STUDIES ON THE ANALYSIS AND STABILITY OF PYRETHRUM EXTRACTS

by

Ian Staniforth B.Sc.

A Thesis submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Council for National Academic Awards)

Kingston Polytechnic 1972

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Mr. G.G. Shone for his advice and encouragement in directing the course of this study, to the Pyrethrum Marketing Board, Kenya for a Research Studentship and for pyrethrum samples, to Mr. L.M. Newall and the staff of the computer unit, to Messrs. D. Holmes and B.J. Saxby for the design and construction of the analogue-to-digital converter, to Mr. F.W. Fifield for neutron activation analyses, to Dr. D.A. Rosie for gas chromatography - mass spectrometry facilities, to the Tropical Products Institute for library facilities, to Mrs M. Stockley for typing the thesis and to my wife, Jackie, for the reproduction of diagrams and for her patience during the preparation of the thesis.

CONTENTS

1. INTRODUCTION

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION.

1.1 Source of the insecticide

1.1.1 Production

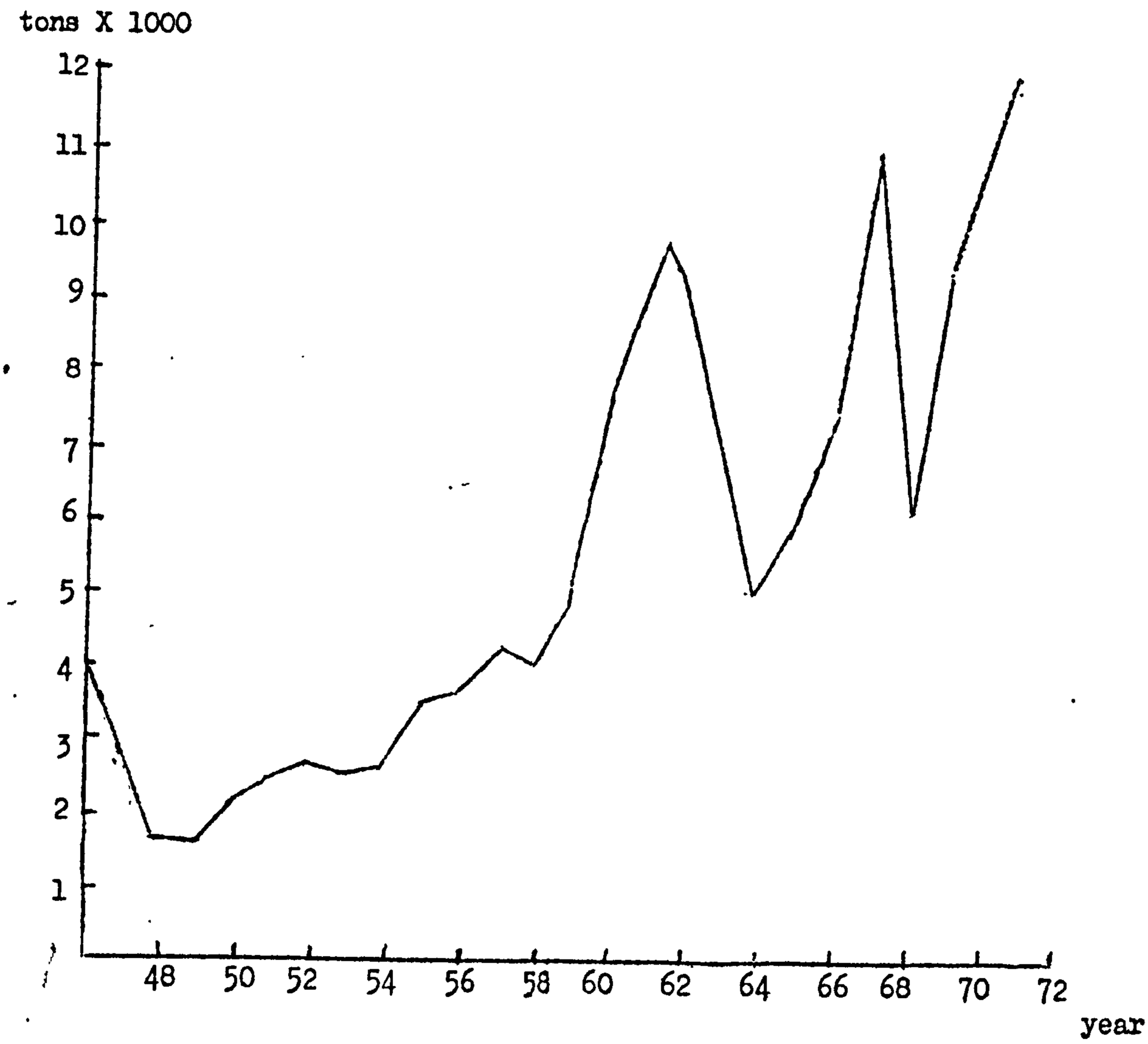
Pyrethrum, more correctly termed Chrysanthemum cinerariaefolium, is a perennial plant of the genus Chrysanthemum which produces a large quantity of white, daisylike flowers. Found in most parts of the World, including Britain, it is probably the earliest known source of insecticide known to mankind; its reported use dates back to 400 B.C.

Although a few other members of this genus also provide a source of insecticide only pyrethrum has gained commercial importance. Its commercial cultivation began in Dalmatia in the early nineteenth century but interruption of supplies during the First World War led to dominance of the World trade by Japan. Japanese trade suffered the same fate however in the Second World War and Kenya, where the plant had been introduced early this century, became the new successor.

Figure 1 shows the expansion of the pyrethrum industry in Kenya in terms of dried-flower production. The post-war expansion in the use of chlorinated pesticides, the rapid changeover from large European-owned farms to cooperative based plot-holdings on achieving independence (1963) and the wide scale introduction of a plant selection scheme (1968) caused temporary setbacks. Over half of the World production is supplied by Kenya and since demand exceeds supply continued expansion is expected.

The climate is of particular importance in the success of commercial cultivation[1,2]. For flower initiation chilling is essential followed by long periods of sunshine and ample rainfall (about 4 inches per month) but with a sufficient dry season for weed

Figure 1 Annual production of dried pyrethrum flowers in Kenya



control. Extreme climatic conditions must be excluded. Areas at high altitude in equatorial regions exhibit the ideal conditions, allowing an 8 to 9 month harvesting season so that one plant yields several crops of flowers per season.

The Kenya highlands, over six thousand feet above sea level, are ideally situated[2]. In addition to a favourable climate the volcanic soils are deep, fertile and well-drained allowing retention of the moisture in dry weather and since harvesting is not mechanised the relatively low labour costs help to maintain competitive prices.

Only the flower heads are picked at the appropriate stage of maturity[2,3], since the greatest concentration of the active principles are located in the achenes of the flower head although their general distribution throughout the plant has long been recognised[4,5]. A guaranteed average content of 1.3 per cent active material in the dried flower head helped to establish Kenya as a major World exporter of dried flowers although flower heads containing greater than 2 per cent are now frequently encountered.

1.1.2 Marketing

Prior to World War II pyrethrum insecticide was marketed in the form of finely ground flowers[6] but with the advent of sprays and fogging devices[7,8] the need for pyrethrum powder diminished except for the manufacture of mosquito coils[9]. The bulk of pyrethrum is now processed in the country of origin and exported as pyrethrum extract, either as a crude dark-brown oleoresin, prepared by multistage counter-current percolation of the dried flowers with light petroleum or, more recently, in a decolourised form produced by extra refining to meet the need for non-staining aerosol formulations[10,11].

Oleoresin concentrate contains approximately 30 per cent insecticidal material but is usually diluted with kerosene for marketing at a standard 25 per cent. The non-insecticidal material is largely of unknown constitution but contains substantial quantities of plant waxes, plant pigments such as chlorophylls and carotenoids, fats and other hydrocarbons[12].

Various methods of purifying oleoresin, to concentrate the active material, have been devised[13]. Dewaxing by low temperature treatment removes some of the extraneous matter, but marketed, non-staining, "Pale Extract" is produced by methanolic extraction of oleoresin at low temperatures followed by decolourisation with activated charcoal[14].

Solvent-free Pale Extract contains approximately 60 per cent active material but is diluted with Shellsol T, a commercial isoparaffinic solvent, to 25 per cent. The use of lower extraction temperatures, about -45°C, and an optimum 5 per cent water in the extraction solvent has recently been shown to give improved purification[15].

The unrivalled method of purification, without regard for economic requirements, is the "Nitromethane concentrate" process developed by Barthel, Haller and La Forge[16]. Three extractions by nitromethane were found to extract 97 per cent of the active material from oleoresin extract and selective adsorption of extraneous matter by activated charcoal yielded an almost colourless solution. The involatile material contained less than 10 per cent non-active matter.

1.2 The active principles

1.2.1. Structure and terminology

Insecticidal activity in pyrethrum has been shown to be due to the presence of six, structurally related, esters termed pyrethrin I, cinerin I, jasmolin I, pyrethrin II, cinerin II and jasmolin II (Figure 2). Identification of these esters as the toxic constituents of the plant was only achieved slowly. The pioneer work of Staudinger and Ruzicka, published in 1924[17], identified the pyrethrins although their assigned structures were later modified. Twenty years later La Forge and Barthel[18] identified two new insecticidally active components of pyrethrum, the cinerins, and as recently as 1966 Godin and co-workers[19-21] determined the structure of the jasmolins.

Due to this historical development the term 'Pyrethrins' became accepted as the collective name for these components with 'Pyrethrins I' and 'Pyrethrins II' distinguishing the two sub-classes. Verbal confusion may often arise and therefore throughout this work the term pyrethrins has been used to denote only pyrethrin I and

Figure 2 Structure of the natural rethrins.

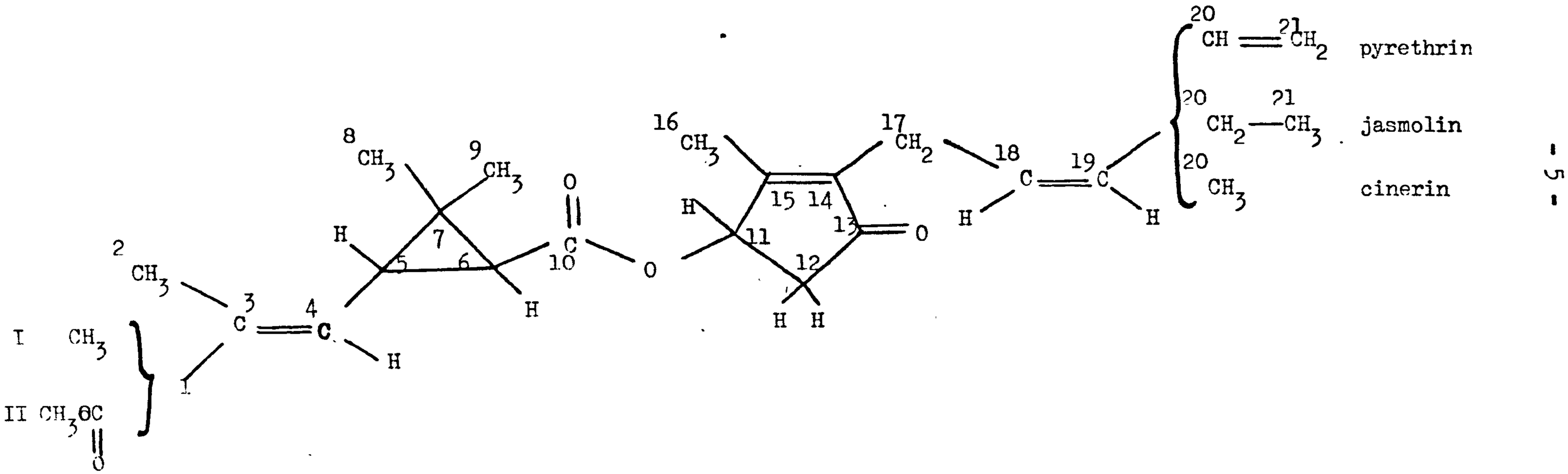
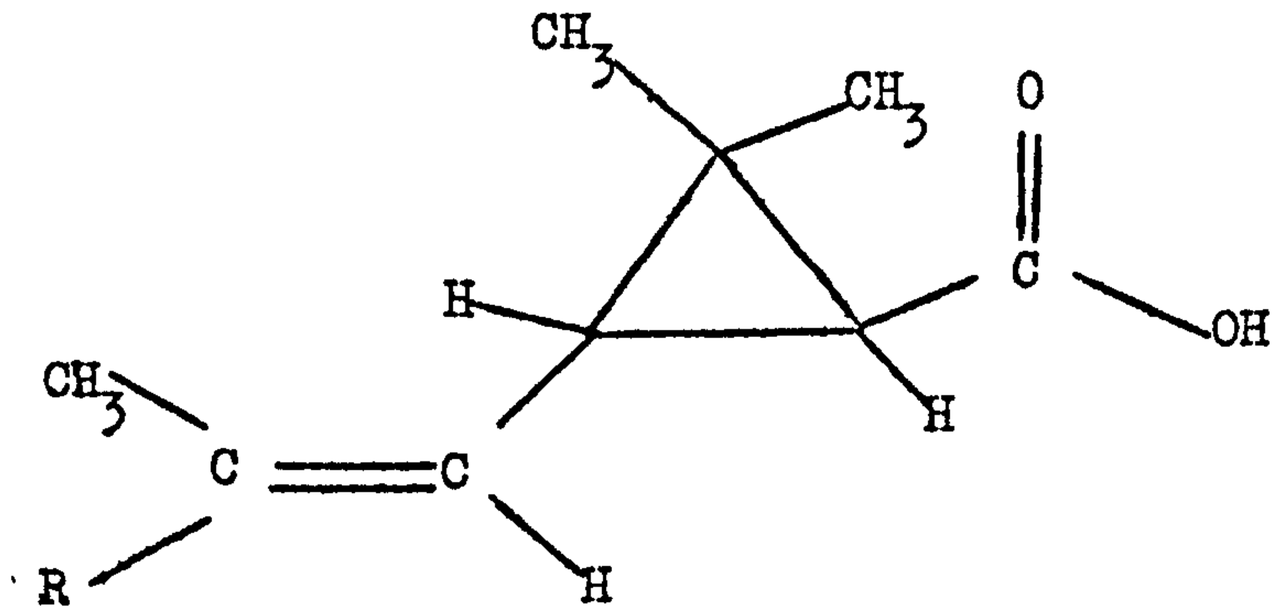
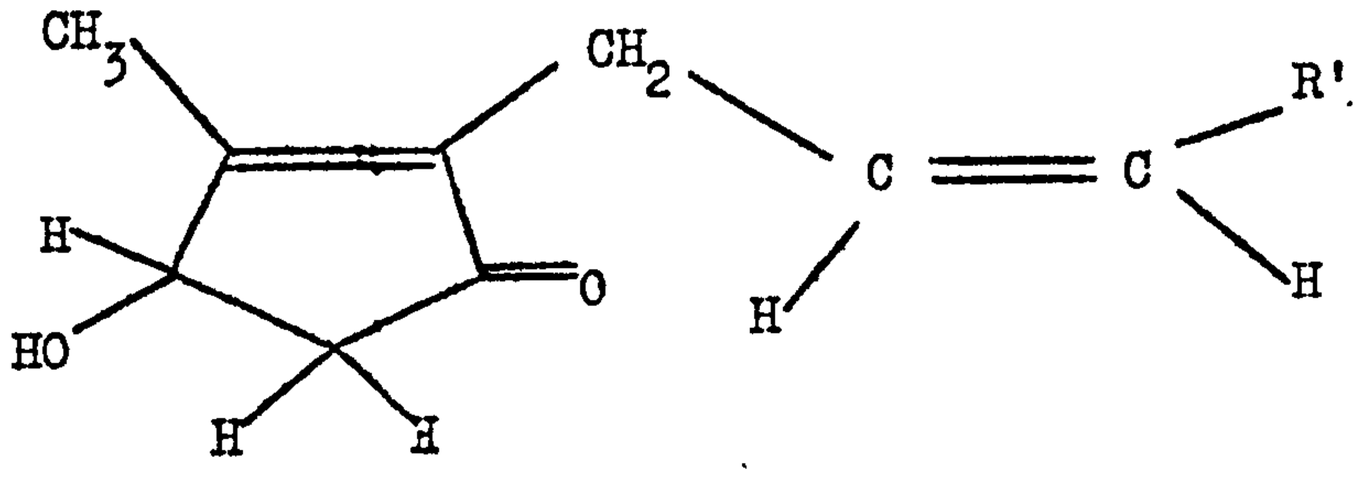


Figure 3 Structure of the naturally derived chrysanthemum acids.



R	
CH_3	chrysanthemum monocarboxylic acid / chrysanthemic acid
$COOCH_3$	pyrethric acid
COOH	chrysanthemum dicarboxylic acid

Figure 4 Structure of the naturally derived rethrolones.



R'	
$CH = CH_2$	pyrethrolone
$CH_2 - CH_3$	jasmolone
CH_3	cinorolone

pyrethrin II collectively (analogous to cinerins and jasmolins) whilst the term 'rethrins', originally suggested as the basic stem by Harper[22], has been used to denote the six compounds collectively. The term 'synthetic rethrins' or 'pyrethroids'[22] signifies compounds with structures and activity similar to those found in nature.

The acid components of rethrin I and rethrin II esters are chrysanthemic or chrysanthemum mono-carboxylic acid and pyrethric acid respectively (Figure 3). Pyrethrolone, cinerolone and jasmolone (rethrolones[22]) (Figure 4) are the alcohol components of the pyrethrins, cinerins and jasmolins respectively.

Both the acid and the alcohol moieties have geometrical and optical isomers. There are therefore sixteen possible isomers for each rethrin, of which only one has been found to occur naturally, the (+)<u>cis</u> rethronyl (+)-<u>trans</u> chrysanthemumate.

The proportion of the six naturally occuring rethrins in the pyrethrum flower varies throughout the plant and between different clones[23] and is also dependent on the maturity of the flower[4]. The ratio of rethrins I to rethrins II may therefore range from about 0.6 to 2.4[23] but composite samples of Kenya flowers usually have ratios between 0.8 and 1.0 (as determined by the A.O.A.C. variant of the official method of analysis, see 1.4). Processing to yield pyrethrum extracts increases the concentration of the rethrins I relative to rethrins II so that the ratio is close to unity and the proportion of each constituent is remarkably constant[24,25]. The proportion in a typical extract is shown in Table 1.

