise oxidative degradation to give either two molecules or one molecule of vitamin A1. Vitamin A1 has λmax at 325nm (n-Hexane). From this piece of information it can be realised that whereas the epiphasic carotenes absorb in the range 400 - 480nm, the more polar (hypophasic) carotenoids, for example, vitamin A1 and lutein absorb in the ultraviolet region where the flavonoid compounds also absorb. So the carotenoids absorb both in the UV and visible regions of the electromagnetic spectrum. The n-Hexane pyrethrum extract absorption peaks at the UV and visible regions were identified to include carotenoid and flavonoid compounds which are yellow pigments as illustrated below.

The various pyrethrum extracts re-extracted in n-Hexane (AF) (Section 2.6.6.4) were scanned in the UV and visible regions to assess their difference in composition. The column chromatography isolate fractions shown from t.l.c. screening to contain carotenoid and flavonoid compounds (Section 2.6.7 - 2.6.10) were similarly scanned in the UV and visible region.

A β-carotene authentic sample was also scanned. Fig. 27(a) and (b), 30 and 31 show the spectra of the various pyrethrum extracts, n-hexane carotenoid fraction, β-carotene and flavonoid isolate fraction all scanned in n-Hexane (AF).

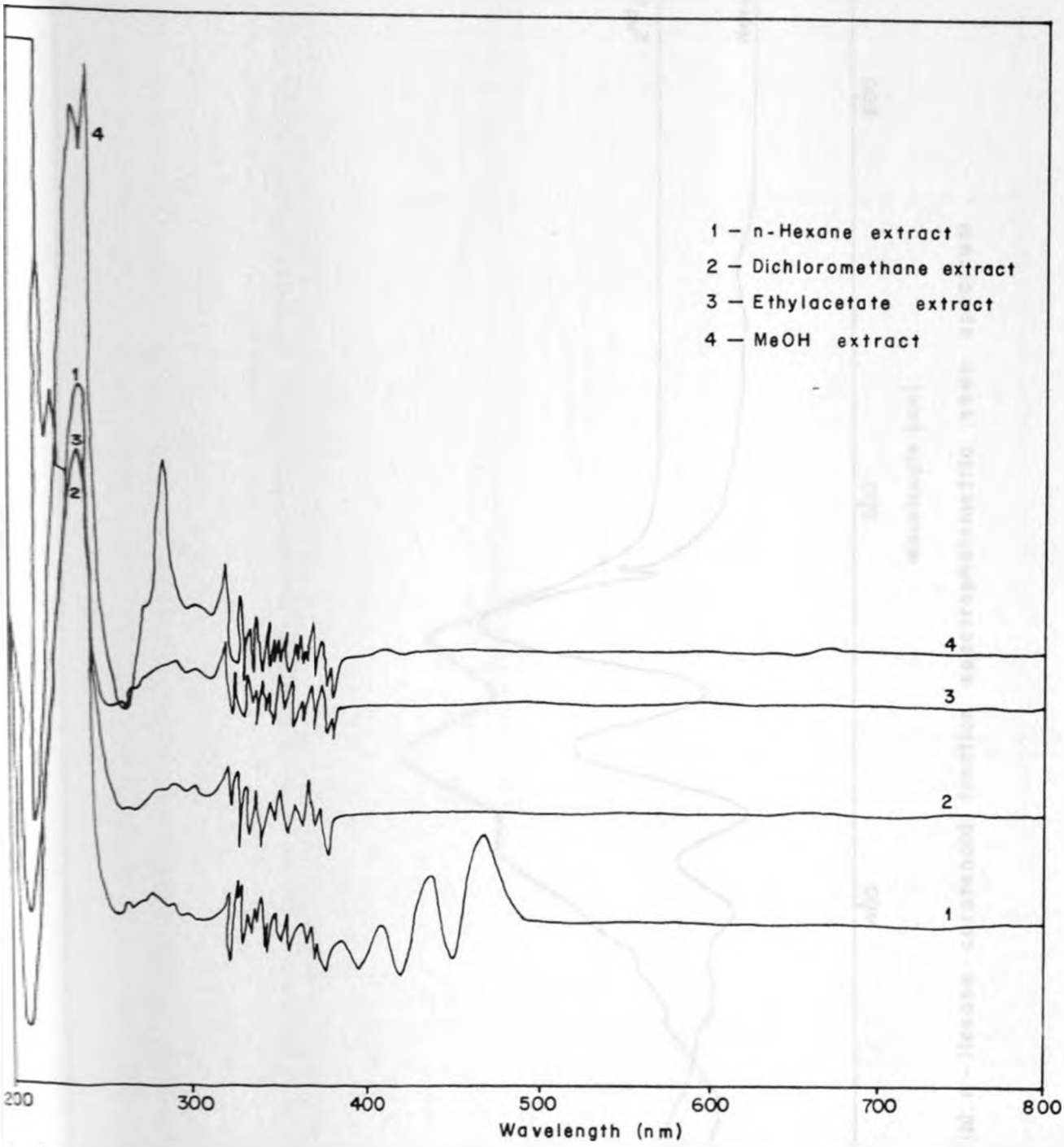


Fig.27(a): Spectrophotometric scan of n-Hexane, dichloromethane, Ethylacetate and Methanol pyrethrum extracts run in n-Hexane (AF) spectra.

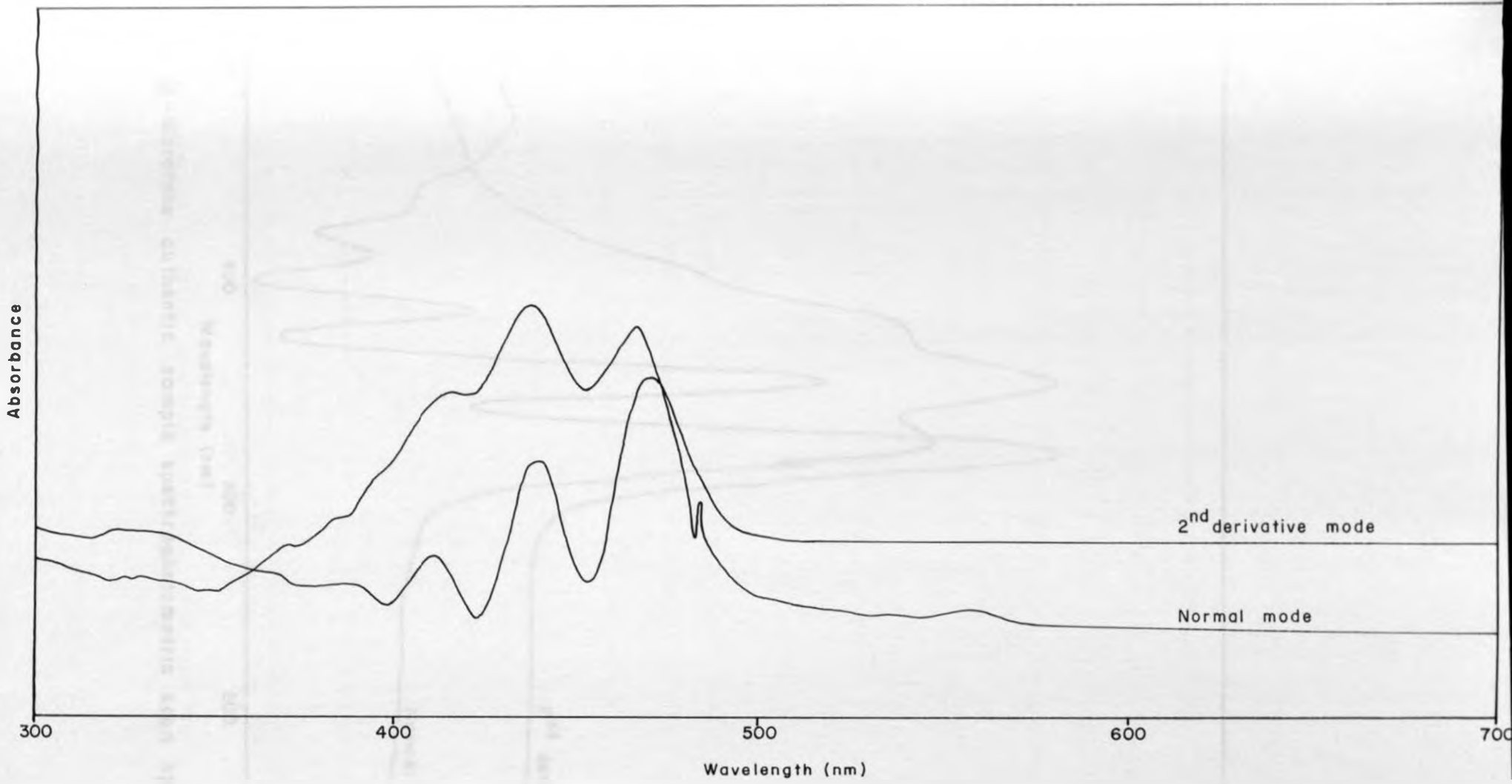


Fig. 27(b): n - Hexane carotenoid fraction spectrophotometric scan spectrum .