Table 1 Proportion of rethrins in a typical extract

proportion as % of rethrins I			proportion as % of rethrins II			rethrins I / rethrins II
cinerin I	jasmolin I	pyrethrin I	cinerin II	jasmolin II	pyrethrin II	
20	11	69	28	10	62	1.0

1.3 Toxicity

1.3.1. Insect response to the natural insecticide

There is conflicting evidence as to the relative insecticidal activity of the individual rethrins[26-28] which has undoubtedly arisen due to inconsistancies in criterion of response, mode of application, solvent media and sample purity.

As contact pesticides, the rethrins primarily act on the central nervous system but initial penetration into the phase associated with the peripheral nervous system incurs vigorous excitation followed by paralysis which is exhibited in the rapid knockdown property that has become characteristic of pyrethroid insecticides. Work on synthetic compounds (see 1.3.4.) has confirmed that the toxic and paralysis properties are independent actions, the former being dependent on the ability to overcome the detoxifying mechanism of the insect and the latter on the speed of diffusion and dissolution of the insecticide in the external phase. Since each of the rethrins exhibits these properties to various degrees the criterion of response is obviously important in assessing data on their relative insecticidal activity.

There are several methods of administering a poison to an insect; (i) feeding as a stomach poison, (ii) by injection, (iii) by topical application, (iv) in an inert dust carrier, (v) as a fumigant and (vi) by means of an aerosol spray. The first is inapplicable to rethrins and although the second is thought to produce

more reliable data it is foreign to that experienced by the insect in the field. Topical application is the most favoured method of assessing insecticides and was used by many of the early workers. Powdered pyrethrum, the early form of marketing the insecticide, the use of thermal fogging devices, mosquito coils and aerosol bombs lends support to the argument that the latter three modes of administering the insecticide yields data more compatible with results found in practice.

Differences in the physical state of the insecticide which is profoundly affected by the solvent media[29] may produce pronounced changes in the relative toxicity of the rethrins especially, it would appear, that of pyrethrin I and pyrethrin II[30]. The solvent itself may also exhibit marked toxicity or synergistic (see 1.3.2.) properties[31].

Bio-assays on the active constituents derived from their natural source suffer from the uncertainty of sample purity. This has been emphasised by the periodic discovery of the rethrins and the incomplete knowledge of the nature of much of the extraneous matter in pyrethrum extracts. The use of their synthetic analogues, following the development of suitable synthetic procedures, often fails to distinguish between each isomeric form (see 1.3.4.).

Many of the conclusions of the early workers must therefore be viewed critically. In general terms the pyrethrins exhibit greater biological activity than the cinerins or jasmolins and the rethrins I (chrysanthemates) are more toxic than the rethrins II (pyrethrates) but the latter appear to exert greater paralytic action[32]. The relative toxicity of pyrethrin II however appears to be greatly susceptible to changes in insect species and experimental conditions so that it may be found reversed relative to pyrethrin I or cinerin I[33,34].

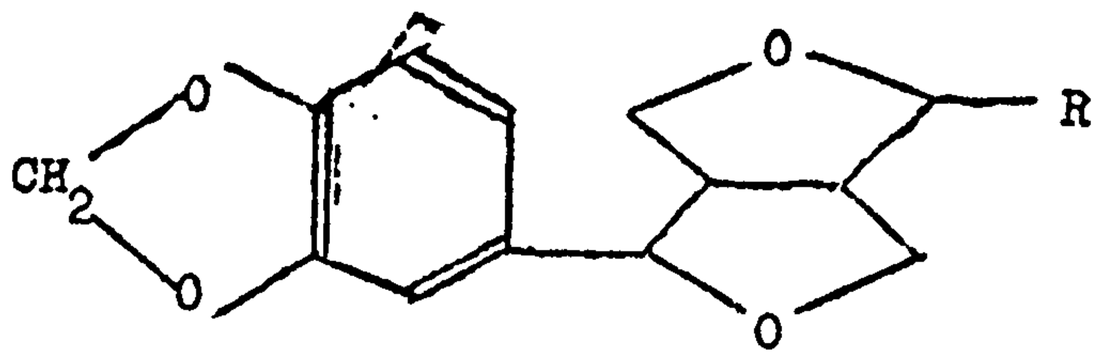
In addition to its toxic and rapid paralysing properties pyrethrum is also an efficient repellent[35-37]. Little is known of this mode of action since it is difficult to distinguish between the various physiological stages. Activation and knockdown closely follow the irritation phase which is associated with the repellent action and therefore the discriminating ability of the insect is soon dominated by the toxic action. Repellency however persists at concentrations below that required for toxicity[38] and is a property not associated with most insecticides.

1.3.2. Synergism

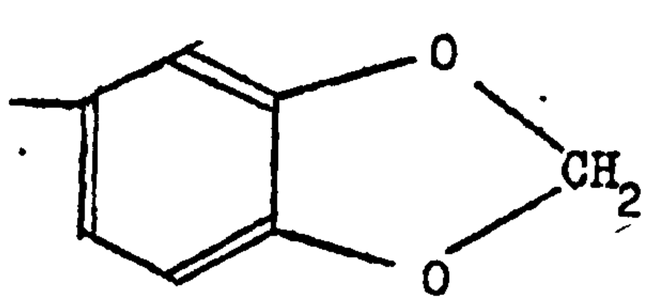
The enhancement of insectidial activity by compounds of little or no insecticidal activity to a value greater than the summed activity of the independent compoundsiis termed synergism. The rethrins have been found to be susceptible to synergism and a large number of compounds have been claimed as pyrethrum synergists[39-41]. One of the first to be discovered and achieve practical importance was sesamin[42] (I) isolated as an active principle of sesame oil[43,44]. This, and the related, more potent sesamolin[45] (II), illustrated the importance of the methylenedioxyphenyl group and formed the basis for the development of pyrethrum synergists; piperonyl butoxide[46] (III) being the most notable.

The degree of synergism varies with each rethrin but in general rethrins I are better synergised than rethrins II[47].

The use of synergists, which are relatively cheaper to obtain, greatly reduces the cost of pyrethrum insecticide formulations as well as enhancing their activity. Since the rate of enhancement decreases with increase in synergist concentration there is an optimum practical ratio of synergist to insecticide based on economic considerations[48]. In formulations for use against houseflies a ratio of approximately 8:1 synergist:rethrins is commonly used.

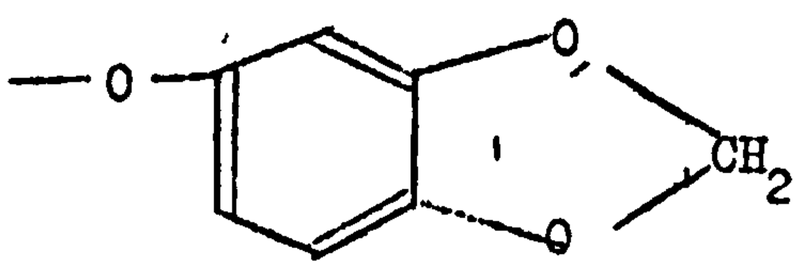


R =



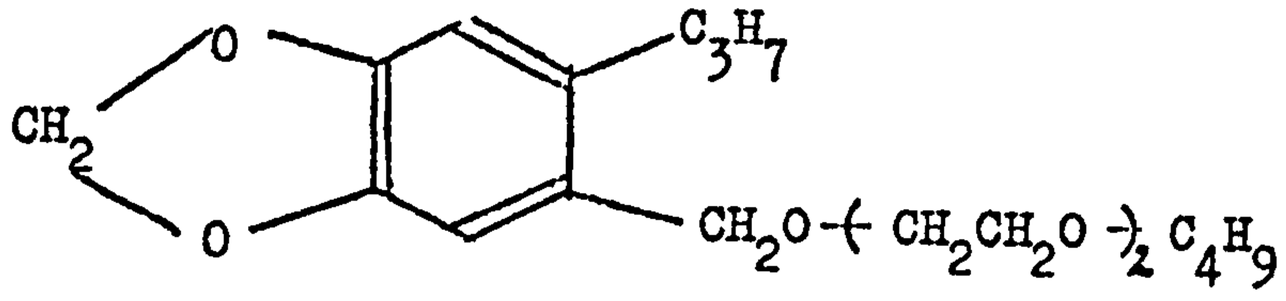
I

sesamin



II

sesamolin



III

piperonyl butoxide

1.3.3. Resistance and mammalian toxicity

Insects attempt to combat the effect of pyrethroid insecticides by a detoxifying mechanism which is the subject of present investigations by Casida and co-workers. Present knowledge indicates that in houseflies one process in the detoxification of rethrins I is the oxidative metabolism of the trans-methyl group of the isobutenyl moiety (Figure 2; C-1) by the housefly abdomen-NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) enzymatic system. The hydroxymethyl compound is formed which, by the action of further enzymes, form a conjugate or is oxidised to the corresponding aldehyde and acid compounds[49,50]. Little if any hydrolysis or modification of the alcohol moiety has been detected. The products of enzymatic oxidation possess only a fraction of the toxicity of the original rethrins.

If a sub-lethal dose is applied, the insect may be initially paralysed but will recover by detoxifying the insecticide before it can reach the central nervous system. Only if the detoxifying mechanism is 'swamped' is the insect killed. Synergists in general, and piperonyl butoxide in particular, apparently act by blocking the initial oxidation of the rethrins by substituting at the active site of the detoxifying enzyme[51]. In methylenedioxyphenyl synergists hydroxylation occurs at the methylene-dioxy moiety.

Insects which successfully survive insecticidal action give rise to resistant strains but resistance to pyrethrum insecticide does not appear to have developed at the same alarming rate as resistance to organo-chlorine and organo-phosphorus insecticides despite its long record of usage[52]. Reported cases of resistance in the field are few and in labcratory studies it is equally rare. Where encountered it is interesting that resistance to synergised rethrins is lower than to thcse unsynergised and, therefore, there is an increase in response of resistant strains to synergised rethrins[53].

The toxicity of insecticides to mammals is a factor which has been given increasing attention in recent years. In the case of pyrethrum insecticide the work reviewed by Jolly and Waterhouse[54] and Malone and Brown[55] has confirmed the view, established over decades of use, that the rethrins are very low in order of chronic mammalian toxicity[56]. It is widely used in public water systems for the control of organisms such as water lice[57,58] and there are reported cases of pyrethrum possessing anthelmintic properties[59].

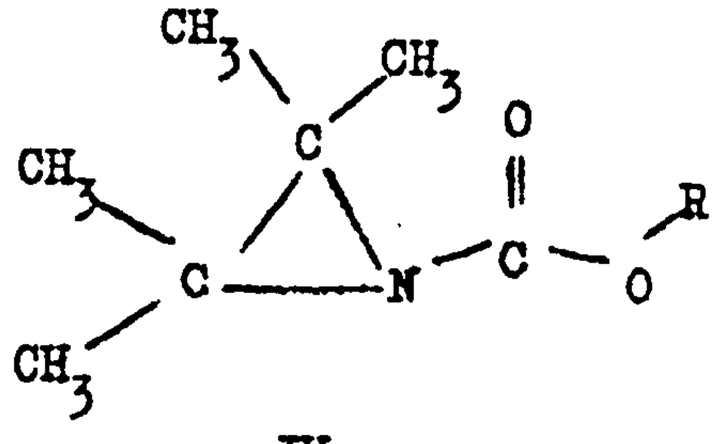
Rethrins may be absorbed through the respiratory tract, gastrointestinal tract and, in small amounts, through the skin but appear to be rapidly metabolised by a similar mechanism to that which operates in some insects[60]. Unrefined pyrethrum extract is known to produce skin irritations in humans but tests with refined extracts

showed negative reaction indicating that the irritant is contained in the extraneous matter of crude extract[61-63].

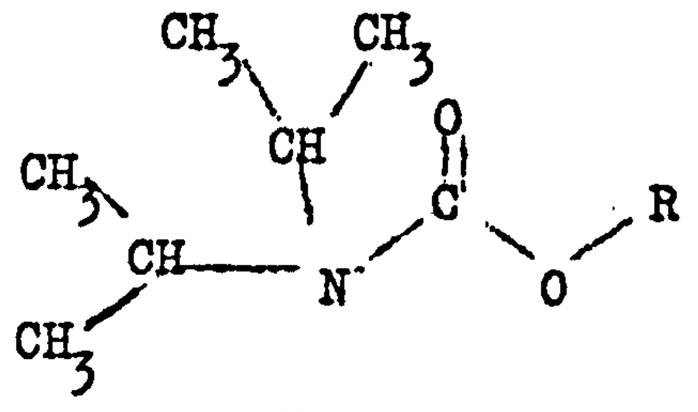
1.3.4. Importance of the stereochemical structure and the development of synthetic analogues

The necessity for the rethrins to adopt the required conformation to enable intimate contact to be made with the receptor is very much in evidence by the sensitivity of their insecticidal action to chemical and stereochemical changes within the molecule. The development of synthetic procedures has enabled this to be extensively studied leading to the development of synthetic, rethrin-like insecticides. Much of this work has been accomplished by Elliott and co-workers at Harpenden and their work and that of earlier workers has been excellently presented in a series of reviews[26,64-66].

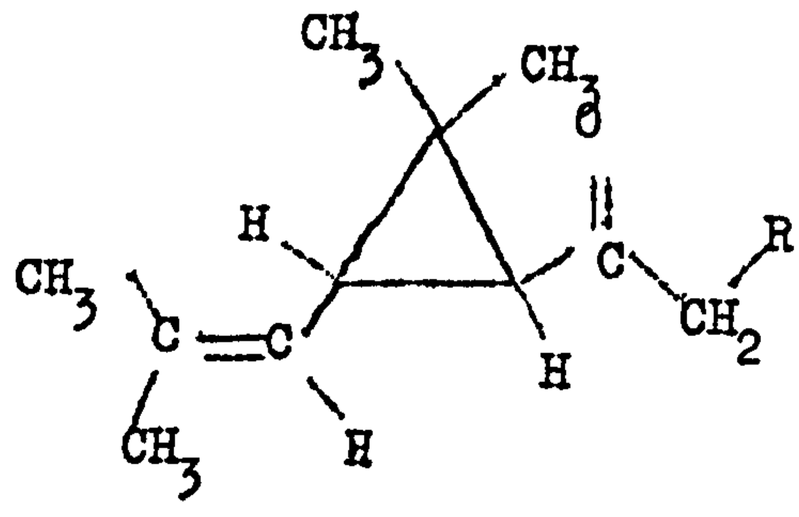
The evidence indicates the importance of two separate centres within the rethrin molecule: An isopropyl skeleton in the acid moiety and an unsaturated side-chain in the alcohol moiety (Figure 2; C-7, 8, 9 and at C-14) appear to be the major features although the component acids and alcohols are inactive. The importance of the ring structures in each moiety appears to be in providing the correct spacial arrangement of these two groups. The gem-dimethyl substituted cyclopropane ring would appear almost unique in this respect, providing an isopropyl group in a definite stereochemical configuration relative to the carboxyl group. Most attempts to replace the ring have led to inactive compounds but recent work by Berteau and Casida[67] has illustrated that a certain degree of activity is maintained when the cyclopropane ring is replaced by the tetramethylaziridino group (IV) or diisopropylamino group (V).



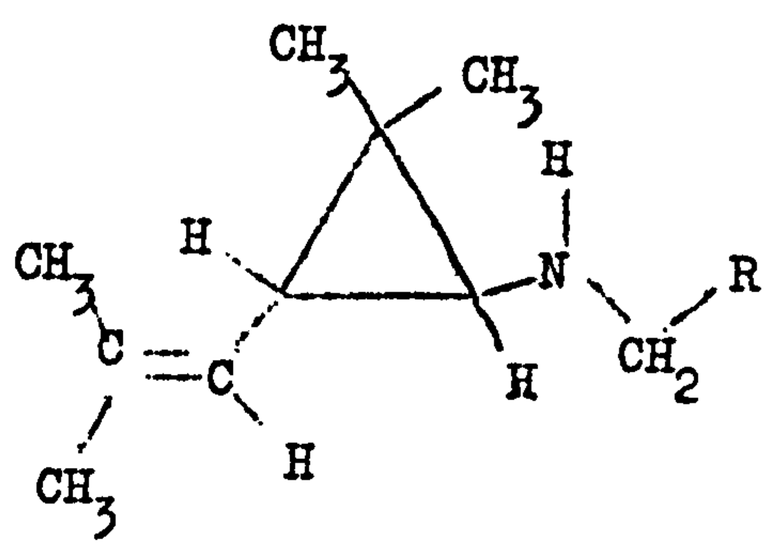
IV



V



VI



VII

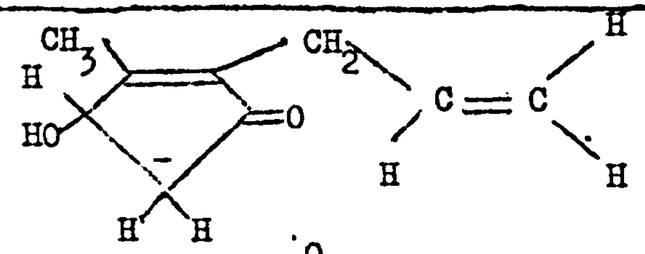
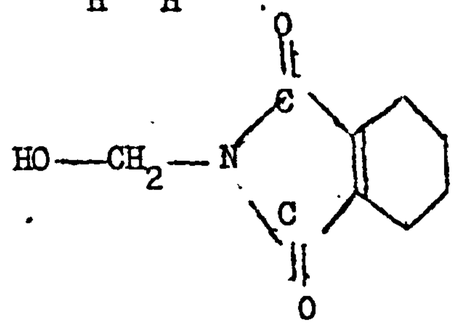
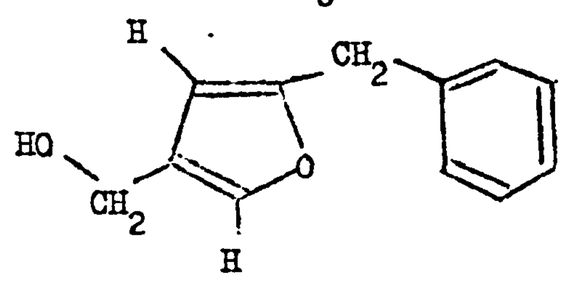
R = rethronyl

Rethrin-like compounds with substituents other than the trans-isobutenyl group in the acid moiety (Figure 2, at C-5) have been shown to possess various degrees of insecticidal activity, some greater than the rethrins, but apparently the ease of detoxification is affected in mammals as well as insects[66]. The ester linkage has been successfully replaced by a ketone (VI) or amino (VII) group[67] and the optimum site of attachment to the cyclopentenolone ring varies with different compounds, further evidence of an optimum stereochemical configuration, but should be remote from the alcohol side chain. The α,β -unsaturated ketonic system (Figure 2; C-13, 14, 15) is not important in itself but probably provides a suitable area of high electron density for interaction at the receptor site. These groups are therefore not essential features of the rethrin molecule for insecticidal activity.