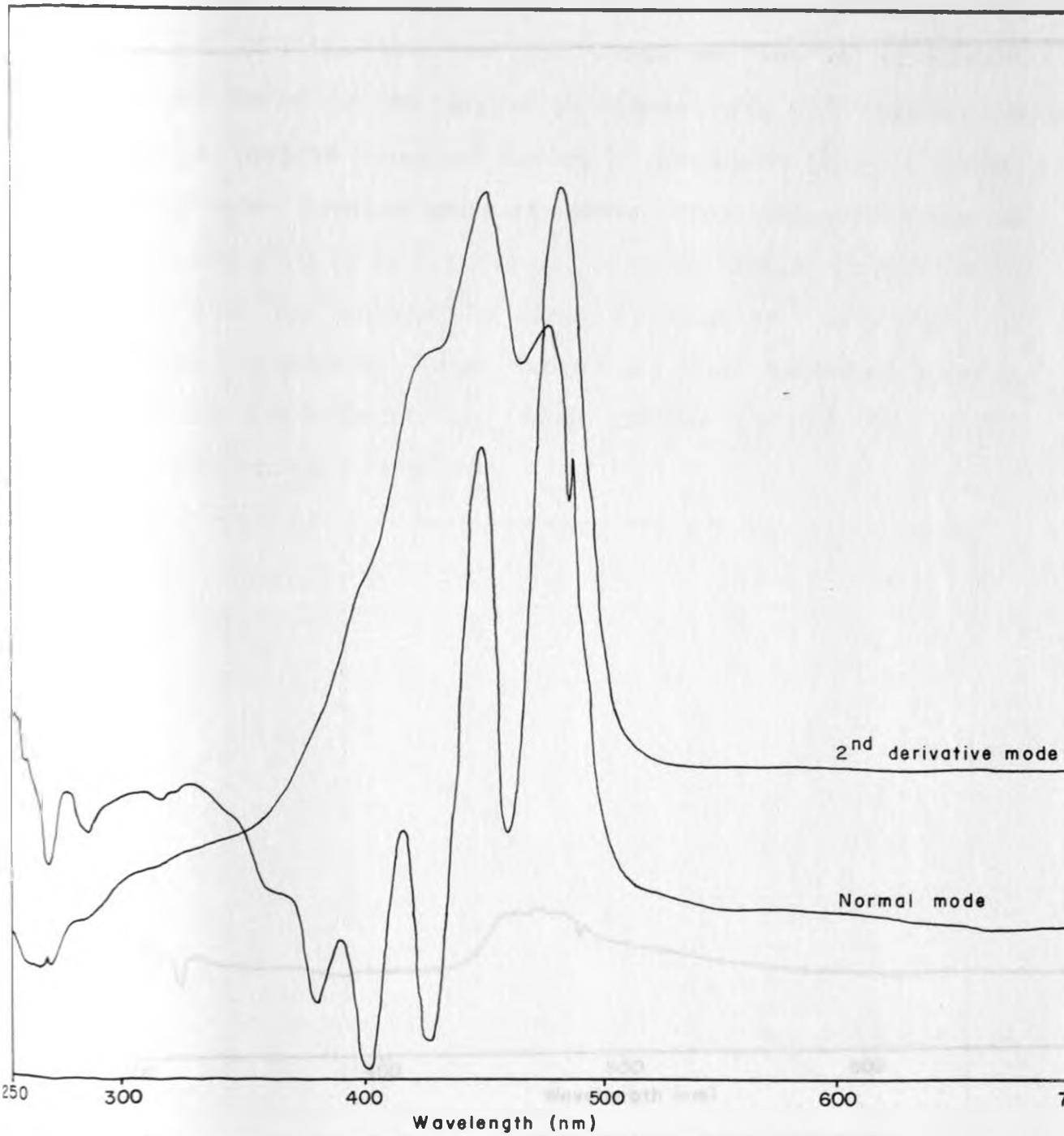


Fig. 28 : β - Carotene authentic sample spectrophotometric scan spectrum .

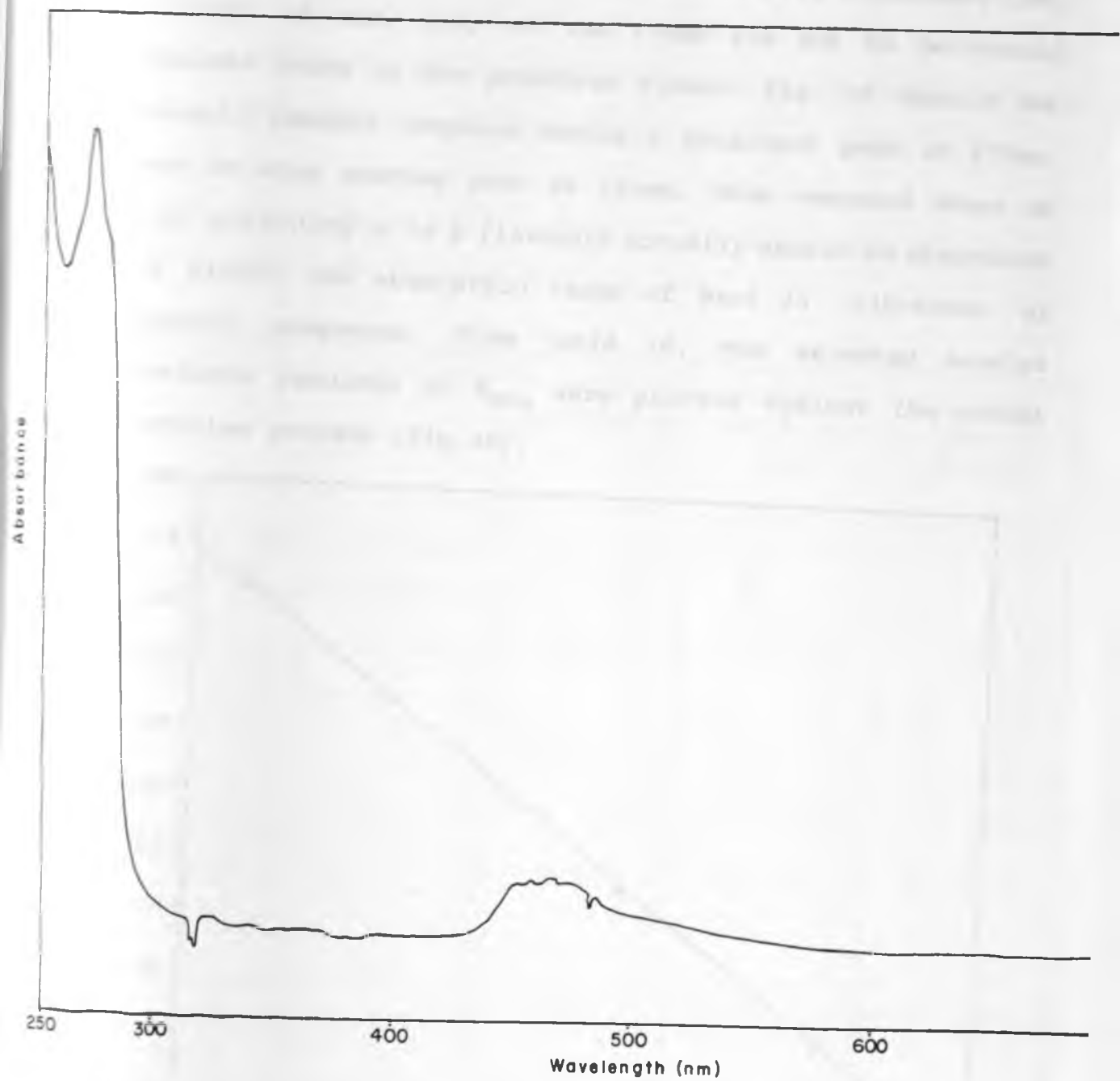


Fig.29 : Flavonoid fraction spectrophotometric scan spectrum.

From the carotenoid fraction scan spectrum (fig. 27a), absorbance peaks were noted at 420, 440 and 470nm. The authentic β-carotene spectrum (fig. 28) showed absorbance peaks at 410, 420, 440 and 470nm. It was concluded from this occurrence that the peaks at 410, 420, 440 and 470nm are due to carotenoid compounds found in the pyrethrum flower. Fig. 29 depicts the flavonoid isolate compound having a prominent peak at 275nm. There is also another peak at 285nm. This compound shown on t.l.c. screening to be a flavonoid actually showed an absorbance peak within the absorption range of Band II (240-380nm) of flavonoid compounds. From table 16, the selected samples absorbance readings at λ_{225nm} were plotted against the actual Pyrethrins content (Fig.30).

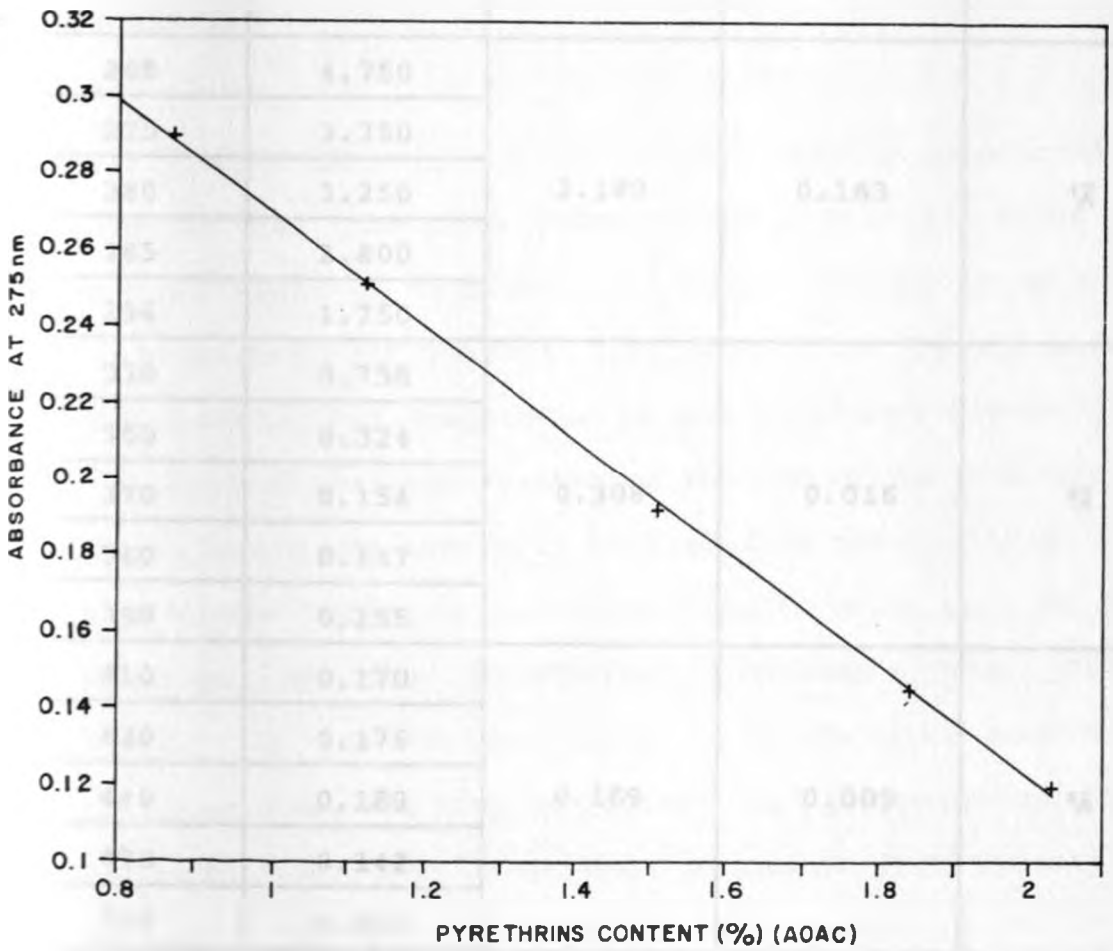


Fig. 30: Selected samples absorbance readings at 275nm correlation with the actual Pyrethrins content .