It has long been established that at least one degree of unsaturation is essential in the cyclopentenolone side chain for rethrin-like activity. The absence of any side-chain removes all toxicity. Compounds with a saturated side-chain exhibit low toxicity although a high knockdown rate is reported to be maintained. Toxicity is increased slightly with an increase in side-chain length to an optimum value of four to five carbon atoms. A double bond at the second carbon atom of the chain (Figure 2; C-18) leaving a methylene group between this and the ring, provides greatest toxicity but if further removed from the ring it is reduced to almost that of the saturated analogue. Substitution on the double bond is unnecessary and therefore the rethronyl analogue with the simplest side chain is that containing the allyl group (VIII). The chrysanthemate of this compound, termed allethrin[68], was the first synthetic rethrin analogue to be commercially produced as a rival to the natural insecticide (see Figure 5). As a rethrin I analogue, 'allethrin I' is a more precise nomenclature. Compounds with branched side-chains have substantially reduced toxicity and addition of alkyl groups to the chain causes a slight decrease but vinyl substitution greatly enhances toxicity. (Hence pyrethrins » allethrin ≥ cinerins ≥ jasmolins).

The importance of the stereochemical requirements is further emphasised by reference to the toxicity of the isomers of the naturally ocurring rethrins. This is found to vary markedly although the major difference is between the corresponding optical isomers whilst the geometrical isomers show only slight difference. The trans-acid esters are of the order of two to four times as toxic as the cis-acid esters but esters of the cis-alcohol are only slightly more toxic than those of the trans-alcohols. In both moieties however the dextrorotatory isomer yields the most potent insecticide. Esters of the (+)-alcohols are four to five times more toxic than

Figure 5 Commercial pyrethroids.

Common name	Chrysanthemate of :	
+ Allethrin, allethrin I	allethrolone	 VIII
+ Neopynamin, + phthalthrin, tetramethrin	N-hydroxymethyl tetrahydrophthalimide	 IX
+ SBP-1382, resmethrin, * bioresmethrin	5-benzyl-3-furyl-methyl alcohol	 X

+ trade name

* name used for the (+)-trans chrysanthemate (the marketed form of the commercial insecticides contains a mixture of the geometrical and optical isomers)

those of the (-)-alcohols whilst (+)-acid esters may be more than fifty times more toxic than the corresponding (-)-acid ester. The esters constituted from the (+)-*trans* acids and (+)-*cis* alcohols therefore have the optimum stereochemistry for maximum toxicity.

A logical development of the work elucidating the structural requirements for rethrin-like toxicity was the synthesis of compounds of greater activity than the natural rethrins and the marketing of synthetic pyrethroid insecticides. Allethrin, as noted above, was the first such compound to be marketed although in general it is less toxic than its natural homologues and its commercial development in the United States was probably prompted by the desire to be independent of the foreign source of natural insecticide. Neopynamin[65] (Figure 5) was the first pyrethroid to approach the activity of the natural rethrins whilst the recently marketed SBP.1382 (Figure 5) has been shown to be twenty times more toxic to houseflies than the natural insecticide[70].

The development and marketing of synthetic pyrethroids would appear to offer new competition to the natural product. However there are several points which illustrate their complementary rather than competitive roles and the need for caution in assessing their relative merits.

First it should be noted that the development of the synthetic products was achieved solely by modification in the alcohol moiety. This removed the source of optical and geometrical isomerism in this part of the molecule but no satisfactory substitute has been found for the acid moiety. The marketed synthetic pyrethroids are therefore a mixture of four isomers and data on their toxicity should not be confused with data on the most active isomer. Commercial allethrin, for example, is approximately one quarter as toxic as (+)-allethronyl (+)-*trans*

chrysanthemate[27] and SBP.1382 has less than two-fifths the toxicity of benzyl-3-furylmethyl (+)-trans chrysanthemate to houseflies[65,69]. The marketing of the most active isomer now that (+)-trans chrysanthemic acid is commercially available will be dependent on the price of the new product.

The degree of increased activity is often further complicated by relating the activity of the synthetic pyrethroid to that of pyrethrin I rather than pyrethrum. Since the composition of the latter may vary between samples the use of pyrethrin I provides a more precise value for the relative activity of the pyrethroid but this may be several times smaller than that related to pyrethrum.

Toxicity ratings will also be dependent on the insect species used and many synthesised rethrins have been found to exhibit activity to only a few species and to be actually less toxic to many others. The commercially available pyrethroids are no exception; a point not always noted in voicing the spectacular claims of marketed products[70]. Neopynamin is as toxic to houseflies as natural pyrethrum but less toxic to other insects[66,71]. Similarly SBP.1382 is twenty times more toxic to houseflies but only one and a half times as toxic to mustard beetles[69] and less toxic if compared to pyrethrin I[66,71].

The attempts to develop the more toxic pyrethroids have been, to some degree, to the detriment of their rapid paralysing properties. Neopynamin exhibits greater knockdown ability to houseflies relative to pyrethrum but SBP.1382 is inferior in this respect and comparison of esters of 5-benzyl-3-furylmethyl alcohol (X) has indicated that toxicity and knockdown responses are not correlated[27].

They do not therefore possess the same broad spectrum of effectiveness as pyrethrum although their greater specificity in insecticidal action may render them more suitable for use in areas not open to the natural insecticide.

The degree of mammalian toxicity is similarly found to be delicately balanced and although many synthesised pyrethroids have even lower mammalian toxicity than natural pyrethrum some, mainly with modified acid moieties, have been found to exhibit increased toxicity[66]. It is notable that the (+)-*cis* chrysanthemate of 5-benzyl-furylmethyl alcohol shows much greater mammalian toxicity than the (+)-*trans* ester[66].

The fact that synthetic products are intrinsically different from the naturally derived insecticide is further emphasised by the degree to which they may be synergised. The toxicity to houseflies of bioresmethrin (Figure 5), the most active isomer in SBP.1382, has not been found to be enhanced using piperonyl butoxide at a toxicant:synergist ratio of 1:1 and racemic and isomeric mixtures showed only slight increase in activity[69]. Further work using pretreatment of the insect with a large dose of a methylenedioxyphenyl synergist enabled the true toxicity to be determined undiminished by detoxification. This confirmed the low synergistic factors for the most apparently toxic compounds whilst pyrethrum was increased by a factor of 200 and pure pyrethrin I by 300[65,66]. The toxicity of the natural insecticide then approaches that of the most potent synthetic product. Although insecticidal formulations containing synergists may not achieve this impressive degree of synergism in practice, it is necessary to note that the toxicity relationship between synthetic pyrethroids and the natural product so often quoted may bear little resemblance to their relative performance in the field.

1.4 Methods of analysis

1.4.1. The mercury-reduction method (A.O.A.C. and P.B.K. variants)

The official method for the analysis of pyrethrum flowers and extracts which is at present in use is based on the alkaline cleavage of the rethrin esters followed by the titrimetric determination of the separated chrysanthemic and chrysanthemum dicarboxylic acids (Figure 3). This enables the rethrin I and rethrin II content, then thought to be solely pyrethrins (see 1.2), to be assessed separately.

The method developed from a procedure put forward by Seil[72] which involved hydrolysis of the extract, precipitation of irrelevant fatty acids as their barium salts, separation of the chrysanthemum acids by steam distillation andtheir estimation by alkali titration. The discovery that some of the chrysanthemic acid is hydrated during steam distillation and remains to be assessed with the di-acid[73] caused this method to be abandoned in part in favour of the 'mercury-reduction' method.

Originally devised by Wilcoxon[74] and modified by Holaday[75] the method differs from that of Seil in the extraction and estimation of chrysanthemic acid. This is separated from the di-acid by extraction with petroleum spirit (b.p. 40 - 60°) and determined by reaction in alkaline solution with aqueous mercuric sulphate solution (Denigès reagent). The mechanism of the estimation reaction is not fully understood but mercurous sulphate is formed via a series of characteristic colour changes. The quantity of mercurous sulphate is determined volumetrically by titration with potassium iodate and related to the rethrin I content of the original sample by an empirical factor arrived at by collaborative work on standard chrysanthemic acid solutions[76]. The rethrin II content is determined by alkali titration as in the Seil method.

There are two main variations of the mercury-reduction method. One, the A.O.A.C. (Association of Official Agricultural Chemists) method[77] is used predominantly in the U.S.A. although it has been increasingly used in Europe in the last decade. The other is the P.B.K. (Pyrethrum Board of Kenya) method[78] and is similar to the methods described by the Tanganyika Extract Company Limited[79], the British Pharmaceutical Codex 1954[80] and the British Veterinary Codex 1953[81].

The principal differences between the major variations of this method lie in the procedure following the precipitation of the fatty acids as their barium salts from the mixture of chrysanthemum acids by addition of barium chloride. The A.O.A.C. method advocates acidification with sulphuric acid causing precipitation of excess barium which is then removed. Evidence of chrysanthemic acid loss by occlusion with the barium sulphate[82] however led to the P.B.K. procedure of acidifying with hydrochloric acid. This difference results in the former procedure yielding values for rethrin I content which are 75 to 85 per cent of those returned by the latter variant.

The A.O.A.C. 9th Edition method is merely the 8th Edition defined more precisely in order to improve repeatability between different analysts.

The 10th Edition includes a modification in the procedure for the purification of chrysanthemum dicarboxylic acid to exclude water-insoluble material which frequently leads to falsely high results[83,84]. This has been found to yield values for rethrin II content which may be only 96 per cent of those returned by unmodified procedures.

1.4.2. The ethylenediamine (E.D.A.) method and the alkoxyl method

The E.D.A. method, originally designed for the analysis of allethrin[85], a synthetic rethrin homologue (see 1.3.4.), has been proposed by Wachs and Hanley[86] as an alternative to the mercury-reduction method for pyrethrum analysis although it is reported to have been in use for quality control for a number of years[86]. The method is based on the selective reaction of ethylenediamine with rethrin esters to form amine salts of the carboxylic acids which are then titrated in pyridine with sodium methylate. The carboxymethyl group of rethrin II esters (Figure 2, C-1) does not react with ethylenediamine under the experimental conditions used therefore the total rethrin content is determined on the basis of the rethronyl ester function alone. It is recommended that a chromatographic purification step be used before analysis to remove extraneous matter which might interfere with the estimation (see 1.4.8.).

It has been suggested[87] that the E.D.A. method be used in conjunction with the alkoxyl method[88,89] for rethrin II determination which depends on the reaction of the methoxyl group of the rethrin II esters with hydriodic acid to form methyl iodide. This is then distilled into pyridine to form an alkyl pyridinium iodide complex which is titrated with tetrabutylammonium hydroxide. From the value for total rethrin content by the E.D.A. method and that for rethrin II by the alkoxyl method the rethrin I content may be found by difference.

1.4.3. The infra-red spectrometric method

A method based on the infra-red absorption characteristics of the chrysanthemic acids derived from the sample and separated by the P.B.K. procedure, has been suggested by Byrne, Mitchell and Tresadern[90,91]. Both acids exhibit a strong absorption at 1110 cms. (9.0 μ), corresponding to the carboxylic C - O stretching vibration, which was shown to obey the Beer-Lambert law

over a considerable range of concentrations. Percentage transmission was related to rethrin I or rethrin II content by calibration graphs of the respective pure chrysanthemum acids.

Direct assessment of total rethrin concentration has been proposed by McTaggart, Thornton and Harford[92] related to a standard pyrethrum extract.

1.4.4. The ultra-violet spectrophotometric method

In contrast to the methods of rethrin analysis which are based on detection of the acid moiety methods have been devised based on the detection of the alcohol moiety of the compounds. The spectrophotometric method of Bechley[93] for total rethrin analysis is in general commercial use for assessing dried pyrethrum flowers and the modified method of Shukis, Christi and Wachs[94] may be used to check extracts. The methods are based on the absorption maximum exhibited by rethrins in the ultra-violet region of the spectrum at about 225 nm. due primarily to the α,β-unsaturated carbonyl configuration in the rethrolone moiety (Figure 2; C-13, 14, 15). The pyrethrins show further absorption in this region due to the conjugated diene configuration in the pentadiene side-chain (Figure 2; at C-14) and the additional α,β-unsaturated carbonyl structure of the acid moiety side-chain of rethrins II (Figure 2; at C-5) produces an additional increase in absorptivity.

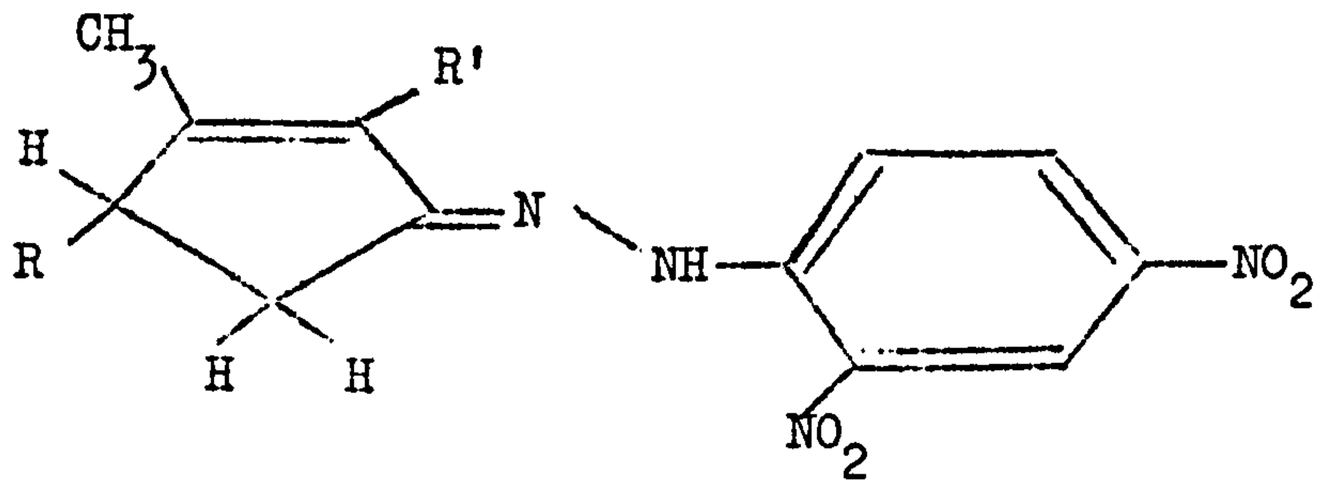
On the basis of published values of the absorptivities of the insecticidally active constituents (exclusive of the then unknown jasmolins) a theoretical value ($E^{1\%}_{225}$ (EtOH) = 1104) was calculated by Ward and Newham for an extract containing pyrethrin I, cinerin I, pyrethrin II and cinerin II in the ratio 3:1:3:1 (w/w)[95]. Pyrethrum samples may be analysed using this value or by direct comparison with standard samples analysed by the official method (1.4.1.).

1.4.5. <u>The dinitrophenylhydrazine (D.N.P.) method</u>

The D.N.P. method of pyrethrum assay also relies on the analysis of the rethrolone moiety for its assessment of rethrin content. The method was first proposed by Moore[96] who demonstrated that the reagent reacted quantitatively with allethrin I (Figure 5) under certain conditions and that the resulting 2,4-dinitrophenylhydrazone (d.n.p.h.) could be freed from concomitant substances by chromatography on alumina. The product was estimated spectrophotometrically.

The method was later modified by Green and Schecter[97] using milder reaction conditions under which the rethrin d.n.p.h. (XI) instead of the rethronyl ethyl ether d.n.p.h. (XII) was formed. This prevented interference from free rethrolones.

Modification of the chromatographic step by Moore[98] enabled rethrin I and rethrin II products to be separated and further improvements by Smith[99] provided separation of the then four known rethrin d.n.p.h.s.



R	R'		
chrysanthemumate	rethrolone moiety	rethrin d.n.p.h.	XI
CH_3 CH_2 O	" side-chain	rethronyl ethyl ether d.n.p.h.	XII

A revised, more rapid method has been suggested by Head for rethrin I and II analysis[100] as well as a method for total rethrin assessment which omits the chromatographic step[101]. This latter method is based on the observation that the forerun of the chromatogram, usually comprising of three bands, was derived from the reagent and not the sample. The amount present in each analysis was therefore constant and may be corrected for by a blank.

Since d.n.p.h.s. exhibit an absorption maximum at wavelengths of about 380 nm. due to the nitro-aromatic system there is no interference by other carbonyl or unsaturated diene functions in the molecule (c.f. ultra-violet spectrophotometric method, 1.4.4.). Each rethrin product therefore produces the same response, that is they possess the same molar absorptivity (E_{377} (EtOH) = 28000)[99].

1.4.6. Other colorimetric methods

Of the colorimetric methods[102-108] there are two which have achieved minor practical significance:

(i) The method of Williams et al, involves heating the sample with phosphoric acid - ethyl acetate (4:1) reagent to yield a red-coloured complex whose absorption is determined spectrophotometrically at 550 nm. The reaction is not given by cinerins or jasmolins and therefore is presumed to involve the pentadienyl side chain of the pyrethrins (Figure 2; at C-14). A positive reaction is given by pyrethrolone. Colour formation is suppressed by synergists and high boiling hydrocarbons. A prior chromatographic purification step is therefore necessary for application to extracts and formulations but the method has been applied to the assessment of rethrin concentration in water supply systems[57] and the rapid assay of dried pyrethrum flowers[109].

(ii) The 'sulphur-colour' method has two variations developed by Levy[107,110,111] and by Brown[108,112-114]. They both estimate the total rethrin content of a sample by heating with sulphur in carbon tetrachloride in an alkali medium to form a red-brown complex. The colour intensity is determined spectrophotometrically at 540 nm. and related to rethrin concentration by the response of a standard extract. The reaction is thought to involve the α,β -unsaturated ketone group of the alcohol moiety (Figure 2; C-13, 14, 15) and interference is shown by ketones and aldehydes. Levy's method however involves harsher conditions which, it has been shown[114], leads to early colour development and a less stable complex and caused discrepancies between the two variants.

1.4.7. Gas-liquid chromatographic (g.l.c.) methods

The development of g.l.c. over the last decade provided a new technique for the analysis of rethrins. Early workers in this field[115-118] were able to identify the rethrin components in the complex chromatograms of commercial extract by comparison with rethrin fractions isolated by other techniques and the jasmolins were first detected by g.l.c.[19,118].

An examination of oleoresin on a non-polar stationary phase (Apiezon L) by temperature-programming from 50°C to 300°C showed the presence of at least fifty components[25,118]. In general therefore the resolution was poor and the chromatograms were complex and not suitable for quantitative measurement.

A vast improvement in the attempts to quantify analysis of rethrins by g.l.c. was made when neopentylglycol succinate (N.P.G.S.) was used as stationary phase[25,119]. This retains much of the more polar material that, on non-polar packings, is eluted with the rethrins and allows examination of pyrethrum extracts without a prior cleaning procedure. Low percentage

loading of stationary phase (~ 1%) and a short column length (~ 1 m.) permit relatively short retention times without resorting to high operating temperatures. This is essential with regard to thethermal instability of the pyrethrins (see 1.5.1.).

The six rethrins elute from gas chromatographic columns in the order cinerin I, jasmolin I, pyrethrin I, cinerin II, jasmolin II and pyrethrin II. On N.P.G.S. stationary phase the jasmolin components are clearly distinguishable although not completely resolved from the respective cinerins, compared to almost indiscernible pre-shoulders to the pyrethrin peaks in the chromatograms from non-polar packed columns.

This g.l.c. method has not been used to determine the absolute rethrin concentration of extracts but has been used with standard extracts, which have been analysed by the A.O.A.C. method, to provide relative results. It may therefore be used as a method for yielding A.O.A.C. values for rethrin I and rethrin II content without recourse to the more tedious and time consuming official method[4,23] and in some cases only the pyrethrin I peak of the chromatogram has been used[57,120] to evaluate the sample by comparison with standard extracts.

A method based on the g.l.c. of the methyl esters of the chrysanthemum acids has been proposed by Beckman and Allen[121] and developed by Head[122]. The acids are derived from the rethrins by the mercury-reduction hydrolysis procedure and then methylated by an ethereal solution of diazomethane. Absolute values for rethrin I and rethrin II content can then be obtained by comparison with the response of standard methyl chrysanthemate and pyrethrate solutions.

1.4.8. Accuracy of the methods

1.4.8.1. 'False' rethrins

Due to the complex nature of pyrethrum extract composition it is apparent that a method of analysis which is not specific for the rethrins may give rise to inaccurate results by inclusion of material which gives a rethrin-like response. Such material may conveniently be termed 'false rethrins'[123,124].

The majority of methods which have been proposed may be classified in general terms as 'acid' methods and 'alcohol' methods on the basis of the portion of the rethrin molecule mainly involved in the detection procedure (Table 2). In 'acid' methods the false rethrins may be taken to include free chrysanthemum acids, chrysanthemum acids combined with other alcohols and other acids and their esters closely resembling these acids which have not been excluded from the estimation process. Similarly rethrolones, free and combined with other acids, and rethrolone-like material will be false rethrins in the 'alcohol' methods.

The presence of free acids in oleoresin, identified as lauric, palmitic, oleic and linoleic acids, was first demonstrated by Ripert[125]. They were also found to be present as esters and recently Head[124,12] has shown that 40 per cent of oleoresin is combined fatty acids which were identified by g.l.c. and their relative proportions determined.

La Forge and Haller[127] isolated fatty acids from the impure semicarbazone of pyrethrin I, suggesting the presence of significant amounts of fatty esters of pyrethrolone, and Acree and La Forge[128] showed that pyrethrolone is combined with palmitic and linoleic acids. Brierley and Brown[123] confirmed the former work by catalytic hydrogenation of rethrin I concentrate containing no

Table 2 Comparison of methods of pyrethrum analysis

Method of analysis	Section ref.	'Acid/ 'Alcohol'	Absolute/ Relative	Rethrin differentiation
Mercury-reduction (A.O.A.C. or P.B.K. variant)	1.4.1.	Acid	Absolute	rethrins I and rethrins II
E.D.A.	1.4.2.	Acid	Absolute	total rethrins } rethrins I and rethrins II
Alkoxy		Acid	Absolute	rethrins II }
Infra-red spectrometric	1.4.3.	Acid	Absolute	rethrins I and rethrins II
Ultra-violet spectrophotometric	1.4.4.	Alcohol	Relative or Absolute	total rethrins
D.N.P.	1.4.5.	Alcohol	Absolute	Variants allow: total rethrins rethrins I and rethrins II pyrethrin I, cinerin I, pyrethrin II, cinerin II
Phosphoric acid colour	1.4.6.(i)	Alcohol	Relative	pyrethrin I and pyrethrin II
Sulphur-colour	1.4.6.(ii)	Alcohol	Relative	total rethrins
G.l.c.:untreated	1.4.7.	Complete molecule	Relative	{rethrins I and relative proportion of each three {rethrins II and relative proportion of each three
hydrolysed		Acid	Absolute	rethrins I and rethrins II

free acids and estimated the presence of non-rethrin rethrolone esters equivalent to about 5 per cent of the rethrin I content as determined by the P.B.K. procedure. They also demonstrated the presence of a similar quantity of non-rethrin esters which gave chrysanthemic acid on hydrolysis.

Most methods however recognise the presence of possible contaminants and include a purification procedure to exclude them. The precipitation of fatty acids as their barium salts in the mercury-reduction method and the exclusion of water-insoluble material from the chrysanthemum dicarboxylic acid fraction in the A.O.A.C. 10th Edition procedure are attempts to do this but their success is dependent on the relative solubilities of true and false rethrin-derived material.

The work of Brown and co-workers has illustrated that a considerable amount of false rethrins is included by the mercury-reduction method of analysis. An extract solution passed through a column of activated alumina was found to lose 10 to 15 per cent of the total rethrin content as determined by the P.B.K. procedure while retreatment caused no further loss and the retained material had no biological activity[129]. Analysis of degraded extracts was found to give better agreement with bioassays when chromatographed on alumina prior to analysis[113].

The mercury-reduction method assumes that the separated chrysanthemum acids used in the estimation procedure are pure. This has been investigated by Brierley and Brown[123,124] who showed that silica chromatographic purification of the respective acid fractions derived by the P.B.K. procedure removed 15 to 20 per cent of the material previously supposed to be the corresponding chrysanthemum acid. In the case of the chrysanthemic acid fraction the material removed by silica chromatography was

shown to be almost entirely byproducts produced during alkaline hydrolysis[123]. An independent examination[130] of the chrysanthemum dicarboxylic acid fraction by paper chromatography indicated the presence of at least five different acids which together constituted between 9 and 18 per cent of the "chrysanthemum dicarboxylic acid", depending on the extract. Infra-red spectrometric results by Brierley and Brown[123,124] and by Mitchell and co-workers[90,91] and g.l.c. results based on the chrysanthemum acid content[122] agreed with these findings.

Since rethrin I results determined by the A.O.A.C. procedure are 75 to 85 per cent of those returned by the P.B.K. variant the former can be held as coincidentally providing the more accurate assessment of the true amount of total chrysanthemic acid (free and combined). The amount lost[82] in the A.O.A.C. procedure is therefore compensated by the inclusion of some material analysing as chrysanthemic acid. A knowledge of the chrysanthemum acid content (free and combined) however does not distinguish that derived solely from the rethrins.

The accuracy of other methods of analysis where absolute rather than comparative results are determined (see Table 2) has inevitably been compared with that of the official mercury-reduction method. Since these have generally been found to yield lower results than either variant of the official method, this is a further indication of its inaccuracy.

Results by the early D.N.P. method[96] on pure samples were 90 per cent of those returned by the official method* and only 60 per cent on some crude oleoresin samples. The discrepancies

* modified A.O.A.C. 7th Edition procedure, equivalent to 8th Edition procedure.

were underlined by analyses of a purified sample of allethrin when D.N.P. analysis yielded 99.7 per cent whilst the official method* produced values of 99.3 per cent (as rethrins I) - plus 8.4 per cent (as rethrins II).

The later modified D.N.P. method[100] was found to give results equivalent to about 79 per cent of both rethrin I and rethrin II values determined by the P.B.K. procedure. Results by the 'rapid' D.N.P. method[101], which omits the chromatographic step, are equivalent to about 86 per cent of the values returned by the P.B.K. procedure for the total rethrin content of crude extracts, but less than 80 per cent of those for refined extracts.

The direct ultra-violet spectrophotometric method gave inconsistant absorptivity values when based on mercury-reduction results of rethrin concentration. Lower values were found for Pale Extract compared to oleoresin[95,100] and similarly for old flowers and extracts as compared to fresh samples[95]. This latter observation infers greater loss of ultra-violet absorbing material and, if it is assumed that the absorption is attributed solely to the rethrins, a greater loss of rethrins than is indicated by the official method.

Chromatographic purification of samples before analysis by both methods provided a more consistant relationship between the two methods. The observed absorptivity values were then 1 to 2 per cent lower than the theoretical value proposed by Ward and Newham[95] and represents lower results for rethrin content than determined by the A.O.A.C. procedure.

* modified A.O.A.C. 7th Edition procedure, equivalent to 8th Edition procedure.

The sulphur-colour method, another 'alcohol' method, found better correlation with bioassays on extracts degraded by irradiation than the mercury-reduction method but it was noted that even this method showed increasing deviation from the bioassays as degradation became pronounced[112].

It has been suggested by Brierley and Brown[123,124] that the true rethrin content of pyrethrum samples is less than that returned by any of the present methods of analysis. Based on their investigation of 'false' rethrins and their assessment of the total chrysanthemum acid content (free and combined) (see pp. 30, 31) they estimated that true values were less than 70 to 75 per cent of those returned by the P.B.K. variant of the official method for both rethrin I and rethrin II results (about 90 per cent of rethrin I values by the A.O.A.C. variant).

Experience of the g.l.c. analysis of extracts led Head to report that the factor used to convert area response into component weight was 10 to 20 per cent lower for rethrins I than for rethrins II[24]. The factors were derived by equating the area response of a standard extract to its rethrin I and rethrin II content as determined by the A.O.A.C. 8th Edition procedure[77]. This difference is possibly due to a difference in responses produced by the rethrins I and II in the detection system (see 2.1.2.1.) but if these are the same then it would indicate that the true rethrin II content is only 80 to 90 per cent of that returned by the A.O.A.C. procedure - assuming the rethrin I result is correct. If the rethrin I result is high then the true rethrin II content will be in further error as shown below:

$$\text{Wt.} = \text{Area} \times \text{Factor}$$

$$\text{True Wt.} = \text{Area} \times \text{True Factor}$$

$$\text{Factor (I)} = R \times \text{Factor (II)}$$

where $R \neq 1$ (= 0.8 to 0.9 - Head[24])

$$\therefore \text{Wt(I)/Area(I)} = R \times \text{Wt(II)/Area(II)}$$

$$\therefore \text{Wt(I)} \times \frac{\text{True Factor (I)}}{\text{True Wt(I)}} = R \times \text{Wt(II)} \times \frac{\text{True Factor (II)}}{\text{True Wt(II)}}$$

If True Factor (I) = True Factor (II) then for an extract supposedly containing equal amounts of rethrins I and II (by official method) -

$$\text{True Wt(II)} = R \times \text{True Wt(I)}$$

The relative inaccuracy of the rethrin I and rethrin II results is therefore in agreement with the conclusions of Brierley and Brown.

1.4.8.2. Limitations of adsorption chromatographic purification

From a comparison of the methods of pyrethrum analysis (see 1.4.8.) it is apparent that at best the A.O.A.C. and P.B.K. variations of the mercury-reduction method can only claim commercial significance where comparative results are of primary importance. The method is empirical in nature and gives repeatable results only by strict adherence to the stated experimental conditions. A report published in 1964 investigated the method and found it to be "inherently unsatisfactory" and recommended its use only as an interim measure[131].

The alternative methods often give greater precision and appear to offer greater accuracy especially where the use of alumina chromatography as a prior purification step is recommended, and all methods, including the mercury-reduction method, would appear to benefit in accuracy if such a step was included. Adsorption chromatography cannot however be guaranteed to remove all false rethrins. The mercury-reduction method has been shown to require chromatography on alumina prior to hydrolysis and then on silica to purify the chrysanthemum acids and would still be

inaccurate by the inclusion of non-rethrin chrysanthemumates and chrysanthemum-like acids. The described E.D.A. and alkoxy methods would similarly analyse non-rethrin chrysanthemumates and similar esters. Results by the other 'acid' methods (Table 2), which are specific for the chrysanthemum acids, would not be inflated by the free acids if alumina chromatography was incorporated in the procedure but chrysanthemumates resembling the rethrins could escape retention on the column.

Results by the spectrophotometric method proposed by Ward and Newham[95] are comparable with results by the A.O.A.C. procedure when samples are chromatographed prior to analysis by both methods. If the A.O.A.C. results are high (see 1.4.8.), This implies that some non-rethrin ultra-violet absorbing material is eluted from the column. Such material has been reported to elute before the rethrins[95,132] but even if this was separately collected there is always the possibility that some of the extraneous matter would elute with the rethrins. The use of alumina chromatography prior to analysis by the 'rapid' D.N.P. method was shown to be equivalent to the chromatographic treatment of the reaction products[101]. The inclusion of carbonyl compounds, especially rethronyl derivatives, by any variant of the D.N.P. method is therefore probable. The other 'alcohol' methods (Table 2) are not only open to the same source of error but since the results are relative to a standard sample they incorporate the errors of the method used to standardise this sample (usually the A.O.A.C. method). In addition the colorimetric methods base their estimation on an empirical reaction of which little is known. This inevitably enlarges the source of errors.

As well as being an incomplete answer to the problem of false rethrins the incorporation of a chromatographic step prolongs the analysis time. The suggested infra-red spectrometric method and the 'rapid' D.N.P. method willingly sacrifice the increased accuracy for such a reason. Furthermore, adsorption chromatography is not readily reproducible and slight variations in the experimental conditions can affect the elution time and the resolution.

1.4.8.3. Errors arising from variations in sample composition

In addition to inaccuracies incurred by false rethrins the majority of methods fail to provide information on the proportion of the active constituents (Table 2) and errors may arise from variations in sample composition. In general the rethrins I and II are converted to a compound or compounds whose response in the detection step is independent of its/their source. The mercury-reduction method, the infra-red spectrometric method and the hydrolysis - g.l.c. method analyse the chrysanthemum acid content derived, theoretically, from the rethrins. In the E.D.A. method and D.N.P. method rethrin derivatives are formed which show responses independent of the rethrin-type.

Fundamentally the majority of methods therefore yield rethrin content in terms of the number of moles of rethrins and an error is incorporated in the methods by using an arbitrary factor to express the result in terms of weight. The mercury-reduction method uses the molecular weights of pyrethrin I and pyrethrin II to relate the chrysanthemic acid and chrysanthemum dicarboxylic acid to rethrin I and rethrin II content respectively. The values are therefore really pyrothrin equivalents. Values by the E.D.A. method for total rethrin content are derived using the average molecular weight or, when used in conjunction with the alkoxy method, the individual pyrethrin molecular weights. A hybrid

factor is used in most of the variants of the D.N.P. method assuming the relative proportions of the rethrins in the sample. The variant designed for individual estimation of the then known rethrins allows the use of their individual molecular weights but procedures which determine rethrins I and II content used a factor based on a pyrethrin to cinerin ratio of 4:1. The 'rapid' variant for total rethrin analysis assumes a pyrethrin to cinerin ratio of 3:1 and an equal rethrin I and II concentration. This compares with the original method of Moore who assumed an equal proportion of pyrethrins and cinerins but a rethrin I to rethrin II ratio of 6:4.

In the methods where rethrin I and II contents are determined the error from this source can be no greater than the difference in the molecular weights of the members of each rethrin sub-class (approximately 4 per cent). It will usually be considerably less and the use of hybrid factors in an attempt to minimise it for pyrethrum samples generally encountered. Where only the total rethrin content is determined deviation from the supposed rethrin I to rethrin II ratio may result in more serious error but for therange experienced in normal flowers and extracts it is claimed[98,101] to be adequately covered by an error of about ± 3 per cent.