It is clearly evident from fig.30 that proportionally the low Pyrethrins content pyrethrum samples have high flavonoid contents. This result re-affirms the picture given by correlation of the specific absorbances with Pyrethrins content (section 3.1.4). It was also found necessary in view of the above results to similarly scan the world standard pyrethrum extract and the results obtained are shown in Table 17.

Table 17: WSPE (1992): 250-700nm spectrophotometric scan (solution : 1.0073g-100ml n-Hexane (AF).

λ(nm)	ABSORBANCE	MEAN ABSORBANCE	SPECIFIC ABSORBANCE ($A_{1cm}^{1\%}$)	ELECTROMA-GNETIC REGION
265	4.750	3.180	0.163	$^1\overline{A}$
275	3.350			
280	3.250			
285	2.800			
294	1.750			
330	0.758	0.308	0.016	$^2\overline{A}$
350	0.324			
370	0.154			
380	0.147			
390	0.155			
410	0.170	0.169	0.009	$^3\overline{A}$
420	0.176			
440	0.189			
470	0.142			
670	0.013			

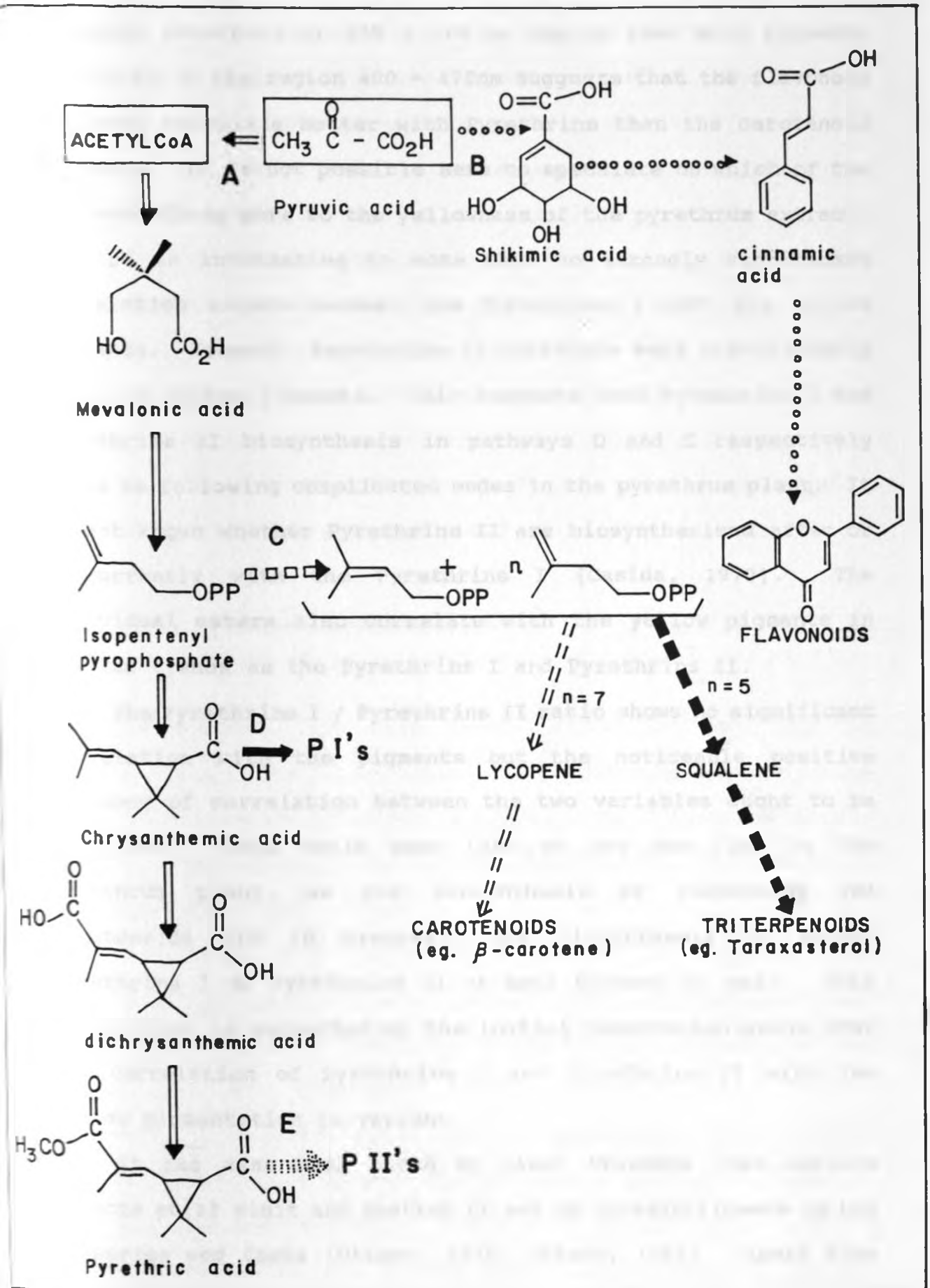
From table 17 it is noticeable that the W.S.P.E. has a very low carotenoid content (${}^{3}A^{1\%}_{1cm}$) as compared to the flavonoid content (${}^{1}A^{1\%}_{1cm}$). Comparing with the pyrethrum flower extract results, the W.S.P.E. records a very high specific absorbance within 265-300nm region than expected. Due to the refinement processes at the factory which give the refined pyrethrum extract from which the WSPE is prepared, WSPE is expected to have very low yellow pigmentation. So it is not clear as to why the WSPE records a high specific absorbance within 265-300nm. A possible explanation is that the additives, specifically BHT, which is an aromatic compound, could also be absorbing within this range of the electromagnetic region.

3.2 DISCUSSION

The results above prompt an insight into the biosynthetic processes in the pyrethrum plant. Scheme 1 which forms an illustrative basis of discussion of these results is an over simplified possible biosynthetic link between the Pyrethrins and the non- insecticidal components in the pyrethrum flower. The scheme shows that both the Pyrethrins and the yellow pigments in pyrethrum flowers are naturally derived from pyruvic acid.

The trends indicated by these results show that as the concentration of the Pyrethrins increases, the yellow pigmentation in the various clonal and varietal pyrethrum flowers diminishes. The results suggest that flavonogenesis and carotenogenesis are not independent processes from Pyrethrins biosynthesis in the pyrethrum flower.

Scheme 1 : BIOSYNTHETIC VIEW POINT.



A better linear correlation between the Pyrethrins with pigments absorbing at 265 - 300 nm region than with pigments absorbing in the region 400 - 470nm suggests that the flavonoid pigments correlate better with Pyrethrins than the carotenoid pigments. It is not possible here to speculate on which of the two contribute more to the yellowness of the pyrethrum extract.

It is interesting to note that no strongly significant correlation exists between the Pyrethrins I with the yellow pigments. However, Pyrethrins II correlate very significantly with the yellow pigments. This suggests that Pyrethrins I and Pyrethrins II biosynthesis in pathways D and E respectively could be following complicated modes in the pyrethrum plant. It is not known whether Pyrethrins II are biosynthesised after or concurrently with the Pyrethrins I (Casida, 1973). The individual esters also correlate with the yellow pigments in similar trends as the Pyrethrins I and Pyrethrins II.

The Pyrethrins I / Pyrethrins II ratio shows no significant correlation with the pigments but the noticeable positive tendency of correlation between the two variables ought to be explained. This could mean that at any one time in the pyrethrum plant, as the biosynthesis of flavonoids and carotenoids are in progress, the biosynthesis of either Pyrethrins I or Pyrethrins II or both proceed as well. This explanation is supported by the initial observation above that the correlation of Pyrethrins I and Pyrethrins II with the yellow pigmentation is variant.

It has also been noted by plant breeders that certain insects still visit and destroy or eat up certain flowers in the nurseries and farms (Ottaro, 1978; Ottaro, 1992). Apart from

the fact that these insects may have adapted themselves to this activity, these results suggest that the insects visit the very bright flowers which have low Pyrethrins content. The bulk of the non-insecticidal components of pyrethrum flowers are undesirable. The yellow pigments in the refined pyrethrum extract of commerce affects the aesthetic quality of the formulated products of the natural Pyrethrins. The triterpenol taraxasterol is a menace in the pyrethrum industry due to its gelling effect. So could it be possible to shut off (refer to Scheme 1) the undesirable pathways B and C so as to favour pathway A in order to achieve flowers of high Pyrethrins content and low non-insecticidal composition either genetically engineered or agronomically cloned?

3.3 Conclusion and recommendations

The results from this investigation indicate that an inverse linear correlation between the Pyrethrins and the yellow pigments exists in pyrethrum flowers. The Pyrethrins show a better linear correlation with pigments at 265 - 300nm region than pigments at 400 - 470nm.

It is possible that there could be certain factors which influence the biosynthetic processes in the pyrethrum plant and this requires further research. It is already reported that the accumulation of β-carotene in *Dunaliella salina* is highly influenced by light intensity, temperature, salinity and nitrogen deficiency levels. These factors have been found to decrease the growth rate of the algae (Erick, 1989). It is possible to control and direct biosynthetic processes despite

economic and other constraints, the future pyrethrum crop improvement programmes ought to involve a biotechnological approach. The recent American indication of production of abundant and cheap Pyrethrins from a biotechnological source in due course could be a possible success in this direction. The role of genetic engineering on pyrethrum farming is already beginning to realize good results in India (Sing and Sharma, 1989). In view of this future competitive environment in the pyrethrum industry, the results cited in this thesis could form a possible working basis for future action in this regard.

An extensive study on various clone and variety samples from different climatic regions ought to be investigated similarly. Correlations between the Pyrethrins and the Pentacyclic triterpenoids particularly taraxasterol also requires investigation. It is possible to develop the above correlation into a quality control parameter as well as utility of the same in future crop improvement programmes to achieve flowers of low pigmentation but high Pyrethrins content either genetically engineered, biotechnologically or agronomically developed. The current processing of the dry flowers in the factory at Nakuru could also be reviewed such that low and high Pyrethrins content flowers are categorised and processed proportionately. This approach could give a refined pyrethrum extract that has relatively low yellow pigmentation and possibly acceptable level of taraxasterol resulting into a gel free commercial product.

REFERENCES

1. Abduazimov, B.K., Abdullac, N.D., Yunusov, A.I, Yagudaev, M.R. and Sidyavin, G.P. (1985). **Kim Priors 2**:199-201. In : SCI (1985)

2. Aggarwal, Y.P. (1990). **Statistical methods**. Sterling private publishers Ltd. PP 141-163.

3. Acree, F. Jr. and Larforge, F. B. (1937). Constituents of pyrethrum flowers. X : Identification of the fatty acids combined with pyrethrolone. **J. Org. Chem. 2** : 308-313.

4. Barton, D.R., Bockman, O.C. and de Mayo, P. (1960). Sesquiterpenoids, part XII: Further investigations on the chemistry of pyrethrosin. **J. Chem. Soc.** 2263

5. Beckley, V.A. (1950). The spectrophotometric estimation of the Pyrethrins. **Pyrethrum Post 2**(1): 23.

6. Bramwel, A. F., Crombie, L., Hemsley, P., Pattenden, G., Elliot, M.,and Janes,N. F. (1969). Nuclear Magnetic resonance spectra of the natural Pyrethrins and related compounds. **Tetrahedron 25**:1727-1740.

7. Brown, N. C., Phipers, R. F. and Wood , M. C. (1954). Pyrethrum Estimation of "False" Pyrethrins in pyrethrum extract. **Pyrethrum post 3**(3): 35

8. Brown, N.C., Phipers, R.F. and Wood, M.C. (1956). " The analysis of Pyrethrins. Part III. A correlation of various Analytical Methods with Bioassays".**Pyrethrum post 4** (1): 24-29

9. Casida, J. E. (1973). Pyrethrum, **The Natural Insecticide.** Academic press, New York. PP 3, 14, 18, 26, 30, 32, 87, 108, 329.

10. Chandler, S.E. (1951). Botanical aspects of pyrethrum. General considerations : the seat of the active principles. **Pyrethrum post 2**(3):1

11. Cottered, G.C. (1950). The insect infestation of groundnuts in Kano and their control. **Pyrethrum Post 2**(1): 27.

12. Chandler, S.E. (1952). Application of Pyrethrum in public health and related fields.**Pyrethrum Post 3**(1):2

13. Crombie, L., Doherty, C.F., Pattenden, G. and Woods, O.K.(1971b). The acid thermal decomposition products of natural chrysanthemum dicarboxylic acid. In : Casida (1973)

14. Desmarchelier, J.M., Bengeston, M. and Sticks, R. (1979). Stability and efficacy of Pyrethrins on grain storage. **Pyrethrum Post 15**(1): 3.

15. Dobson, O.C. (1958). Insecticides and public health. **Pyrethrum Post 4**(4): 6.

16. Doskotch,R.W., El-Faraly, F.S. and Gafford, C.D. (1971). sesquiterpene lactones from pyrethrum flowers. **Can. J.Chem. 49**: 2103

17. Elliot, M and Stenphenson, H. (1966). " Pyrethrol-Taraxasterol". A note. **Pyrethrum post,8** (3): 27-28

18. Elliot, M. and Janes, N.F. (1973). "Chemistry of the Natural Pyrethrins. In: Pyrethrum, **The Natural**

Insecticide. J.E. Casida, Ed. Academic Press, New York.

19. Erick, T.V. (Ed) (1989). Biotechnology of vitamins, pigments and growth factors. Enselvier applied science, London and New York. PP 15-19.

20. Finar, I.L. (1975). Organic chemistry vol. 2 : Stereochemistry and the chemistry of Natural products. Longman UK Ltd. England. PP 463-491.

21. Fisher, A.R. and Yates, F. (1963). Statistical tables for biological, agricultural and medical research 6th ed. Longman. PP 63

22. Fukushi, S. (1952). Studies on the components of the unsaponifiable matter of the wax of *Chrysanthemum cinerariaefolium*. **J. Agric. Chem. Soc. Jap. 26**:1-2. In : Casida (1973) PP 31.

23. Furniss, S.B., Hannaford, A.J., Smith, P. W.G., Tatchell, A.R. (eds) (1989). Vogels Textbook of Practical Organic Chemistry, Longman Group, UK Ltd. pp 233.

24. Githinji, P.M. (1973). The effects of drying temperature and drying time on Pyrethrins content on pyrethrum flowers. **Pyrethrum Post 12** (2): 77 - 82.

25. Gladinger, C.B. (1936). " Pyrethrum flowers " 2nd ed. PP 400-404. Mclaughlin Gormly King, Mineapolis Minesota.

26. Gladinger, C.B.(1945). "Pyrethrum flowers" supplement to 2nd ed. PP 3, 97. Mclaughlin Gormly King.

Mineapolis Minesota.

27. Glenie, J.B. and Harborne, J.B. (1972). "Flavonoids of Pyrethrum". **Pyrethrum post 11**(3): 82-84

28. Glynne, J.G.D. (1956). A new use of synergised Pyrethrins in crop protection. **Pyrethrum Post 4**(1): 34.

29. Glynne, J.G.D. (1960). Studies on the photolysis of pyrethrum. **Ann. Appl. Biol. 48**(1): 352-362

30. Goodwin, T.W. (ed)(1976). Chemistry and Biochemistry of plant pigments. Vol 1, 2nd edition . Academic press London, New York. PP-739.

31. Greening, H.G.(1979). Chemical control on insects infesting farm grain and harvesting machinery. **Pyrethrum Post 15**(2): 48.

32. Griffin, C.S. (1973). Mammalian toxicology of pyrethrum. **Pyrethrum Post 12**(2): 50.

33. Hagley, E.A.C. (1959). Pyrethrum. A note on its horticultural applications. **Pyrethrum Post 5**(1): 23.

34. Head, S.W. (1963a). An examination of the effect of the picking methods on the Pyrethrins content of dry pyrethrum flowers. **Pyrethrum post 7**(2): 3-9.

35. Head, S.W.(1969). The pyrethrum extract constituents **Ph.D. Thesis.** University of Nairobi. PP 31-80

36. Head, S.W. (1973). "Composition of Pyrethrum Extract and analysis of pyrethrum " In: Pyrethrum,**The Natural insecticide** J.E. Casida, Ed. Academic

Press, New York.

37. Hogsett, J.N., Kacy, H.W. and Johnson, J.B.(1953). Analysis of commercial allethrin. Determination of allethrin content by reaction with ethylenediamine. **Anal.Chem.25**: 1207-1211.

38. Holmstead, R.L. and Soderlund, D.M.(1978). "Separation and Analysis of Pyrethrins by combined gas-liquid chromatography-chemical ionization mass spectrometry." **Pyrethrum post, 4**(3): 79-82.

39. Izuru, Y. (1973). Mode of action of synergists in enhancing the insecticidal activity of pyrethrum and pyrethroids. In : Casida (1973) PP 195-210.

40. Jeffrey, G.H., Bassett, J., Mendham, J. and Denney, R.C. (1989). Vogel's text book of Quantitative Chemical analysis. ELBS Longman 5th ed. PP 137-138, 144-146, 842.

41. Ikahu, J.M.K. and Ngugi, C.W. (1990). Investigations into yield losses in some pyrethrum clones through picking of flowers at improper stages of development, part II. **Pyrethrum Post 17**(3): 101-104.

42. Kamau, S.M. (1990). "Quantitative determination of Pyrethrins by rapid high performance liquid chromatographic analysis". **Pyrethrum post 8**(1): 7-10

43. Katsuda, Y. and Tikamoto T. (1958). Studies on the degradation of Pyrethrins. **Botyo-Kaguku.23**: 60-63. In : Casida (1973). PP 32

44. Kawano, Y., Yanagiha, K., Miyamoto, T. and Yamomoto, I. (1980). Examination of the conversion products of Pyrethrins and allethrin formulations, exposed to sunlight by Gas liquid chromatography and mass spectrometry (MS). **J. chromat. 198** (3): 317-328

45. Kemp, w. (1991). **Organic spectroscopy.** 3rd edition. Macmillan Education Ltd. Hampshire. pp. 244 - 247

46. Kenya News Agency (KNA) (1993). The Standard newspaper. The Standard Ltd, Nairobi (K), 27th Feb, PP3 column 6.

47. Lepelley, R.H.(1973). The start of pyrethrum growing in kenya. **Pyrethrum post 12**(1):32.

48. Mabry, T.J., Markham, K. R. and Thomas, M. B. (1970). **The systematic identification of flavonoids.** Spring - verlag. New York. pp. 41- 61

49. Marshall, R.A.G. (1971). " Colorimetric method for the determination of Iron in pyrethrum extracts" **Analyst** (London) **96**(1146): 675-678.

50. Mitchell, W. (1953). Note on recent change in the A.O.A.C.method for the Assay of Pyrethrins. **J.Sci.Food Agric. 5**: 246-248.

51. Miskus, R.P. and Andrews, T.L.(1972). Stabilization of thin films of Pyrethrins and allethrin.

J. Agri. Food Chem. 20:313-315.

52. Munro, R.J. (1961). An experiment on the drying of pyrethrum flowers. **Pyrethrum Post 6**(2): 25-29.

53. Ngugi, C.W. and Ikahu, J.M.K. (1991). The effect of drying temperature on Pyrethrins content in some pyrethrum clones. **Pyrethrum Post 18**(1): 19-21.