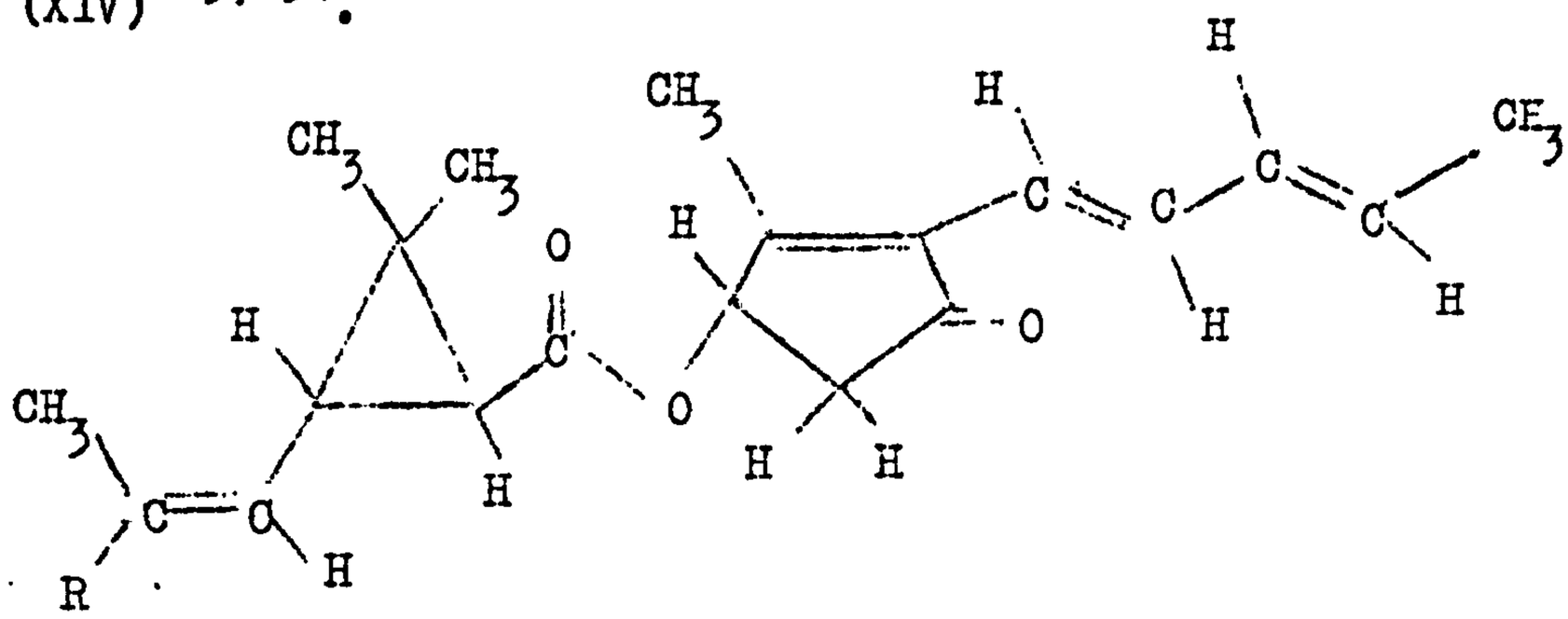
In the ultra-violet spectrophotometric method proposed by Ward and Newham[95], where response is not independent of rethrin-type, variation in sample composition will incur a more serious error, especially a variation in the pyrethrin to cinerin ratio. In calculating their theoretical specific absorption value a pyrethrin to cinerin ratio of 3:1 and equal quantities of rethrins I and II were assumed and an error of ± 4 per cent was claimed to cover all but the 'abnormal' samples. Furthermore

they claimed that a method which penalises a sample of low pyrethrin content will be more in accord with bioassays (see 1.3.1.).

1.5 Stability

1.5.1. Thermal instability

Early investigations into the chemistry of pyrethrum showed that biological activity decreased on heating[17,133]. Brown and co-workers[113] demonstrated that at temperatures in excess of 200°C pyrethrum extracts incurred serious loss of activity and separation of thermally degraded material by displacement chromatography yielded two new components postulated to be isomers of the pyrethrins, termed isopyrethrin I (XIII) and isopyrethrin II (XIV)[113,134].



R = CH_3 isopyrethrin I XIII

R = $COOCH_3$ isopyrethrin II XIV

The isopyrethrins, having partial double-bond character between the cyclopentenolone ring and the pentadienyl side chain, have restricted rotational and flexing freedom of the whole side chain with respect to the plane of the ring. This restriction in conformation is reflected in the low toxicity and, more particularly, in the low knockdown effect of the isopyrethrins

relative to their natural isomers[134].

Thermal degradation is of great significance in the problems encountered in the drying of pyrethrum flowers[135], distillation of pyrethrum extract to produce refined extract[136] and in the design of thermal fogging machines[137]. It may be monitored by an increase in the light absorbance of a solution of the insecticide measured at 270 nm. and a corresponding decrease at 230 nm[138]. The ratio of these in a fully isomerised sample is 0.62 compared to 0.08 in a natural, undegraded sample.

1.5.2. Photo- and autoxidative decomposition

Loss of insecticidal activity by the action of air and/or light on whole flowers, ground flowers and refined and unrefined extracts of pyrethrum has been the subject of many investigations. The most serious losses occur by the combination of air and light although degradation is observed in the presence of either of these agents. In the case of flowers observed degradation in an inert atmosphere and darkness[139] has been attributed to enzyme activity[140]. Opposing views have developed as to the mode of degradation involved and as to the effectiveness of stabilisers.

Campbell and Mitchell[141] concluded that the pentadienyl side chain of the alcohol moiety (Figure 2; at C-14) was involved in the mode of degradation following studies on the exposure of highly purified pyrethrum concentrate (Nitromethane Extract; see 1.1.2.) to daylight at 30°C for 3 months. An amber-coloured, resinous deposit was formed constituting about one half of the original sample. It was insoluble in petroleum spirits or refined kerosene and insecticidally inactive. Hydrolysis of this material yielded chrysanthemum mono- and di-carboxylic acids and therefore polymerisation appeared to be confined to the keto-alcoholic

portion of the molecule.

This view was supported by Freeman[142] following studies on pyrethrins and cinerins, isolated from pyrethrum extract, and allethrin I. The samples, contained in an evaporating dish to a depth of 1/8 inch, were subjected to irradiation by ultra-violet light at 40°C for 10 hours (stirred at hourly intervals). The rate of degradation decreased in the order pyrethrins, cinerins, allethrin I and the latter was apparently unaffected in the short time required to cause degradation in the pyrethrins. Changes in the infra-red spectra corresponded to chemical changes occurring only in the side chain of the alcoholic moiety and unsaturation in this part of the molecule was considered to be the major cause of their instability to ultra-violet light.

Brown and Phipers[132] irradiated samples, exposed as thin films, with tungsten light and concluded that modifications occurred in both the acid and alcohol portions of the molecule. Decolourised extracts exhibited a slower rate of degradation in the acid moiety compared to crude oleoresin but no change in the rate of alcohol moiety modification, illustrating the pronounced effect that the plant colouring pigments have on extract deterioration. Further work by Brown[143] suggested that the pyrethrins were less stable than the cinerins and the rethrins I were less stable than the rethrins II. The trans-methyl group of the isobutenyl moiety therefore renders the acid moiety side chain more prone to oxidation than the methyl-carboxy group in pyrethrates. The results however have been queried by Freeman[142] since the conclusions were necessarily based on the results of two independent methods of analysis for rethrins, that is, an 'acid' and an 'alcohol' method (see 1.4.8.). Freeman claimed

that the solubility of polymerised rethrins would invalidate the comparison of results and he was forced to conclude that "the statement concerning the attack by light upon the acid portion of the molecule is probably incorrect", although decolourisation undoubtedly improves stability. The photolysis studies of Glynne Jones[144] confirmed this latter point.

The work of Head and co-workers[145] apparently added to further confusion by demonstrating that even dewaxed and decolourised extracts, when exposed as thin films to sunlight (containing, at an altitude of over 6,000 feet, a larger proportion of ultra-violet radiation), gave products derived from modifications in the acid moiety. The conclusions were based on g.l.c. analysis (see 1.4.7.) of degraded samples compared with isolated rethrin components of pyrethrum extract which were similarly treated. Comparison of the relative retention volumes of the rethrins and their major degradation product indicated a relationship common to each rethrin I. This was held as conclusive evidence for modifications in the acid moiety, the alcoholic portion remaining unchanged.

Photoxidation of four synthetic chrysanthemates, including pyrethrin I and allethrin I, by irradiation with a sunlamp of thin films in air yielded at least eleven products separated by two-dimensional thin-layer chromatography (t.l.c.)[146]. Both acid and alcohol modifications were noted and the alterations in the acid moiety were independent of the nature of the alcohol moiety. Since each pyrethroid studied degraded at different rates, pyrethrin I being the most labile, it is evident that some portion of the alcohol moiety undergoes decomposition during irradiation. With pyrethrin I and allethrin I, the alcohol moiety was the most susceptible to modification, while in others lacking an olefinic

side chain in the alcoholic moiety, the acid moiety was modified most rapidly.

There is therefore much evidence for the instability of the rethrins in air and light but comparisons are made difficult by the inconsistancies in irradiation conditions, methods of analysis and sample purity. The work of Chen and Casida[145], utilising synthetic rethrins of high purity, probably gives a more accurate assessment of their instability and the mode of degradation. Similarly the source and spectral range of the light used may affect the mode of degradation and in order to rationalise the apparent contradictions it is necessary to start with the premise, first suggested by Nasir[147], that there are three distinct chemical inactivation reactions:

(i) a rapid direct photolysis by absorption of light in the range 210 - 250 nm. which is independent of the presence of oxygen;

(ii) a slower and more general light catalysed decomposition by light above 250 nm;

(iii) a much slower oxidation reaction which is independent of the presence of light.

The absorption spectrum for the rethrins reaches a maximum at approximately 230 nm.[148] arising from a π to π^* transition attributed to the conjugated ene-one system in the cyclopentenolone moiety and supplemented, in the case of the pyrethrins by a similar transition in the conjugated diene system of the side chain. It might therefore be expected that a non-oxidative mode of degradation involving modifications of the alcoholic moiety would occur by direct absorption of light in this region of the spectrum.

In studies utilising sunlight it is probable that the small proportion of the ultra-violet light below 250 nm. in solar radiation is largely responsible for the rapid deterioration of rethrin samples.

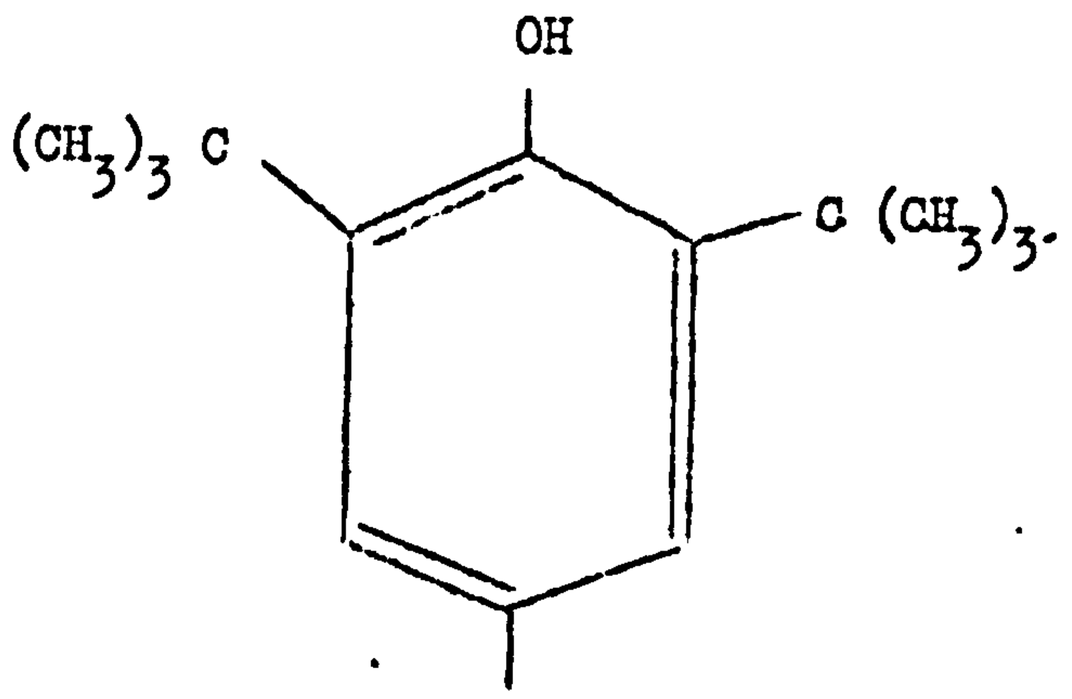
The role of chlorophyll-type pigments in extracts as photosensitizers is now generally acknowledged and their presence will greatly affect the course of degradation causing oxidative breakdown in the acid moiety to predominate.

When the major site of deactivation is in the alcohol moiety the chrysanthemate and pyrethrate of a particular alcohol may be expected to exhibit little or no difference in stability whilst the pyrethrolone esters will be considerably less stable than those of cinerolone and jasmolone. When acid moiety modifications predominate, the rethrins I will be less stable than the rethrins II.

1.5.3. Stabilisation

Stabilisation of pyrethrum powders and extracts is of great importance to curtail the financial losses sustained by the industry as well as prolong their activity in the field. A number of patents describe the use of most of the well established groups of antioxidants[149,150] and the marketed product usually contains the antioxidant, 4-methyl-2,6-ditertbutyl phenol (butylated hydroxy-toluene, B.H.T.) (XV) chosen[151] for its commercial availability and its recognised low mammalian toxicity.

Many stabilisers were thought to act by virtue of their light absorbing properties since they were found to absorb in the ultra-violet region of the spectrum but the evidence[144] indicates that most of them act primarily as a direct or indirect chemical inhibitor.



butylated hydroxy toluene (B.H.T.)

XV

Similar conclusions were arrived at by Brooke[152] in an investigation into the stabilising effect of synergists. All the synergists studied, including piperonyl butoxide (III), only absorbed light of wavelength less than 380 nm. but improved stability was also achieved when using light of the visible region only or in the dark, indicating that the stabilising effect was that of an antioxidant.

This is in accordance with the work of Head and co-workers[145] on the stabilising effect of piperonyl butoxide on crude and refined extracts. The synergist was shown to improve the stability of a partially dewaxed sample which was considered to degrade by oxidation of the acid moiety. No significant stabilisation was conferred on the more stable Pale Extract sample.

1.6 Present and future prospects

1.6.1. The merits of the natural insecticide

The post-war development and expansion of the relatively cheap and more persistant organo-chlorine and organo-phosphorus insecticides was a setback to the expansion of the Kenya pyrethrum industry (see Figure 1). However an increasing awareness over the last decade of the long-term effects of 'residual' pesticides

on animals by concentration in the body have resulted in their falling into disrepute[153]. The low mammalian toxicity (see 1.3.3.) and rapid insecticidal action (see 1.3.1.) of pyrethrum and the development of pyrethrum synergists (see 1.3.2.) have helped to encourage growth in the industry and maintain competitive prices whilst the emergence of strains of insect species resistant to organo-chlorine compounds has demonstrated the fallibility of these insecticides. The importance of pyrethrum in the control of insects therefore appears to be increasing and the merits of this natural insecticide have recently been summarised by J.B. Moore[154].

The lack of persistance of pyrethrum insecticidal formulations due to rethrin instability, considered by some to be a major disadvantage, may even be considered an asset in many respects. With relation to environmental pollution their advantage over the residual insecticides is now accepted. Even relative short-term persistance, of the order of a few weeks, may have an undesirable effect on the balance between the pest and predator populations. This may be due to direct toxic action on the predators or the virtual elimination of the host and may result in a later, more serious infestation. The absence, to date, of resistant strains of insect species (see 1.3.3.) may also be attributed to the lack of pyrethroid persistance and the use of non-persistant formulations in the recently developed fine-particle dispensers allows them to be sprayed from aircraft in uncertain wind conditions, free of the possibility of long range contamination.

In certain applications rethrin instability in air and light is not a problem. In the protection against grain infestation or of packaged foodstuffs formulations have been found to provide a relatively long period of activity[155] since treated cartons are often enclosed within an outer casing and stored in relatively

dark conditions. When the concentration of the active rethrins has eventually been reduced by degradation the repellency property of pyrethrum (see 1.3.1.) maintains protection for several months[156].

In addition to the present markets for pyrethrum, new application techniques and the use of stabilisers to maintain stability in the open for two to three days, have opened new outlets in the horticultural and agricultural fields whilst the revival of the mosquito coil as a relatively low priced insect repellent makes an increasing demand on pyrethrum production.

In production, an improvement programme is well under way in replacing flowers of low rethrin content (< 1.2 per cent of the dried flower head) with clonal material which produce a high yield of flowers possessing a high rethrin content. The guaranteeing of an average of 1.3 per cent rethrins was considered a hallmark in the production of pyrethrum but yields of the order of 2 per cent are now frequently encountered[157,2]. Further clonal development has been undertaken based on the propagation of the plant discovered in 1966 whose flowers contained 3.21 per cent rethrins[158].

Recent investigations into the activity of mosquito coils have indicated it to be related to a high rethrin I to rethrin II ratio and a high pyrethrin I content[159,160]. Other work has indicated that rethrin I rich extracts provide a greater mortality rate with houseflies[32]. On the basis of this work it has been suggested that propagation of clones with a high rethrin I to rethrin II ratio may improve the activity of products in general and mosquito coils in particular.

In addition to producing a greater yield of rethrins per acre plant selection and breeding will also lead to a relative reduction in extraction costs whilst the work of Pattenden *et al*[161]

on the biosynthesis of rethrins may, in the long term, lead to a greater control over their production in the plant.

1.6.2. The present study

Air transportation of refined pyrethrum extract would shorten delivery times and reduce transport costs. At present refined extracts containing about 60 per cent rethrins are diluted at the factory to about 25 per cent for storage (see 1.1.2.). To make airfreight a commercial proposition transportation of a concentrated form is necessary. However it was thought possible that the stability of extracts decreased with increase in rethrin concentration[28], based on impressions founded on the effect produced by 'simulated' conditions of degradation. The majority of information has been derived from studies on the stability of thin films and/or the use of 'forced' degradation techniques such as artificial ultra-violet irradiation. The extent to which data from these studies may be applied to bulk extracts under normal storage and transportation conditions is questionable in as much as the mode of degradation has been shown to be highly susceptible to changes in sample purity and experimental conditions (see 1.5.).

The purpose of this study therefore was to examine the s stability of refined pyrethrum concentrates stored under ambient conditions over long periods of time, to ascertain the effect of any contaminant extraneous matter on the stability and, in the event of instability being confirmed, to develop suitable stabilisation procedures.