54. Odinga, W. A. (1992). Personal communication.

55. Odinga, W. A. (1992). 1992 World Standard Pyrethrum extract. **Pyrethrum post 18**(14): 121-125.

56. Official Methods of Analysis (1984). AOAC 14th ed. Arlington V.A., pp 101-103.

57. Otieno, D.A and Pattenden, G.(1979). Degradation of natural Pyrethrins. **Pyrethrum post, 15** (2): 30-37

58. Otieno, D.A., Jondiko, I.J., McDowell, P.G. and Kezdy,F.J. (1982). Quantitative analysis of the Pyrethrins by HPLC. **J. Chromat. Sci. 20**: 566-570.

59. Otieno, D.A. (1983). "Quantitative analysis of the Pyrethrins by HPLC". **Pyrethrum post 15** (3): 71

60. Ottaro, W.G.M.(1978). The relationship between the ploidy level and certain morphological characteristics of *Chrysanthemum cinerariaefolium* vis. **Pyrethrum post 14**(1): 10-15.

61. Ottaro, W. G. M. (1992). Personal communication.

62. Pandita, P.N. (1990). **Pyrethrum Post 17** (3) : 111

63. Parlevliet, J.E. (1975). Breeding Pyrethrum in Kenya. **Pyrethrum Post 13 (2):** 47-54.

64. Parlevliet, J.E. (1975). The genetic variability of the yield component in the Kenyan Pyrethrum population. **Pyrethrum Post 13 (1):** 23-28.

65. Pattenden, G. (1970). Some studies on the biosynthesis of the Pyrethrins. **Pyrethrum Post 10** (4): 2

66. Price, R.W. (1960). Sulfoxide - a pyrethrum synergist. **Pyrethrum post 5(3):** 5-11

67. Pushpa, K. , Asha, G.,Rathone, A.K., Mukul, S., Sneh,S. and Usha, G. (1979). Investigations into possible new sources of Pyrethrins. **Pyrethrum post 15**(1): 9-10.

68. Pyrethrum Bureau (1987). Formulating pyrethrum manual. PP 4, 5, 8, 11, 18, 36, 43-46.

69. Rickett, F.E. (1972). Preparative scale separation of Pyrethrins by liquid-liquid partition chromatography. **J. Chromatography 66:** 356-360

70. Rose, W. G. and Haller, H.L. (1937). Constituents of pyrethrum flower XI. **J. Org. Chem. 2:** 484-488.

71. Sawicki, R.M. (1962a). Insecticidal activity of the pyrethrum extract and its four insecticidal constituents against house flies .
II Synergistic activity of piperonyl butoxide

with the four constituents. **J.Sci. food. Agr. 13:** 260-264.

72. Sawicki, R.M. (1962b). III Knockdown and recovery of flies treated with pyrethrum extract with and without piperonyl butoxide **J. Sci. food. Agr. 13:** 286-292.

73. Sawicki, R.M. and Thain, E.M. (1962). " Insecticidal activity of pyrethrum extract and its four insecticidal constituents against houseflies. IV. Knock down activities of the four constituents. **J.Sci. Food. Agri: 13:** 292 -297.

74. Shashida, Y., Nakata, H. Shimomur, H., and Kagaya, M. (1983). **Phytochem. 22**(5): 1219-1222.

75. Singh, S.P. and Sharma, T.R. (1989). Genetic improvement of pyrethrum for selective divergence,Heterosis and potential hybrid clones. **Theor.A. Gen.** 78.

76. Smith, H.J. (1959). A modified 2,4- dinitrophenyl hydrazone method for the determination of "Pyrethrins I" and " Pyrethrins II". **J.Sci. Food Agric. 10:** 260-267.

77. Sofowora, A. (1984). Medicinal plants and Traditional Medicines in Africa. John Wiley and sons. New York. pp. 173 and 194.

78. Stahl, E. and Pfeifle, J.(1966). Thin layer and gas liquid chromatography of pyrethrum based insecticides. **Pyrethrum post 8**(4): 8-9

79. Staudinger, H. , Muntwyler, O., Ruzicka, L., and Seibt, S. (1924). Insektentotende stoffe. VII. **Acta 7**:390-406. In : Casida (1973).

80. Tuikong, A.R. (1979). Pyrethrum Breeding in Kenya: A historical account. **Pyrethrum post 15**(4):113-117.

81. Wanjala, B.W.(1990). Performance of low-altitude, unimproved pyrethrum clones grown in Kisii, Kenya. **Pyrethrum post 18** (2): 61-64

82. Wanless, G.C., King, W.H. and Ritter, J.J. (1955). Hydrocarbons in pyrethrum cuticle wax. **Biochem. J. 95** : 684-690. In : Casida (1973) PP.31

83. Ware, G.W., Freeman, W.H. and Co. (eds)(1983). Pesticide theory and application. San Francisco.

84. Weaving, A.J.S. and Sylvester, N.K. (1967). Pyrethrum as a insect repellent, part II. A laboratory technique for its evaluation as a mosquito repellent, and the influence of formulation on persistence. **Pyrethrum post 9**(1): 31-35

85. Whitehead, D.L. and Bowers, W.S. (Eds). (1981). Natural products for innovative pest management. Pergamon press vol 2 : 93-121.

APPENDIX I

Analysis of Pyrethrum

There exist a number of chemical methods for the determination of Pyrethrins out only one the 'mercury reduction' method forms the basis for the following detailed analytical procedures.

Method 1 - The Pyrethrum Board of Kenya Powder and Flower Method (AOAC)

Based on the "Official Methods of Analysis of the Association of Official Agricultural Chemists, 8th Edition, 1955" pp. 68-70 in short, the A.O.A.C. 8th (revised 1984). As its title implies, this is used for the assessment of pyrethrum flowers and powder.

METHOD OF ASSAY

Reagents

(a) *Deniges reagent

Mix 5g yellow HgO with 40ml H2O, and while stirring, slowly add 20ml H_2SO_4; then add additional 40ml H_2O and stir until completely dissolved. Test for absence of mercurous Hg by adding few drops of (b) to 10ml and titrating with (c) as under method 1, 'Pyrethrins I', line beginning "Add 3ml HCl..."

(b) Iodine Monochloride Solution

Dissolve 10g KI and 6.44g KIO_3 in 75ml H_2O in glass-stoppered bottle; add 75ml HCl and 5ml $CHCl_3$, and adjust to faint iodine colour (in $CHCl_3$) by adding dilute KI or KIO_3 solution. If much iodine is liberated, use stronger solution of KIO_3 than 0.01M at first, making final adjustment with 0.01 M solution. Keep in the dark and readjust when necessary.

(c) Standard Potassium iodate solution - 0.01 M. Dissolve 2.14 pure KIO_3, previously dried at 105°C, in H_2O and dilute to 1 litre. 1ml of this solution = 0.0057 g. "Pyrethrins I" and needs no further standardisation.

(d) Petroleum ether

Is a fraction boiling between 40° and 60°C from which the aromatic hydrocarbons have been removed.

(e) Hydrochloric acid

Is concentrated acid of the British Pharmacopoeia containing not less than 35 per cent w/w and not more than 38 per cent w/w of HCl.

(f) Solution of sodium chloride (British Pharmacopoeia, 1953)

Is a saturated solution of sodium chloride in distilled water.

(g) Other reagents should comply with the requirements of the British Pharmacopoeia and British Pharmaceutical Codex. These are equivalent to "Analytical Reagent" standards.

(h) Phenolphthalein - 1g phenolphthalein in 100ml alcohol.

Preliminary Treatment

Extract sample containing 20-75 mg 'Pyrethrins I' (12.5-20g) in Soxhlet or other efficient extraction apparatus for 7 hours with petroleum ether. After extraction is complete, evaporate petroleum ether to ca. 40ml, stopper flask, and place it in refrigerator at 0-5°C at least two hours or preferably overnight. Filter cold extract through cotton plug, saturated with cold petroleum ether, in stem of funnel, and collect filtrate in 250ml Erlenmeyer. Add 20ml chilled petroleum ether to extraction flask. With rubber policeman, dislodge resinous material in flask, swirl contents without allowing liquid to warm up appreciably, and filter through cotton wool. Repeat operation twice, using 10ml portions chilled petroleum ether. Add several glass beads and remove solvent on a water bath, without attempting to heat residue long enough to remove last traces of solvent. Add 15-20ml 0.5 N alcoholic NaOH to flask containing pyrethrum extract, connect to reflux condenser, and boil gently 1-1.5 hours.

'Pyrethrins I'

Transfer to 600 ml beaker and add sufficient H_2O to bring volume to 200ml. Add few glass beads, or preferably use boiling tube, and boil down to 150ml. Transfer to 250 ml volumetric flask and add 1 g filter-cel and 10 ml. 10% $BaCI_2$ solution. Do not shake before diluting to volume. Dilute to volume, mix thoroughly, filter off 200 ml, neutralize with H_2SO_4 (1+4), using 1 drop phenolphthalein, and add 1 ml. acid in excess.

If necessary to hold solution overnight at this point, leave in alkaline condition. Filter through 7 cm paper, coated lightly with suspension of filter-cel in H_2O on buchner, and wash several times with H_2O. Transfer to 500ml. separator and extract with two 50ml portions petroleum ether. Wash extractions with two or three 10ml. portions H_2O and filter petroleum ether extractions through cotton plug into clean 250ml. separator. Wash cotton with 5 ml petroleum ether. Extract petroleum ether with 5 ml. petroleum ether. Extract petroleum ether with 5ml. 0.1 N NaOH, Shaking vigorously. Drain aqueous layer into 100ml. beaker, wash petroleum with 5ml. H_2O or with additional 5ml. 0.1 N NaOH, and add this to beaker. Add 10ml. of the Deniges reagent and let it stand for 1 hour in the dark at 25 ± 2°C. Add 20 ml. alcohol and precipitate HgCl with 3 ml. saturated NaCl solution. Warm to ca. 60°C and filter through small paper, transferring all precipitate to paper, and wash with 10ml. or more hot alcohol. Wash with 2 or more 10 ml. portions hot $CHCl_3$ and place paper and contents in 250ml. glass stoppered Erlenmeyer flask. Add 30ml HCl and 20 ml H_2O and cool: and 6 ml. $CHCl_3$ or CCl_4 and 1 ml. of the ICl solution and titrate with the KIO_3 solution, shaking vigorously after each addition

until no iodine colour remains in $CHCl_3$ or CCl_4 layer. Take as end point when red colour disappears from $CHCl_3$ or CCl_3 layer. From the volume of the standard KIO_3 solution used in titration calculate % 'Pyrethrins I'.

KIO_3 reacts with mercurous Hg to form mercuric Hg and iodine; further addition of KIO_3 in presence of HCl oxidises iodine to ICl:

$$2Hg_2Cl_2 + 4ICl = 4HgCl + 2I_2 \text{ (iodine)}$$

$$2I_2 + 6HCl = KCl + 3H_2O$$

(Addition of ICl does not change volume relationship between mercurous Hg and KIO_3 solution and aids in determining end point in titration of small quantities of Hg).

'Pyrethrins II'

If necessary, filter aqueous residue from petroleum ether extraction from 'Pyrethrins I' determination through gooch. Concentrate filtrate to ca. 50 ml and transfer to 500ml separator. (Acidify with 10ml HCl and saturated with NaCl throughout following extractions). Extract with 50ml ether, drain aqueous layer into second separator and extract again with 50ml ether. Continue extracting and draining aqueous layer, using 35 ml for the third and fourth extractions. Combing 4 ether extractions, drain and wash with three 10 ml portions saturated NaCl solution. Filter ether extractions through cotton plug into 500 ml Erlenmeyer and wash the cotton wool with additional 10 ml ether. Evaporate ether on a water bath and remove any fumes of HCl with current of a air and continued heating. Dry for 10 minutes at 100°C. Add 2 ml

neutral alcohol and 20 ml H_2O and heat to dissolve acid. Cool, filter through gooch if necessary, add 1 or 2 drops phenolphthalein, and titrate with 0.02 N NaOH, of which 1 ml = 0.00374 g 'Pyrethrins II'

NOTES

1. The flower gristing procedure used at the Pyrethrum Board of Kenya laboratories, Nakuru, is as follows:
   (a) The whole original sample, irrespective of size, is gristed in a Wiley grinding mill to pass a 2mm aperture screen.
   (b) After thorough mixing, the coarsely ground material is quartered down to give a sub-sample of suitable size.
   (c) This sub-sample is reground in the Wiley mill to pass 1 mm aperture screen. The powder should be sufficiently fine to pass a No. 16 B.S.S. sieve or a No. 18 U.S. standard sieve.
2. The quantities given in brackets, first line of Preliminary treatment are too large for high grade East African flowers. The PBK laboratories normally use 5,8 or 10 g of sample.
3. In the calculation, make an allowance for the aliquot taken at the Barium sulphate precipitation stage.

Appendix II

Method II - Pyrethrum Board of Kenya Extract Method (PBK)

(September,1954)

Based on a British Pharmacopoeia method for pyrethrum extract.

METHOD OF ASSAY

Reagents:

a) Solution of mercuric sulphate (British pharmacopoeia, 1953). Mix 5 g of yellow mercuric oxide with 40 ml. of water, and while stirring, add 20 ml. of sulphuric acid (conc.); add 40 ml. of water and stir until completely dissolved.

b) Strong solution of iodine monochloride (British Pharmacopoeia, 1953). Dissolve 6.44 g of potassium iodate and 10 g of potassium iodide in 75 ml. of water; add 75 ml. of hydrochloric acid and shake until a clear solution is obtained; add 5 ml. of chloroform and them add M/20 potassium iodate dropwise, shaking vigorously, until the chloroform becomes colourless. (Note: Strong solution of iodine monochloride should be kept in a stoppered bottle, protected from light and stored in a cool place).

c) **Potassium iodate, M/100** (British Pharmaceutical codex,1949) is an aqueous solution of potassium iodate containing, in 1,000 ml., 2.14 g. of KIO_3.

d) Petroleum ether is a fraction boiling between 40°C and 60°C. from which the aromatic hydrocarbons have substantially removed. Commercial preparations having an aromatic content not greater than 0.025% (measured as benzene by ultraviolet absorption) are satisfactory. Both n and iso-hexane with the same maximum level of aromatic ''impurities'' are also suitable solvents.

e) Hydrochloric acid is concentrated acid of the British Pharmacopoeia containing not less than 35 per cent w/w and not more than 38 per cent w/w of HCl.

(f) Solution of Sodium Chloride (British Pharmacopoeia, 1953) is saturated solution of sodium chloride in water.

(g) Other reagents should comply with the requirements of the British Pharmacopoeia and British Pharmaceutical Codex. These are equivalent to "Analytical Reagent" standards.

(h) 1 g phenolphthalein in 100ml alcohol.

Preliminary Treatment

Weigh a quantity of extract sufficient to contain 0.12-0.13g. of total Pyrethrins into a 150ml lipped stoppered conical flask. Dilute with 50ml of aromatic-free petroleum ether (boiling range 49° - 60°C) and add 1g of filter-Cel. Mix thoroughly and keep at room temperature (ca. 20°C) for two hours.

Filter through a gooch crucible, prepared with asbestos and washed first with alcohol (95%) and then with aromatic-free petroleum ether into a 500 ml conical flask. Wash the flask and

filter with successive small quantities of petroleum ether until the running are colourless.

Remove all but the last traces of petroleum either on a water bath. Do not use a current of air. Add 20 ml N/2 alcoholic KOH and boil under reflux for 1.5 hours.

Pyrethrins I

Transfer the solution from the preliminary treatment to a 600ml. beaker rinsing the flask with water. Dilute to approximately 200ml. with water. Concentrate to about 150 ml by heating, avoiding loss by frothing and cool. Transfer to a 250 ml graduated flask; and 1 g of filter-cel and 10 ml of 10% Barium chloride solution, swirl gently, allow the precipitate to settle and test the supernatant liquid with a few drops of Barium chloride solution if necessary; do not shake before diluting to volume; adjust to 250ml with water, mix thoroughly and allow to stand for 10 minutes. Filter 200 ml of the mixture into a measuring flask and transfer to a 500 ml separating funnel, neutralise with hydrochloric acid using phenolphthalein solution as indicator, and add a further 1 ml of hydrochloric acid; add 5 ml of sodium chloride solution and 50 ml of petroleum ether and shake vigorously for not less than one minute; allow to separate; filter the petroleum ether layer through a loose plug of cotton wool into a second separator.

Repeat the extraction of aqueous layer and precipitate with two further portions of 50ml. and 25 ml. of petroleum, ether, filtering the petroleum ether extracts through the same plug of

cotton wool into the second separator; wash the combined petroleum ether extract with three portions, each of 10 ml. of water. Combine the aqueous layer and the three washing and retain for the determination of Pyrethrins II. Shake the mixed petroleum ether extracts vigorously with 5 ml 0.1N sodium hydroxide for not less than one minute,allow to separate and transfer the aqueous layer to a 250 ml tipped stoppered conical flask; wash the petroleum ether layer with two portions, each of 2.5ml of distilled water and add the washing to the aqueous layer; add 10ml of mercuric sulphate solution and allow to stand for one hour at 25° ± 2°C in the dark. Add 20 ml of acetone and 3 ml of sodium chloride, stir, heat to boiling and allow the precipitate to settle, decant and filter through paper: wash the precipitate in the flask with 10 ml of hot acetone and two portions, each of ml of hot chloroform, decanting and passing the washing through the same filter. Transfer the filter paper to the precipitation flask, add 30 ml of hydrochloric acid and 20 ml of water, and cool; add 20 ml of chloroform and 1 ml of strong iodine monochloride solution and titrate, shaking vigorously, with M/100 potassium iodate until the chloroform has no pink colour. Repeat the process, omitting the pyrethrum extract, and calculate the amount of 'Pyrethrins I' from the difference between the two titration; each ml of M/100 potassium iodate is equivalent to 0.0057 g of 'Pyrethrins I'.

'PYRETHRINS II'

Evaporate the aqueous solution from the assay for Pyrethrins 1 to 50ml., cool,filter paper and wash the filter with small portions of water, not exceeding 30 ml in all; acidify with 10 ml

of hydrochloric acid. and saturate with sodium chloride. Extract with four successive portions of 50 ml.,25 ml and 25 ml of solvent ether; wash the combined ethereal extracts with three portions, each of 10 ml of saturated salt solution. Transfer the ethereal extracts to a conical flask, evaporate over a water bath and dry at 100°C for ten minutes, removing any hydrochloric acid fumes with a stream of air. Dissolve the residue in 2 ml. of neutral alcohol (95%), add 20 ml. of water (well boiled and subsequently cooled) and warm, if necessary, to complete solution, titrate with N/50 sodium hydroxide using phenolphthalein solution as indicator. Repeat the determination, using the aqueous layer and washing obtained in the blank determination for 'Pyrethrins I' and calculate the amount of 'Pyrethrins II' from the difference between the two titrations: each ml. of N/50 sodium hydroxide is equivalent to 0.00374 g. of 'Pyrethrins II'.

* **NOTE:** Chrysanthemum monocarboxylic acid reacts with Deniges reagent to form a series of colours beginning with phenolphthalein red, which gradually changes to purple, then to blue, and finally to bluish green. Colour reaction is very distinct with 5 mg. monocarboxylic acid and quantities as low as 1 mg can usually be detected. Therefore, no 'Pyrethrins I' should be reported if colour reaction is negligible. When lethanes are present, after washing HgCl precipitate with alcohol and $CHCl_3$ wash once more with alcohol and then several times with hot water.