2. RESULTS AND DISCUSSION.

2.1 Rethrin analysis

2.1.1. Assessment of the applicability of current methods of analysis

The inaccuracies of the methods of rethrin analysis that are currently available (see 1.4.8.) may be tolerable in commerce or quality control where consistancy is of paramount importance and sample composition varies little. The degree of inaccuracy may be constant for samples from the same source and with a similar history, but this assumption may not be valid where extracts of different purity are being compared and especially when degraded samples are being analysed. The proportion of false rethrins (see 1.4.8.1.) may be considerably increased in partially degraded samples since slight modifications to that part of the molecule which is not used in the determination would not be detected (see 1.5.) and such compounds would probably not be removed by adsorption chromatography (see 1.4.8.2.). This is exemplified by the fact that the jasmolins were not detected by any of the adsorption chromatographic procedures for isolating the rethrins but were eluted with the pyrethrins (see 1.2.). Further, in the study of degraded extracts samples considered abnormal with regard to sample composition may be frequently encountered and errors associated with deviation from the normal may then become more significant (see 1.4.8.3.).

It is because reproducibility rather than accuracy has been considered the criterion of a successful method that the present situation has therefore developed. It must also be concluded that the methods available, as proposed, are not satisfactory for the study of pyrethrum extract stability.

Ideally an unequivocal method needs to be highly specific and to estimate for each active ester separately. This is important in view of the differences in stability and the apparent incompatability of the conclusions formed by previous workers on rethrin stability may be partially attributed to inadequate methods of analysis (see 1.5.).

The direct g.l.c. method (see 1.4.7.) may be considered to approach this ideal but its dependence on a standard extract analysed by another method prevents it from being used as a method for the determination of absolute rethrin content (see Table 2). It appeared however that this method was the most suitable for studying pyrethrum extract deterioration in respect of rethrin content but required further development.

2.1.2. Gas-liquid chromatographic analysis using an internal standard

2.1.2.1. Detector calibration

For quantitative results by g.l.c. analysis the chromatographic detector requires calibration. In the method of rethrin analysis by g.l.c. proposed by Head[24,25] this is achieved by chromatographing a standard extract before and after each sample. This procedure however requires quantitative injection, that is, injection of precisely the same quantity of sample as standard. The degree of accuracy to which this can be achieved is open to question, especially in the case of liquid samples. The attainment of high accuracy by quantitative injection is almost certainly a personal ability and not necessarily maintained by different analysts. Unless both sample and standard are chromatographed at least in duplicate there is no way of checking the validity of an analysis. The method therefore requires a minimum of four chromatographic runs per analysis, preferably more. During the course of this work it was experienced that quantitative injection could not be relied

upon, being dependent upon the quantity injected, the volatility of the solvent, the syringe characteristics and age of injection- port septum and this was confirmed by a number of associates.

The relationship between detector response and quantity of component may be alternatively determined by calibration against an independent compound which is then incorporated in the sample at a known concentration as an internal standard. The response factor is then relative to the marker component. Except where the sample handling required to add the internal standard is difficult, repeatability is good. This procedure removes the necessity of constant volume injection and the calibration factors are not significantly altered by slight changes in operating conditions.

Theoretical calculation of response factors has been attempted for many classes of compounds, especially for use with flame ionisation detectors (F.I.D.) where the response has been found to be related to the carbon content of the component being detected[162-164]. This may be expressed by equation (i).

$$\text{response factor (F)} = \frac{\text{molecular wt. of component (M)}}{12 \times \text{no. of carbon atoms per molecule}} \qquad \text{(i)}$$

It therefore follows that the relative response factor relating two components is given by equation (ii).

$$F_{ab} = \frac{M_a \times C_b}{M_b \times C_a} \qquad \text{(ii)}$$

$$\text{where } F_{ab} = \frac{W_a \times R_b}{W_b \times R_a} \qquad \text{(iii)}$$

= the relative response factor of component a to component b

W_a = the weight of component a of molecular weight M_a containing C_a carbon atoms per molecule and producing response R_a

W_b = the weight of component b of molecular weight M_b containing C_b carbon atoms per molecule and producing response R_b

From equations (ii) and (iii) it can be seen that the relative molar responses are directly proportional to the carbon number of the molecule. Since the effect on the relative response factor produced by an increase in molecular weight is largely offset by the increase in carbon content, compounds of the same homologous series of molecular weight greater than about 100 yield the same response per unit weight. That is, the relative response factors are unity.

The relationship between relative molar response and carbon number however appears to have limited application or are only correct to a first approximation[162,165]. It strictly applies only to normal paraffins and hydrocarbons within the same homogogous series. Less satisfactory results are found for oxygenated compounds[162,165-167]. In certain cases carbonyl carbon atoms have been taken to produce no response to F.I.D.[168] but it has been shown that in higher molecular weight compounds they may give up to a full carbon atom response[167]. Furthermore, the presence of oxygen atoms gives a more substantial rate of change of relative weight per cent of carbon with increment of methylene groups in the case of acids and esters than in hydrocarbons of corresponding chain lengths but this is only significant for low molecular weight compounds.

The use of factors has therefore been considered unnecessary (i.e. F = 1) within molecular classes except for low members[165,169]. The theoretical determination of factors relating compounds of a closely similar molecular class may only approximate to these findings and for dissimilar compounds only experimentally determined relative response factors are applicable and are usually found not to be unity[170].

2.1.2.2: Choice of internal standard for rethrin analysis

The rethrins I are similar in molecular structure (Figure 3) from a chromatographic detection (F.I.D.) viewpoint and may be expected to have relative response factors close to unity for each member (see 2.1.2.1.). Similarly the relative response factors of each of the rethrins II will be close to unity. Table 3 shows the theoretical relative response factors of the individual rethrins calculated from equation (ii). These are within ± 2 per cent of unity which is within the accuracy of chromatographic detection[171]. From either a theoretical or empirical viewpoint therefore no serious error is introduced by assuming an equal response per unit weight for each of the rethrins I and rethrins II respectively and was in fact assumed by Head in determining the relative proportion of each rethrin in pyrethrum samples[24.25].

Since the individual rethrins were not easily available (see 2.4.) for calibration against a marker component the use of a component of similar structure to the rethrins suggested itself whereby the relative response factor could be reasonably assumed to be unity. Allethrin I (Figure 5), the commercially available allyl homologue of rethrins I, was therefore chosen as standard for rethrin I analysis.

Table 3 Theoretical relative response factors of the natural rethrins and allethrins

Rethrin	Molecular weight	No. of C atoms	Relative to	Weight response factor
allethrin I	302.4	19	allethrin I	1.00
cinerin I	316.4	20	"	0.99
jasmolin I	330.4	21	"	0.99
pyrethrin I	328.4	21	"	0.98
allethrin II	346.4	20	allethrin II	1.00
cinerin II	360.4	21	"	0.99
jasmolin II	374.4	22	"	0.98
pyrethrin II	372.4	22	"	0.98
allethrin II	346.4	20	allethrin I	1.09
cinerin II	360.4	21	pyrethrin I	1.10
jasmolin II	374.4	22	"	1.09
pyrethrin II	372.4	22	"	1.08

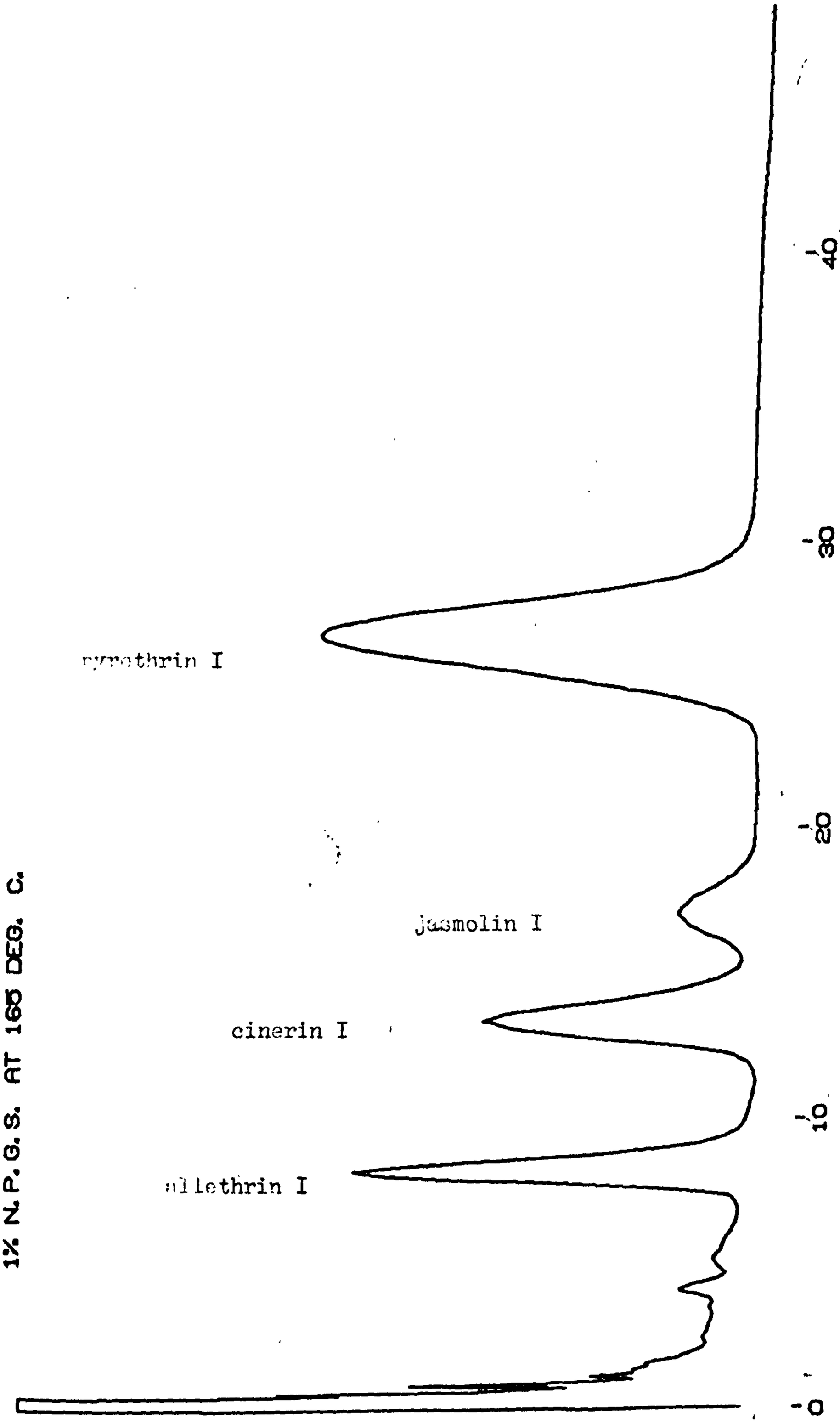
allethrin I = allethronyl chrysanthemate (1.3.4; Figure 5)

allethrin II = allethronyl pyrethrate (2.1.2.4; XVI)

Allethrin I was found to elute prior to cinerin I, as may be predicted by comparison with its natural analogues, and to be completely resolved from cinerin I on columns packed with 1 per cent N.P.G.S. stationary phase (Figure 6). Examination of extract chromatograms showed the presence of no component eluting in this region which would interfere with the evaluation of the allethrin I response. This is not an essential requirement however and a correction could be made to allow for the elution of minor components.

Figure 6 Gas-liquid chromatogram of the rethrins. I.

1% N.P.G.S. AT 165 DEG. C.



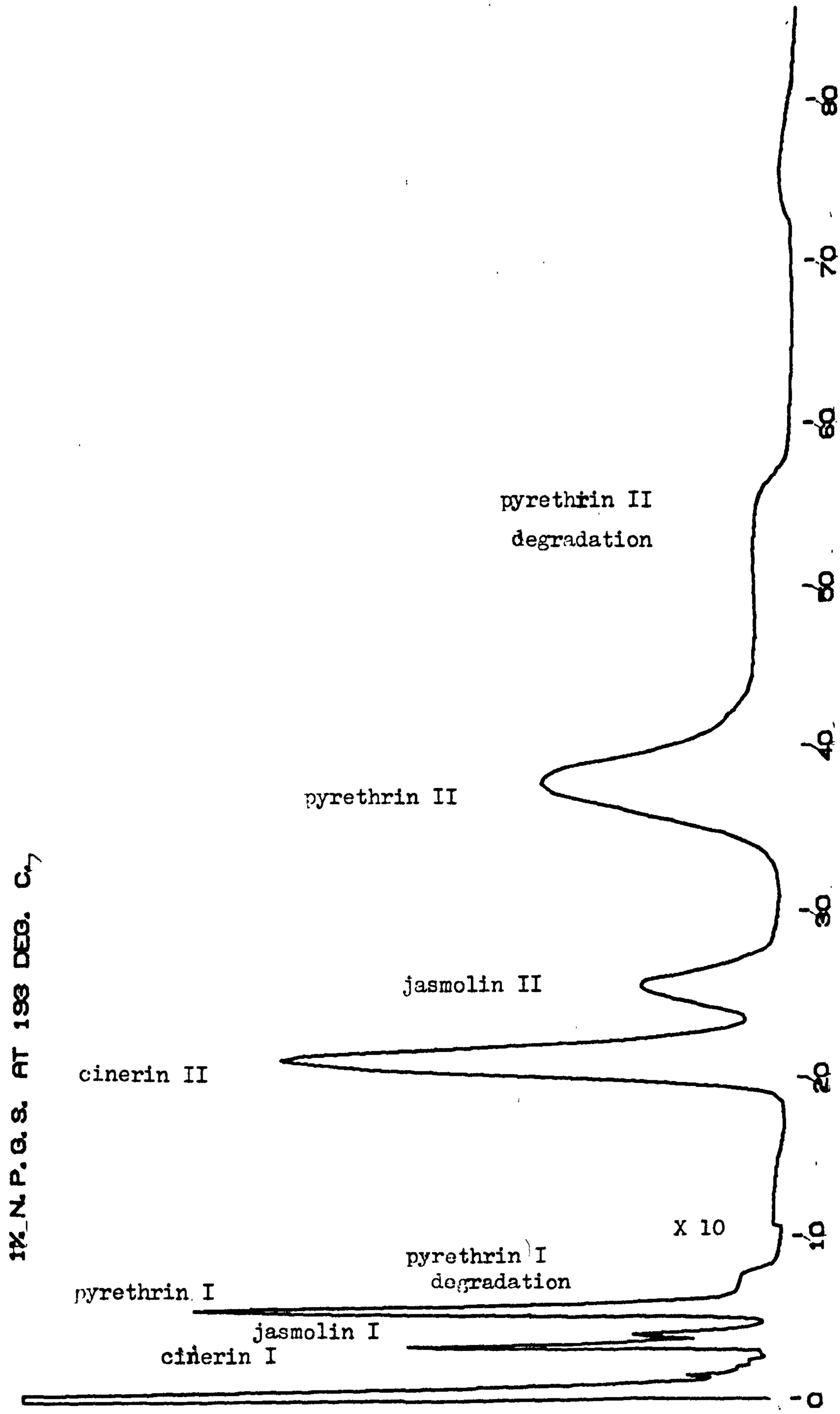


Figure 7 Gas-liquid chromatogram of the rethrins II.

2.1.2.3. Conditions of chromatographic analysis

The optimum conditions of analysis, chosen to produce the best resolution of cinerin and jasmolin components without an inhibitory long analysis time, were found to be with a carrier gas flow rate of 14 to 20 mls. per run, depending on the individual column characteristics, and a column temperature of 165°C (Figure 6).

Isothermal chromatograms at this temperature were unsuitable for rethrin II analysis so with the unavailability of a chromatograph with adequate temperature programming facilities at the commencement of this work a second isothermal run was necessary at a higher temperature for rethrin II analysis. An optimum temperature of 193°C was chosen (Figure 7).

The chromatographic analysis times were of the order of 35 mins. for rethrins I (pyrethrin I elution - 25 mins.) and 60 mins. for rethrins II (pyrethrin II elution - 40 mins.). These could be shortened at the expense of cinerin-jasmolin resolution by an increase in column temperature or carrier gas flow rate but this could not be recommended for accurate analysis. The use of temperature programming would allow shorter chromatographic analysis times and would make possible the analysis of both rethrin I and rethrin II by evaluation of one chromatogram but its use in conjunction with the proposed method would require extremely fine control of the carrier gas flow rate. This is reduced with increase in temperature and since the response is related to the rate of carrier gas through the detector[163] the relative responses of the rethrins to the internal standard would not remain constant unless it was first established that only small changes in carrier gas flow rate were experienced and that the flow rate had been optimised[163] with regard to the effect of such

Table 4 G.l.c. data of rethrin analysis on 1% N.P.G.S. columns

	Equivalent chain length (n-alkanes)	Retention times relative to cinerin I	
		Present work	Head et al[145]
allethrin I	24.8	0.64	0.72
cinerin I	26.2	1.00	1.00
jasmolin I	26.7	1.23	1.29
pyrethrin I	28.0	1.85	1.73
cinerin II	32.6	7.3	7.16
jasmolin II	33.1	8.8	9.04
pyrethrin II	34.3	13.15	13.13

no. of theoretical plates 550 H.E.T.P. 1.8 mm.