APPENDIX III

CHEMICAL NOMENCLATURE

P1 $\underset{\sim}{5a}$ -(1S)-2-methyl-4-oxo-3-(Z)-penta-2,4-dienyl cyclopent-2-enyl (IR)-trans-chrysanthemate

C1 $\underset{\sim}{4a}$ -(1S)-3-[(z)-bute-2-enyl]-2-methyl-4-oxo-cyclopent-2-enyl (IR)-trans-chrysanthemate

J1 $\underset{\sim}{3a}$ -(1S)-2-methyl-4-oxo-3-[(z)-penta-2,4-dienyl]cyclopent-2-enyl(IR)-trans-chysanthemate

P2 $\underset{\sim}{5b}$ -(1S)-2-methyl-4-oxo-3-[(z)-pentan-2,4-dienyl] cyclopent-2-enyl pyrethrate

C2 $\underset{\sim}{4b}$ -(1S)-3-[(z)-but-2-enyl]-2-methyl-4-oxocyclopent-2-enyl pyrethrate

J2 $\underset{\sim}{3b}$ -(1S)-2-methyl-4-oxo-3-[(z)-pent-2-enyl pyrethrate

Chrysanthemic acid $\underset{\sim}{1}$ 3-Isobutenyl-2,2-dimethyl-1-cyclopropane carboxylic acid

($C_{10}H_{16}O_2$, RMM = 168)

Jasmololone $\underset{\sim}{3}$ - 4-Hydroxy-3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one

Cinerolone $\underset{\sim}{4}$ - 2-(2-Butenyl)-4-hydroxy-3-methyl-2-cyclopenten-3-methyl-2-cyclopenten-1-one ($C_{10}H_{14}O_2$, RMM = 166)

Pyrethrolone $\underset{\sim}{5}$ - 4-Hydroxy-3-methyl-2-(2,4-pentadienyl)-2-cyclopentenone

BHT - 2,6-di-Butyl-4-methyl phenol or 2,6-ditertiary Butyl paracresol.

Appendix IV

Analytical raw data

Note: All the replicate data shown here were Q-tested and acceptable at 95% confidence limit. This was the data used to generate the spectrophotometric and AOAC results given in Chapter 3.

1: Spectrophotometric data
2: AOAC data

1 : SPECTROPHOTOMETRIC DATA

Sample	Absorbances at λ225nm			
	Lot 1			
1	0.444	0.443	0.447	0.446
2	0.610	0.609	0.609	0.608
3	0.695	0.696	0.694	0.695
4	0.804	0.805	0.808	0.807
5	0.857	0.858	0.863	0.862
6	0.888	0.887	0.891	0.890
7	0.891	0.890	0.890	0.889

8	0.903	0.904	0.902	0.903
9	0.906	0.907	0.910	0.909
10	0.925	0.926	0.931	0.930
11	0.929	0.928	0.932	0.931
12	0.938	0.937	0.937	0.936
13	0.936	0.937	0.935	0.936
14	0.978	0.979	0.982	0.981
15	0.995	0.996	1.001	1.000
16	1.019	1.018	1.022	1.021
17	1.022	1.021	1.021	1.020
18	1.071	1.072	1.070	1.071
19	1.107	1.108	1.111	1.110
20	1.198	1.199	1.204	1.203
	Lot 2			
1	0.446	0.444	0.445	0.445
2	0.608	0.608	0.610	0.610
3	0.694	0.696	0.697	0.693
4	0.809	0.803	0.803	0.809
5	0.862	0.862	0.858	0.858
6	0.890	0.888	0.889	0.889
7	0.891	0.892	0.889	0.888
8	0.905	0.900	0.904	0.902
9	0.910	0.905	0.906	0.911
10	0.930	0.929	0.927	0.926
11	0.931	0.928	0.930	0.930
12	0.938	0.939	0.936	0.935
13	0.938	0.934	0.937	0.935
14	0.982	0.977	0.978	0.983
15	1.000	0.999	0.997	0.996
16	1.021	1.019	1.020	1.020
17	1.022	1.023	1.020	1.019
18	1.069	1.073	1.072	1.070
19	1.107	1.106	1.102	1.112
20	1.203	1.202	1.200	1.199

1 : SPECTROPHOTOMETRIC DATA : ABSORBANCES AT VARIOUS WAVELENGTHS

Sample	265nm		275nm		280nm		285nm		294nm	
1	0.788	0.778	0.710	0.713	0.624	0.625	0.585	0.584	0.512	0.508
	0.784	0.782	0.714	0.711	0.624	0.623	0.583	0.584	0.514	0.518
2	0.880	0.888	0.691	0.707	0.626	0.628	0.568	0.566	0.448	0.452
	0.884	0.884	0.698	0.700	0.626	0.624	0.562	0.570	0.458	0.454
3	0.934	0.932	0.731	0.742	0.709	0.709	0.640	0.641	0.507	0.503
	0.928	0.930	0.749	0.738	0.710	0.708	0.638	0.637	0.505	0.505
4	0.963	0.959	0.702	0.698	0.647	0.651	0.580	0.584	0.460	0.464
	0.965	0.969	0.700	0.700	0.650	0.648	0.581	0.583	0.460	0.456
5	0.932	0.928	0.732	0.735	0.684	0.688	0.616	0.617	0.477	0.479
	0.930	0.930	0.736	0.733	0.686	0.682	0.616	0.615	0.481	0.483
6	1.070	1.079	0.754	0.752	0.689	0.683	0.604	0.606	0.450	0.455
	1.069	1.078	0.748	0.758	0.686	0.686	0.609	0.601	0.450	0.445
7	0.999	0.105	0.728	0.727	0.673	0.667	0.594	0.597	0.457	0.458
	1.009	1.003	0.728	0.729	0.663	0.669	0.596	0.593	0.457	0.456
8	0.970	0.966	0.709	0.709	0.655	0.645	0.578	0.570	0.433	0.429
	0.974	0.978	0.709	0.709	0.650	0.650	0.574	0.574	0.428	0.434
9	0.917	0.913	0.695	0.693	0.641	0.639	0.570	0.574	0.432	0.433
	0.916	0.914	0.695	0.697	0.643	0.637	0.570	0.566	0.436	0.435
10	0.948	0.944	0.705	0.706	0.624	0.626	0.592	0.596	0.480	0.478
	0.946	0.946	0.704	0.705	0.628	0.626	0.593	0.595	0.481	0.477
11	0.865	0.863	0.693	0.689	0.600	0.603	0.560	0.557	0.469	0.468
	0.866	0.862	0.690	0.692	0.604	0.601	0.556	0.559	0.469	0.470
12	0.927	0.928	0.666	0.668	0.605	0.603	0.558	0.550	0.419	0.418
	0.926	0.926	0.665	0.669	0.607	0.601	0.554	0.554	0.420	0.419
13	0.908	0.906	0.630	0.636	0.584	0.585	0.529	0.520	0.393	0.389
	0.907	0.907	0.638	0.628	0.584	0.583	0.520	0.511	0.383	0.387
14	0.810	0.808	0.652	0.656	0.583	0.581	0.530	0.529	0.421	0.425
	0.808	0.808	0.650	0.658	0.584	0.580	0.532	0.534	0.420	0.426
15	0.969	0.697	0.663	0.664	0.607	0.605	0.544	0.546	0.396	0.393
	0.968	0.968	0.664	0.665	0.613	0.615	0.342	0.540	0.396	0.399
16	0.850	0.855	0.613	0.615	0.569	0.567	0.518	0.519	0.421	0.420
	0.860	0.855	0.612	0.616	0.568	0.568	0.518	0.518	0.422	0.421
17	0.872	0.869	0.639	0.639	0.590	0.589	0.525	0.528	0.407	0.405
	0.870	0.873	0.639	0.639	0.589	0.588	0.529	0.526	0.409	0.411
18	0.894	0.892	0.670	0.650	0.601	0.599	0.529	0.529	0.398	0.397
	0.890	0.892	0.655	0.655	0.600	0.600	0.529	0.529	0.397	0.397
19	0.946	0.940	0.642	0.638	0.590	0.588	0.533	0.535	0.424	0.423
	0.943	0.941	0.640	0.644	0.589	0.389	0.533	0.521	0.420	0.421
20	0.928	0.924	0.704	0.705	0.605	0.608	0.530	0.534	0.408	0.414
	0.926	0.926	0.706	0.705	0.602	0.605	0.536	0.532	0.410	0.412

1 : SPECTROPHOTOMETRIC DATA : ABSORBANCES AT VARIOUS WAVELENGTHS

Sample	330nm		350nm		370nm		390nm	
1	0.215	0.219	0.160	0.165	0.154	0.152	0.170	0.178
	0.219	0.223	0.158	0.153	0.148	0.150	0.174	0.174
2	0.254	0.250	0.202	0.200	0.216	0.218	0.259	0.260
	0.256	0.260	0.200	0.198	0.218	0.220	0.259	0.258
3	0.246	0.245	0.187	0.185	0.165	0.159	0.250	0.246
	0.248	0.247	0.187	0.189	0.164	0.160	0.248	0.248
4	0.340	0.346	0.170	0.180	0.128	0.132	0.260	0.257
	0.348	0.338	0.174	0.176	0.134	0.130	0.258	0.261
5	0.270	0.280	0.197	0.200	0.186	0.188	0.210	0.212
	0.275	0.275	0.205	0.202	0.187	0.187	0.208	0.214
6	0.306	0.304	0.190	0.191	0.158	0.162	0.175	0.175
	0.305	0.305	0.182	0.180	0.166	0.164	0.175	0.176
7	0.257	0.258	0.157	0.159	0.158	0.159	0.173	0.177
	0.258	0.258	0.163	0.161	0.158	0.157	0.174	0.176
8	0.174	0.182	0.130	0.126	0.154	0.142	0.149	0.150
	0.188	0.180	0.136	0.120	0.148	0.148	0.149	0.148
9	0.370	0.364	0.220	0.217	0.198	0.198	0.245	0.251
	0.366	0.368	0.217	0.214	0.198	0.200	0.248	0.248
10	0.296	0.288	0.164	0.168	0.114	0.108	0.244	0.246
	0.290	0.294	0.160	0.164	0.112	0.110	0.244	0.242
11	0.273	0.267	0.192	0.198	0.169	0.173	0.188	0.189
	0.261	0.267	0.194	0.196	0.169	0.165	0.188	0.188
12	0.275	0.283	0.171	0.170	0.164	0.167	0.170	0.175
	0.279	0.271	0.173	0.174	0.176	0.173	0.170	0.165
13	0.249	0.248	0.172	0.168	0.163	0.157	0.156	0.154
	0.257	0.258	0.161	0.179	0.160	0.160	0.157	0.153
14	0.228	0.232	0.230	0.231	0.105	0.112	0.225	0.229
	0.234	0.230	0.234	0.233	0.117	0.110	0.227	0.227
15	0.278	0.275	0.160	0.166	0.103	0.112	0.100	0.104
	0.276	0.279	0.158	0.168	0.119	0.110	0.102	0.102
16	0.261	0.259	0.194	0.195	0.176	0.175	0.186	0.186
	0.263	0.257	0.194	0.193	0.175	0.174	0.186	0.187
17	0.224	0.224	0.173	0.171	0.135	0.147	0.142	0.140
	0.223	0.224	0.170	0.174	0.136	0.146	0.141	0.141
18	0.260	0.263	0.172	0.171	0.184	0.182	0.203	0.206
	0.263	0.266	0.172	0.173	0.186	0.188	0.203	0.200
19	0.302	0.301	0.205	0.198	0.179	0.177	0.186	0.189
	0.302	0.302	0.201	0.208	0.178	0.178	0.185	0.189
20	0.270	0.268	0.158	0.156	0.103	0.102	0.087	0.087
	0.269	0.269	0.159	0.155	0.103	0.104	0.087	0.086

1 : SPECTROPHOTOMETRIC DATA : ABSORBANCES AT VARIOUS WAVELENGTHS

Sample	410nm		420nm		440nm		470nm		670nm	
1	0.211	0.213	0.200	0.200	0.194	0.193	0.150	0.154	0.040	0.040
	0.209	0.211	0.201	0.199	0.193	0.192	0.146	0.150	0.043	0.037
2	0.308	0.308	0.274	0.273	0.247	0.251	0.187	0.181	0.076	0.075
	0.307	0.309	0.274	0.275	0.251	0.255	0.184	0.184	0.076	0.077
3	0.221	0.215	0.196	0.199	0.196	0.200	0.151	0.155	0.079	0.083
	0.218	0.218	0.193	0.196	0.196	0.190	0.153	0.153	0.075	0.079
4	0.182	0.178	0.163	0.157	0.162	0.160	0.125	0.123	0.060	0.063
	0.178	0.174	0.160	0.160	0.162	0.160	0.128	0.120	0.060	0.057
5	0.283	0.279	0.228	0.229	0.106	0.109	0.057	0.053	0.076	0.077
	0.282	0.280	0.229	0.230	0.108	0.105	0.055	0.055	0.075	0.076
6	0.228	0.226	0.196	0.199	0.144	0.151	0.078	0.085	0.056	0.057
	0.229	0.225	0.196	0.193	0.156	0.149	0.081	0.088	0.056	0.055
7	0.219	0.215	0.197	0.195	0.175	0.173	0.124	0.128	0.037	0.035
	0.217	0.217	0.198	0.194	0.174	0.174	0.124	0.120	0.036	0.036
8	0.149	0.147	0.118	0.122	0.075	0.085	0.047	0.045	0.031	0.042
	0.148	0.148	0.124	0.120	0.080	0.080	0.046	0.046	0.049	0.038
9	0.321	0.324	0.279	0.278	0.226	0.228	0.140	0.143	0.073	0.075
	0.323	0.320	0.280	0.279	0.228	0.230	0.144	0.141	0.074	0.074
10	0.193	0.197	0.180	0.183	0.191	0.193	0.150	0.146	0.049	0.050
	0.194	0.196	0.182	0.179	0.191	0.189	0.148	0.148	0.049	0.048
11	0.210	0.211	0.194	0.193	0.172	0.170	0.129	0.130	0.051	0.049
	0.208	0.207	0.195	0.194	0.168	0.174	0.129	0.128	0.050	0.050
12	0.300	0.301	0.264	0.267	0.248	0.250	0.177	0.175	0.040	0.042
	0.299	0.300	0.268	0.265	0.248	0.246	0.176	0.176	0.041	0.041
13	0.241	0.244	0.213	0.211	0.167	0.166	0.098	0.105	0.065	0.063
	0.243	0.240	0.212	0.212	0.168	0.167	0.108	0.101	0.064	0.064
14	0.203	0.201	0.189	0.185	0.199	0.191	0.152	0.150	0.058	0.056
	0.205	0.203	0.186	0.188	0.195	0.195	0.148	0.154	0.058	0.060
15	0.142	0.144	0.125	0.123	0.090	0.093	0.063	0.062	0.045	0.046
	0.142	0.140	0.124	0.124	0.098	0.095	0.633	0.064	0.047	0.046
16	0.159	0.152	0.146	0.150	0.128	0.126	0.092	0.094	0.052	0.051
	0.161	0.168	0.148	0.148	0.127	0.127	0.088	0.018	0.052	0.053
17	0.132	0.132	0.105	0.106	0.102	0.098	0.037	0.038	0.043	0.041
	0.132	0.132	0.106	0.107	0.104	0.100	0.037	0.036	0.040	0.044
18	0.242	0.244	0.219	0.215	0.182	0.178	0.125	0.123	0.034	0.035
	0.244	0.246	0.216	0.218	0.180	0.180	0.124	0.124	0.034	0.033
19	0.203	0.209	0.171	0.165	0.110	0.112	0.070	0.071	0.051	0.053
	0.206	0.206	0.161	0.167	0.110	0.108	0.070	0.069	0.050	0.054
20	0.102	0.104	0.075	0.074	0.040	0.038	0.020	0.018	0.040	0.042
	0.108	0.098	0.076	0.075	0.039	0.039	0.019	0.019	0.040	0.038

2 : AOAC CHEMICAL ANALYSIS DATA

Note: The titration factor (TF) values were determined on daily basis

Sample		Weight (g)	Titre Volume (cm^3)		Titration Factor (TF)
			PI (0.01M KIO_3)	PII (0.02N NaOH)	
1	A	8.1274	3.95	9.45	1.0058
	B	9.1060	4.25	10.55	
	C	8.0361	3.60	8.40	1.0429
	D	9.0739	4.60	10.25	
2	A	8.0312	6.20	9.35	1.0083
	B	9.0792	7.25	11.00	
	C	8.0064	6.05	9.00	1.0292
	D	9.0020	7.30	10.85	
3	A	8.0881	7.50	9.15	1.0376
	B	9.0255	8.60	11.00	
	C	8.0010	7.30	9.35	1.0265
	D	9.0630	8.80	11.35	
4	A	8.0147	8.75	10.50	1.0430
	B	9.0063	10.10	12.20	
	C	8.0827	8.75	10.85	1.0428
	D	9.0257	10.25	12.40	
5	A	8.0673	11.05	8.90	1.0430
	B	9.0017	12.40	9.80	
	C	8.0067	9.80	8.65	1.0081
	D	9.0008	12.50	10.50	
6	A	8.0850	6.15	16.65	1.0293
	B	9.0811	7.15	19.05	
	C	8.0748	6.00	16.30	1.0374
	D	9.0738	7.25	19.10	
7	A	8.0062	8.20	13.65	1.0280
	B	9.0531	9.55	15.80	
	C	8.0290	8.10	13.35	1.0428
	D	9.0346	9.65	15.75	
8	A	8.0424	10.70	10.10	1.0376
	B	9.0026	12.25	11.70	
	C	8.0827	10.65	9.95	1.0426
	D	9.0256	12.40	11.85	
9	A	8.0467	10.30	11.10	1.0376
	B	9.0600	11.85	12.90	
	C	8.0255	10.15	10.85	1.0428
	D	9.0751	11.95	13.05	
10	A	8.0058	10.10	11.50	1.0293
	B	9.0653	11.70	13.55	
	C	8.0003	10.00	11.55	1.0068
	D	9.0016	11.75	13.75	
11	A	8.0004	7.30	16.15	1.0058
	B	9.0003	8.45	18.55	
	C	8.0056	7.20	15.65	1.0290
	D	9.0650	8.65	18.45	

Sample		Weight (g)	Titre Volume (cm^3)		Titration Factor (TF)
			PI (0.01M KIO_3)	PII (0.02N NaOH)	
12	A	8.0256	9.10	13.15	1.0429
	B	9.0752	10.60	15.35	
	C	8.0466	9.05	13.10	1.0375
	D	9.0599	10.70	15.50	
13	A	8.0828	8.60	14.10	1.0430
	B	9.0258	9.90	16.10	
	C	8.0146	8.45	14.30	1.0081
	D	9.0062	10.00	16.80	
14	A	8.0290	8.00	16.15	1.0430
	B	9.0348	9.25	18.55	
	C	8.0061	7.85	16.15	1.0278
	D	9.0530	9.40	19.05	
15	A	8.0748	8.15	16.50	1.0376
	B	9.0738	9.40	18.90	
	C	8.0849	8.05	16.45	1.0291
	D	9.0810	9.55	19.25	
16	A	8.0068	7.75	17.80	1.0083
	B	9.0009	8.95	20.40	
	C	8.0672	7.70	17.20	1.0429
	D	9.0016	9.10	19.95	
17	A	8.0148	8.45	16.80	1.0293
	B	9.0728	9.80	19.40	
	C	8.0146	8.30	16.45	1.0428
	D	9.0061	9.85	19.20	
18	A	8.0011	10.55	14.65	1.0267
	B	9.0631	12.20	17.00	
	C	8.0880	10.55	14.50	1.0374
	D	9.0253	12.30	16.95	
19	A	8.0065	6.30	21.30	1.0293
	B	9.0021	7.35	24.30	
	C	8.0311	6.20	21.65	1.0081
	D	9.0791	7.50	225.25	
20	A	8.0360	9.60	19.10	1.0430
	B	9.0738	11.10	21.95	
	C	8.1273	9.60	19.90	1.0056
	D	9.1059	11.25	23.05	