(relative to pyrethrin II)

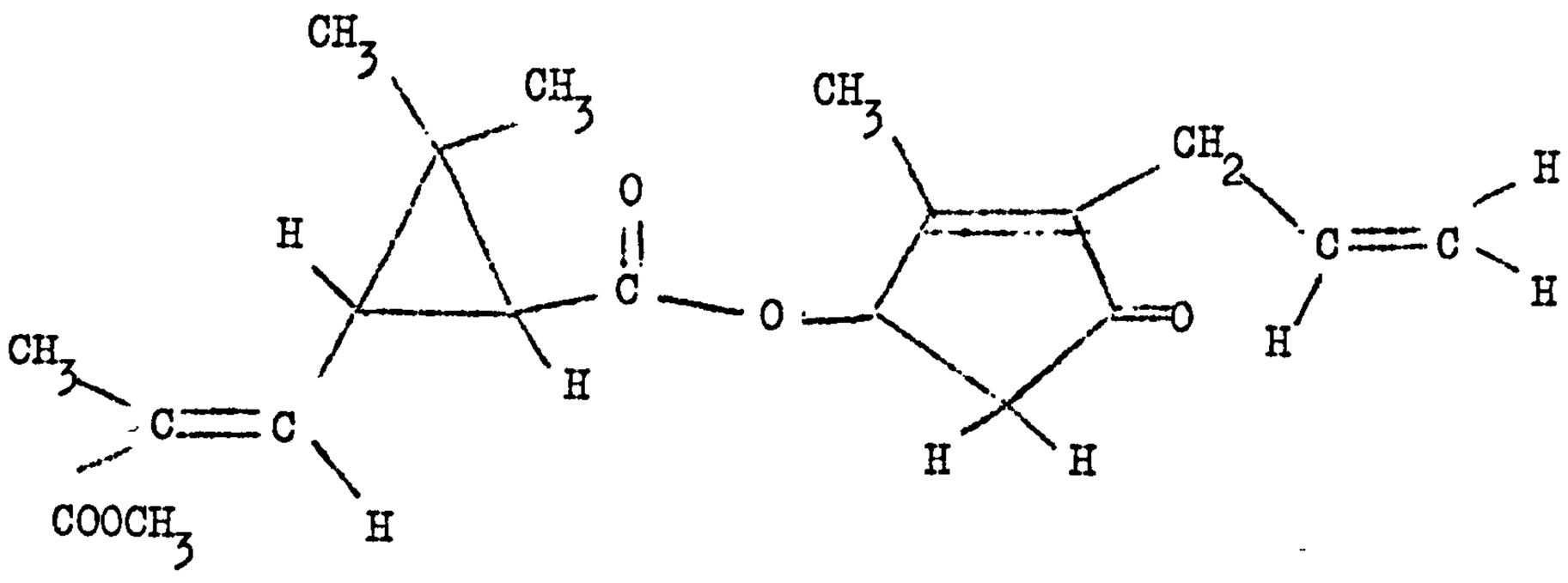
changes on the detector response. Step-wise temperature programming between rethrin I and rethrin II elution, that is, isothermal analysis during the elution of each of these groups of components, would similarly require two internal standards (see 2.1.2.4.), one for each isothermal section, due to the change in carrier gas flow rate.

Data defining the characteristics of the chromatographic columns used throughout this work are given in Table 4. The equivalent chain lengths, with respect to n-alkanes, could not be defined more precisely since the values appeared to depend slightly upon the age of the column (see 2.1.2.8.). This can only be attributed to a gradual modification of the polarity of the stationary phase. The retention times of the rethrins relative to each other would not be so sensitive to such a change and the determined values were in good agreement with the published data

of Head et al[145]. The number of theoretical plates, and hence the height equivalent to one theoretical plate (H.E.T.P.), were similarly found to be affected by the ageing of the column and was reflected in the deterioration in resolution. A freshly prepared column generally contained approximately 550 theoretical plates (H.E.T.P. ~ 1.8 mm.) relative to pyrethrin II, but when discarded (see 2.1.2.8.) the number was about 100 (H.E.T.P. ~ 10 mm.).

2.1.2.4. Internal standard for rethrin II analysis

Ideally allethronyl pyrethrate (XVI) (conveniently termed allethrin II) would be the choice of internal standard for rethrin II analysis by comparison with rethrin I analysis but unlike allethrin I it is not commercially available. As an interim measure therefore rethrin II responses were related to that of pyrethrin I since allethrin I was not always adequately resolved from cinerin I and the solvent front at this temperature.



allethronyl pyrethrate

(allethrin II)

XVI

Table 5 Purity of reconstituted allethrins by the D.N.P. method of analysis[101]

	Sample wt. (mg.)	Net absorbance	% purity	
allethrin I	37.8	1.732	99.0)	99.7
	32.7	1.521	100.4)	
allethrin II	35.3	1.638	100.1)	99.2
	31.1	1.414	98.3)	

In using pyrethrin I as the internal standard for rethrins II there arose the problem of the appropriate relative response factor. As shown by Table 3 the theoretical value of the factors of rethrins II relative to pyrethrin I are of the order of 1.09 based on equation (ii). No data appears to be available concerning the relative response factors of diesters to monoesters but since the difference between these classes of compounds involves an oxygen moiety, equation (ii) may not strictly hold. Since all the rethrins are not structurally dissimilar however and are of relatively high molecular weight it is not improbable that the relative response factors are much closer to unity (see 2.1.2.1.).

2.1.2.5. Experimental relative response factors of rethrins II to rethrins I

The experimental relative response factor of rethrins II to rethrins I was determined as the relative response factor of allethrin II to allethrin I. These were reconstituted from allethrolone and chrysanthemic acid and pyrethric acid (see 2.4.). Their purity was checked by the rapid D.N.P. method of analysis[101],

the chromatographic purification step being unnecessary since this was included in the preparation procedure. This was found to be 99.7 per cent and 99.2 per cent for allethrin I and allethrin II respectively (Table 5). In addition, chromatographed portions of each of the allethrins showed no trace of allethrolone, the chrysanthemum acid or the acid chloride intermediate.

Two samples containing allethrin I and allethrin II in known proportions by weight were chromatographed on a 1 per cent N.P.G.S. column. Their respective area responses were evaluated by a digital integrator (see section 2.2.). The results are shown in Table 6. These indicate that the relative response factors of rethrins II to rethrins I as calculated from equation (ii) (Table 3) are in serious error and within the limits of experimental error the factor may be taken as unity. A more precise determination of the value of this factor by averaging numerous sets of results would be of no great asset due to the degree of variability of detector response[171] which at best appears to be of the order of ± 2 per cent.

2.1.2.6. Thermal degradation of pyrethrins during analysis

Examination of chromatograms from isothermal analysis at 193°C indicated that the majority exhibited significant tailing of the pyrethrin components, especially pyrethrin II. The degree of assymmetry of the pyrethrin peaks varied between columns and with age of column but in many cases the component peak exhibited a definite shoulder which was generally more pronounced with pyrethrin II than with pyrethrin I (Figure 7). It also appeared to increase with an increase in retention time.

Table 6 Relative response factor of allethronyl pyrethrate (allethrin II, A II) to allethronyl chrysanthemate (allethrin I, A I)

Chromatograph	No. 1		No. 2		No. 1		No. 2	
Wt. of A I	2.88 mg				4.63 mg			
Wt. of A II	8.73 mg				16.22 mg			
Wt. ratio (II/I)	3.03				3.505			
A I response*	7800	13406	11713	22270	16275	13396	16550	17833
A II response*	23128	42137	35990	67525	56937	46135	60844	63784
Response ratio (II/I)	2.965	3.143	3.073	3.032	3.498	3.444	3.676	3.577
Factor II/I	1.021	0.964	0.987	1.000	1.001	1.016	0.954	0.981

* digital response units - see section 2.2.

From the known thermal instability of the pyrethrins (see section 1.5.1.) it was possible that these components were undergoing isomerisation during the course of their elution through the chromatographic column. A systematic investigation of this phenomenon was therefore undertaken to examine the effect of temperature and carrier-gas flow rate (control of retention time) on the proportionation of area of the pyrethrin II peak and the shoulder. The area of the shoulder was taken to be that remaining from the total area after deduction of the area of a symmetrical pyrethrin II peak. From the results shown in Table 7 it is immediately apparent that the assymmetry is progressively more significant at higher temperatures and longer retention times.

Table 7 Thermal degradation of pyrethrin II on the gas-chromatographic column

	1	2	3	4	5	6
Column temp. (°C)	193	193	193	185	175	175
Carrier gas flow rate (mls/min)	20	18	14	20	20	20
PII retention time (mins)	23	32	50	35	68	52
shoulder + PII as % of rethrins II	62.4 61.1 61.7	59.4 59.7 59.6	60.0 61.2 60.6	59.1 58.3 58.7	65.6 61.8 63.7	60.2 60.1 60.1
shoulder/PII	0.270 0.281 0.276	0.508 0.496 0.502	0.673 0.668 0.670	0.225 0.221 0.233	0.191 0.204 0.198	*

PII = pyrethrin II

* negligible amount - general assymetry of component peaks - see text

At 193°C the area of the shoulder markedly increased relative to the pyrethrin II peak area with decrease in carrier-gas flow rate (Table 7; cols. 1, 2 and 3). A decrease in column temperature while maintaining a constant carrier-gas flow rate produced a slight decrease in the proportion of shoulder area to pyrethrin II area (Table 7; cols. 1, 4 and 5) but any advantage gained by the lower temperature was obviously offset by the corresponding increase in elution time.

Where the elution time was corrected by an increase in flow rate (Table 7; cols. 2 and 4, 3 and 6) relative to the decrease in temperature a much greater degree of tailing was exhibited at the higher temperature while at 175°C and a pyrethrin II retention time of less than one hour the pyrethrin II peak, showed only a slight degree of assymmetry which was assessed as being no greater than that exhibited by the other components due to the poor analysis conditions.

In each chromatogram however it was noted that the combined area of pyrethrin II and shoulder was constant relative to the cinerin-jasmolin combined peak areas within the errors associated with baseline construction and is expressed in Table 7 as the percentage of rethrins II.

Changes in the pyrethrin I shoulder in the chromatogram were not able to be quantitatively defined because the relative area is small. An increase in retention time by either reduction in carrier-gas flow rate or column temperature produced broadening of the pyrethrin I peak to an extent whereby the shoulder became lost under the major peak. At temperatures less than 175°C no significant assymmetry of the pyrethrin I peak could be noted (Figure 6).

This was therefore held as strong evidence in support of the view of thermal degradation of the pyrethrins on the chromatographic column. Since it was found with experience that the degree of degradation varied between different columns the post-pyrethrin shoulders were included in the area of the respective pyrethrins for quantitative assessment of the chromatograms. The relative response factors of the isopyrethrins would not be expected to be significantly different from those of the respective pyrethrins (see 2.1.2.1.).

2.1.2.7. Analysis of thermally degraded extracts

In an attempt to confirm the view that the pyrethrin content is partially isomerised during g.l.c. analysis at temperatures in excess of 175°C, thermally degraded extracts were examined. Elliott[172] reported that thermal isomerisation is only extensive when pyrethrolone and its derivatives are subjected to temperatures of 200°C or above but then reported[172] appreciable isomerisation of pyrethrin II during distillation under reduced pressure at 190°C. Brown et al[113], in their original investigation of this phenomenon, heated extract samples at 200°C for half an hour while the kinetic work of Goldberg et al[136] indicated 60 per cent isomerisation after half an hour's heating at 195°C; complete after 2½ hours. The rate of isomerisation was followed spectrophotometrically by the fall in absorbance at 230 nm. and rise at 270 nm.

The sample chosen for this investigation was a Pale Extract concentrate containing about 40 per cent rethrins as determined by the A.O.A.C. procedure (section 1.4.1.) (14 to 15 per cent each pyrethrin). Samples of the extract, which had been heated for various time intervals in an open flask fitted with a reflux condenser in an oil bath maintained at a temperature of 195 ± 5°C, were analysed by spectrophotometry and by g.l.c. (1% N.P.G.S. stationary phase at 193°C).

Table 8 Spectrophotometric analysis of heated extracts

Heating time (mins.)	Wt (gms.)	λ_{max} (nm)	d_{max}	% * rethrins	d_{270}	d_{230}	$\frac{d_{270}}{d_{230}}$
0	2.2685	227	0.82	40.9	0.06	0.80	0.075
10	3.1036	228	0.96	34.6	0.19	0.95	0.200
15	2.7328	228	0.82	33.6	0.155	0.81	0.192
40	2.7941	230	0.61	24.7	0.29	0.61	0.475
60	2.7914	231	0.67	25.1	0.32	0.665	0.481
90	2.8145	230	0.605	24.4	0.275	0.605	0.455
120	2.9806	232	0.585	22.0	0.30	0.58	0.517

* Calculated using $E^{1\%}_{max}$ (EtOH) = 1104 - a theoretical value[95]

λ_{max} - wavelength of maximum absorption

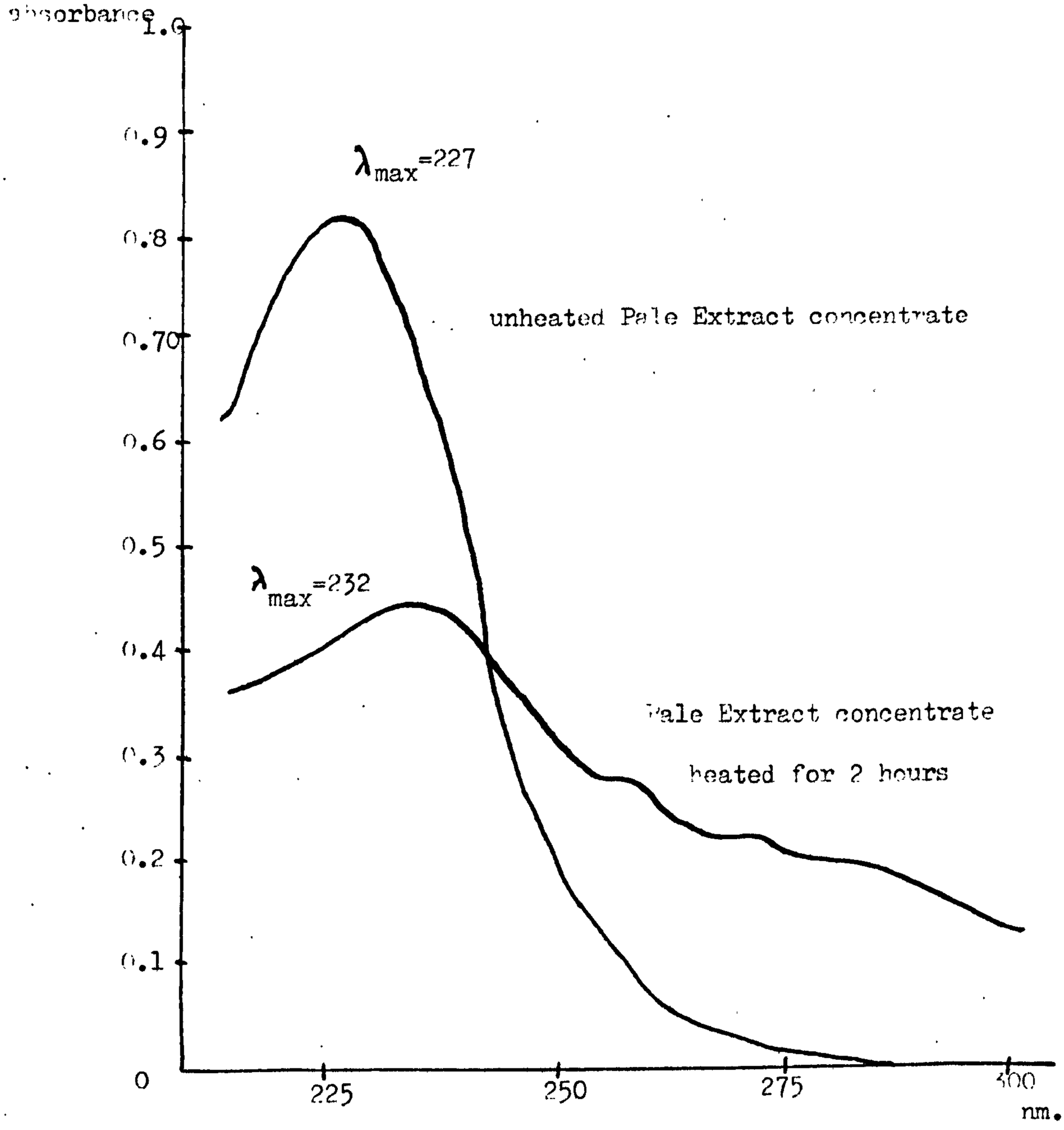
d_{max} - maximum absorption

d_{270} - absorption at 270 nm.

d_{230} - absorption at 230 nm.

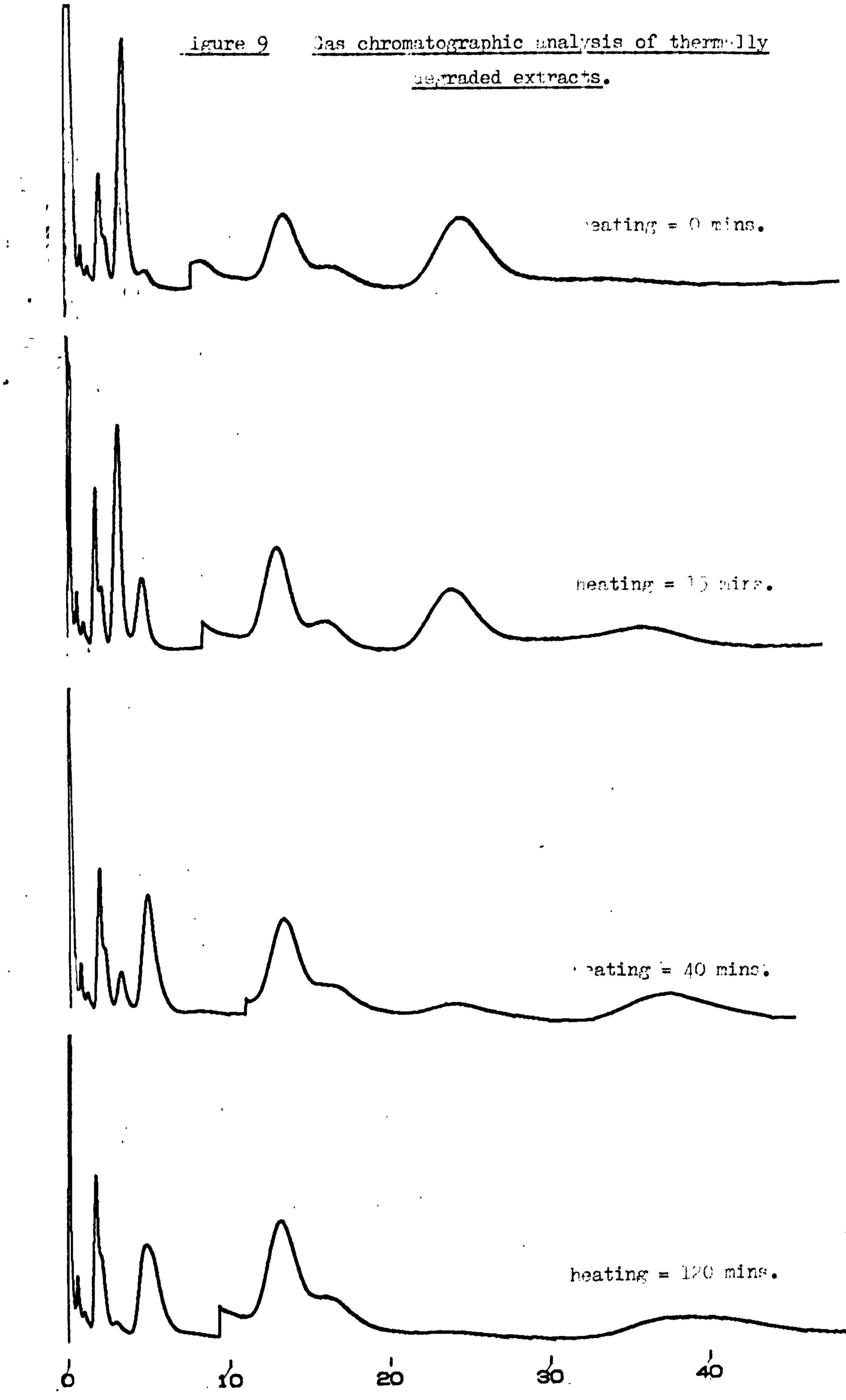
Spectrophotometric examination (Table 8) showed a gradual shift of wavelength of maximum absorption from 227 nm. in the unheated extract to 232 nm. in the sample heated for 2 hours. This is due to the diminishing contribution of the conjugated diene system of the cyclopentenyl side chain (Figure 2; at C-14) ($\lambda_{max} \sim 220$ nm.) so that the conjugated carbonyl system (Figure 2; C-13, 14, 15), augmented by the methyl group (Figure 2; C-16), ($\lambda_{max} \sim 235$ nm.) dominates. This is reflected in the higher value for the wavelength of maximum absorption for rethrins II compared with rethrins I where the conjugated carbonyl system in the acid moiety (Figure 2; C-1, 3, 4) contributes to the

Figure 8 Comparison of the absorption spectra of normal and thermally degraded extracts of equivalent concentration.



reference = absolute alcohol

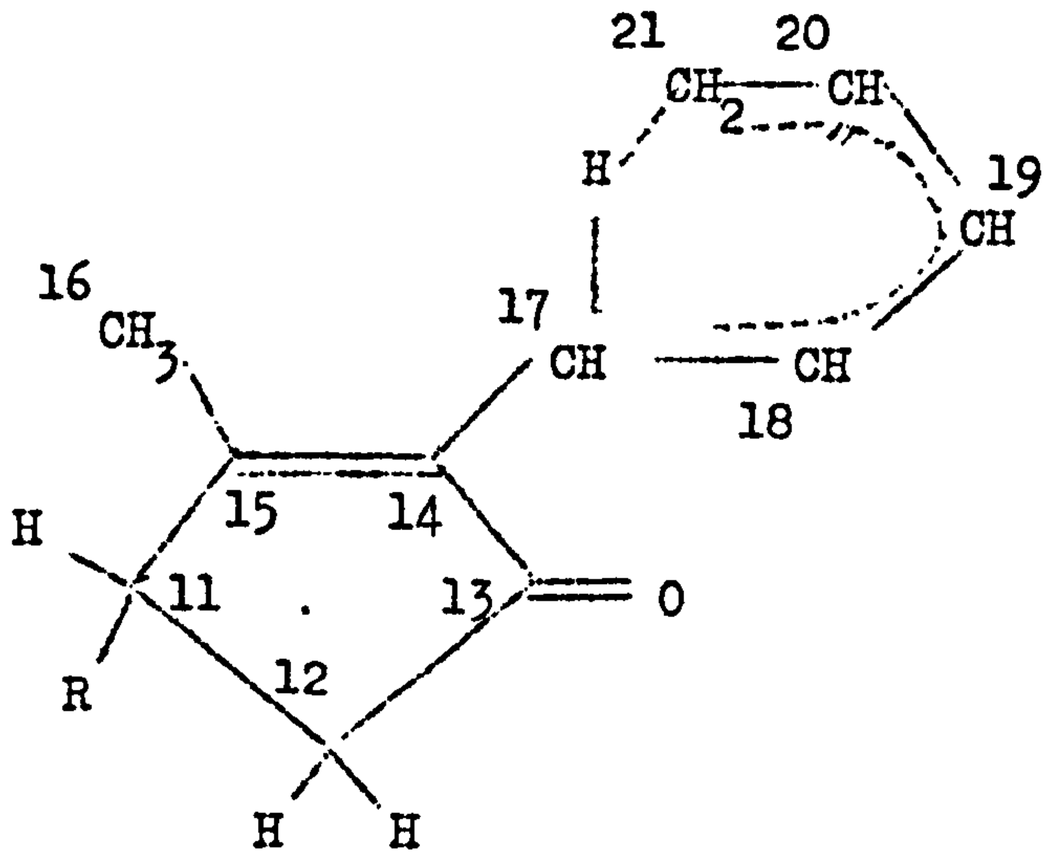
igure 9 Gas chromatographic analysis of thermally degraded extracts.



absorption of the former[21,33,95,173]. There was also a general fall in the rethrin content, as determined by the spectrophotometric method of Ward and Newham[95], with an increase in the period of heating indicating rethrin degradation and a rise in the ratio of absorbances at 270 nm. and 230 nm. indicating the formation of the isopyrethrins (XIII and XIV).

The chromatograms from g.l.c. examination of these samples showed an increase in the relative concentration of the components eluting as post-pyrethrin shoulders and an accompanying decrease in pyrethrin concentration. Four of the chromatograms are reproduced in Figure 9. These show conclusively that the post-pyrethrin shoulders are thermal artefacts of the pyrethrins, probably the respective isopyrethrins.

The cinerins and jasmolins showed no apparent thermal modification in accordance with reports by previous workers[113,172]. It has been concluded[172,174] that a cis-pentadienyl side chain is required so that the rearrangement may proceed via a cyclic intermediate (XVII). The rate determining step is the removal of the hydrogen atom from the first carbon atom of the side chain (C-17). It has been shown that neither the keto group (C-13) nor the endocyclic double bond (C-14) are involved in the rearrangement and the reaction is found not to occur with pyrethrolone derivatives having a trans configuration in the side chain[172,174].



R = chrysanthemumate

XVII

2.1.2.8. Colum deterioration

The degree of pyrethrin isomerisation and loss of cinerin-jasmolin resolution became greater with the age of a particular column. It was considered possible that this was due to the retention of extraneous matter by the column producing, over a number of analyses, a modification of the stationary phase. Much of this retained material would be initially deposited in the early section of the column although possibly eluted gradually through the column.

An attempt to extend the life of a column was made by coupling a preliminary column, two or three inches in length and packed with the same material as the main column, between the main column and the injection port. This pre-column was exchanged periodically (after 5 to 10 analyses) and appeared to increase the life of the main column.

The column continued to deteriorate however over a period of time, probably due to stationary phase bleed which, at such a relatively low loading, would produce significant modification to the column characteristics by exposing active sites in the stationary phase support. Columns were therefore arbitrarily rejected when the jasmolin components eluted only as shoulders to the respective cinerins.

2.1.2.9. Purity of the internal standard

Two samples of allethrin I were used throughout this work as internal standards for the g.l.c. analyses of pyrethrum extracts. The first, sample A, had been purified by adsorption chromatography on alumina while the second, sample B, was ordinary commercial grade allethrin. Commercial allethrin is normally of about 90 per cent purity, the usual impurities being chrysanthemic acid (Figure 3) and chrysanthemic acid chloride, the reagent and intermediate employed during the reconstitution process (see 2.4.). Allethrolone (VIII) is rarely present since the acid chloride is used in excess but other impurities arising from the synthesis of these components have been reported[98].

For quality control in the production of allethrin I two methods of analysis appear to have been used, namely the E.D.A. method (see 1.4.2.) and the D.N.P. method (see 1.4.5.) although most of the methods of pyrethrum analysis are applicable. The former is an 'acid' method and chrysanthemic acid derivatives may be expected to cause interference while the latter, an 'alcohol' method, will be susceptible to interference by rethronyl and ketonic impurities (see 1.4.8.). Since both methods include a chromatographic purification procedure however, most of these impurities, if present, will be removed prior to the estimation procedure.

The D.N.P. method, as described by Head[100], and, for comparison, the 'rapid' variant[101], which excludes the chromatography of the d.n.p.h.s, were used to analyse the samples of allethrin I. The results by either variant (Table 9) showed no difference within the limits of experimental error for either sample indicating that any impurities gave no positive reaction with the reagent. Sample A was substantially pure (99.65%) and was subsequently taken as being 100 per cent allethrin I. Sample B contained 91.5 per cent allethrin.

Qualitative examination by t.l.c. on silica indicated several minor components in sample B (allethrin I, R_f = 0.66) one of which corresponded to chrysanthemic acid (R_f = 0.30) but no component corresponded to allethrolone (R_f = 0.08). Sample A gave only one spot (R_f = 0.66) and the allethrin I d.n.p.h. from both samples also gave only one spot (R_f = 0.73).

G.l.c. (10% Apiezon L stationary phase) of the allethrin I samples showed some minor components in sample B. One of these eluted with the same retention time as chrysanthemic acid but no component in sample B corresponded to allethrolone.

No attempt was made to purify the bulk of sample B since no component was found to produce a significant response on eluting from columns containing 1% W.P.G.S. stationary phase which would interfere with the responses of the natural rethrins. Rethrin analysis results were therefore corrected for the purity of the internal standard.

Table 9 Purity of allethrin I samples (internal standards for rethrin analysis)

		Sample wt. (mg)	Net absorbance	% allethrin I		
Sample A						
	a	36.4	1.689	100.1		99.9
	b	32.5	1.500	99.7	99.65	
*	c	26.6	1.208	98.0		99.4
*	d	36.5	1.703	100.8		
Sample B						
	e	35.9	1.493	89.8		91.35
	f	35.3	1.523	92.9		
*	g	24.5	1.030	90.7	91.5	
*	h	33.6	1.451	93.2		91.7
*	j	37.4	1.578	91.1		

* 'rapid' variant

2.1.2.10. Repeatability of the proposed method

The repeatability of a method is best demonstrated by repetitive analyses on individual samples and by varying the experimental conditions sufficiently to cover possible variations encountered during general use of the method. In order to indicate the repeatability of the developed method an initial investigation was undertaken using a Nitromethane Extract sample (see 1.1.2.). This was analysed three times, each analysis result being based on the average of two chromatograms for each of the rethrin I and rethrin II determinations (usually, for a single analysis, three or more chromatograms are used). The results

Table 10 Precision test of the proposed method - using a Nitromethane Extract sample

wt. (mgs.)		% of RI				responses	% RI		% of RII			responses	% RII£		% RI
AI*	Ext.	CI	JI	PI		RI / AI			CII	JII	PII	RII / PI			
276.6	292.4	22.4	8.6	69.0	69.4	4.19	8.19	8.22	27.7	9.1	63.2	0.914	5.21	5.12	13.34
		21.8	8.4	69.8		4.22	8.25		29.2	10.7	60.1	0.883	5.03		
175.6	77.7	21.0	8.4	70.6	70.2	1.73	8.07	8.095	28.5	9.1	62.4	0.897	5.10	4.985	13.08
		21.9	8.3	69.8		1.74	8.12		31.2	10.3	58.5	0.858	4.87		
214.8	98.0	21.2	8.9	69.9	70.2	1.77	8.03	8.075	29.9	9.2	60.9	0.950	5.38	5.255	13.33
		20.4	9.1	70.5		1.79	8.12		30.4	8.3	61.3	0.905	5.13		
Mean		21.5	8.6	69.9		-	8.13		29.5	9.4	61.1	0.901	5.12		13.25
Standard deviation		0.7	0.3	0.6		-	0.08		1.3	0.9	1.7	0.031	0.13		0.15
Coeff. of variation		0.034	0.037	0.008		-	0.010		0.043	0.094	0.027	0.034	0.026		0.011
95 % confidence limits		0.8	0.3	0.6		-	0.19		1.3	0.9	1.7	0.032	0.37		0.36

* % concn. of allethrin I soln. = 2.067

£ % RII calculated using mean concns. of RI and PI

Table 11 Precision test of the proposed analysis method - using the World Standard Extract sample.

% concn. of AI soln.	wt. (mgs.)		% of RI responses				% RI	% of RII responses				% RII	% R
	AI*	Ext.	CI	JI	PI	RI / AI		CII	JII	PII	RII / PI		
1 1.592	278.74	210.15	21.1	8.2	70.7	5.36	10.35	27.8	8.6	63.6	0.853	6.24	16.59
1.764	206.73	63.01	22.3	8.4	69.3	1.97	10.6	26.1	9.7	64.2	0.802	5.92	16.52
1.764	186.42	131.79	20.8	8.2	71.0	4.59	10.5	31.7	10.3	58.0	0.790	5.88	16.38
2.642	121.98	96.32	21.3	7.9	70.8	3.50	10.7	29.9	8.8	61.3	0.808	6.11	16.81
1.808	138.87	88.90	19.0	8.9	72.1	4.20	10.85	27.2	9.2	63.6	0.826	6.48	17.33
1.808	314.30	126.88	21.5	8.2	70.3	2.53	10.43	30.7	12.8	56.5	0.805	5.91	16.34
Mean			21.0	8.3	70.7	-	10.57	28.9	9.9	61.2	0.814	6.09	16.66
Standard deviation			1.1	0.3	0.9	-	0.18	2.2	1.5	3.2	0.022	0.24	0.37
Coeff. of variation			0.053	0.040	0.013	-	0.017	0.076	0.155	0.053	0.027	0.039	0.022
95 % confidence limits			1.15	0.35	0.95	-	0.19	2.3	1.6	3.4	0.024	0.25	0.39

* Purity of allethrin I = 91.5 %

are shown to have a fairly high degree of precision (Table 10) although the relatively greater value for the coefficient of variation of the relative response of rethrins II to pyrethrin I (Table 10) indicated low precision for the rethrin II chromatogram evaluation and therefore the source of greatest error. The relative proportion of the six rethrins indicated by the twelve chromatograms showed a relatively high degree of precision with those of rethrins II rating worse than those of rethrins I. These however will be greatly susceptible to ageing of the column (see 2.1.2.8.) when resolution is generally found to deteriorate.

These results were confirmed by subsequent work. Where analyses were duplicated the results showed excellent agreement within the range of error demonstrated by this investigation.

The repeatability of the method was examined in greater detail (in the course of demonstrating the accuracy of the method, see 2.1.2.11.) when a sample of World Standard Extract[175] (a blend of different Pale Extracts) became available. These results, shown in Table 11, confirmed the conclusions of the earlier investigation.

2.1.2.11. Accuracy of the proposed method

A method of analysis may possess good repeatability but yield inaccurate results because precision, to which repeatability is linked, can only allow for variable errors whereas a constant bias will be undetected. It is therefore necessary to make the distinction between accuracy and precision when assessing a method of analysis. The former may be defined as the difference between the average result and the true result whilst precision is a measure of the variation among a set of results[176].

Ideally a method of analysis requires accuracy and good repeatability but the former is often sacrificed to some extent in favour of the latter. In the development of the proposed method of rethrin analysis it was intended to improve upon the accuracy of current methods (see 1.4.8. and 2.1.1.) while maintaining, if not improving, the repeatability.

Inaccuracies in the proposed method may arise from three general sources, namely sample preparation, chromatogram evaluation and choice of calibration factors. Preparation of the sample to be chromatographed, by mixing the extract sample with the internal standard in a known proportion by weight, will incur errors of a fraction of a per cent if relatively large quantities are weighed on an accurate balance. One gram, for example, weighed on an accurate five-figure balance has an error of less than 0.01 per cent. If the internal standard is added in the form of a solution the extract sample can be simultaneously suitably diluted to suit the limits of the chromatographic detection[24,25] while preparation of the solution of the standard is similarly a source of negligible error. Human error of manipulation or variable bias in the balance will generally be indicated by the precision of the results. The main source of error in sample preparation is the determined purity of the internal standard (see 2.1.2.9.).

Sources of error in chromatogram evaluation arise from incompletely resolved components and interference by other components to the response of the component of interest. Accurate quantitative determination of each of the six rethrins would, in most cases, be hindered by the incomplete resolution of the cinerins and jasmolins but these pairs of components may usually be estimated together when the pyrethrins, being the most toxic and most labile (see 1.3. and 1.5.), are of primary

interest or only the rethrin I and rethrin II content is required. Construction of the baseline and accurate evaluation of the start and end of component peaks provided a greater problem particularly when the columns were old. Comparison of results from analyses on old and freshly prepared columns (e.g. Table 11; analyses 1 and 3) indicated that no serious error was incurred and separate evaluations of a single chromatogram based on the extreme position of baseline construction support this claim. Rethrin II chromatogram evaluations were hindered by the thermal degradation of the pyrethrins on the column but the inclusion of the area of the thermal degradation products was shown to yield results consistant with those involving only a small amount of degradation (see 2.1.2.6.). The inaccuracies of area measurement are discussed in 2.2.1. but generally, where area response is determined as opposed to its estimation on the basis of linear parameters, the precision of the area evaluation results reflect the accuracy of this stage of the procedure.

The use of an internal standard removed a serious source of inaccuracy, that of quantitative sample injection (see 2.1.2.1.), in the weight-response calibration. The choice of relative response factor and the possible errors involved were discussed in sections 2.1.2.2., 2.1.2.4. and 2.1.2.5.

The results of rethrin analysis by this method were therefore estimated to be accurate to at least $\pm$ 5 per cent. The larger proportion of this error was associated with the rethrin II results and the major source of error undoubtedly lies in the construction of the chromatogram baseline. It should be emphasised that this value must not be associated with the repeatability of the method.

Table 12 Rethrin analyses of the World Standard Extract by different methods

	P.B.K.[78]	A.O.A.C.[77]			U.V.[95]	E.D.A.[86]	D.N.P.[100]	Proposed Method
		8th Ed.	9th Ed.	10th Ed.				
Rethrins I	13.3	11.5	11.4	11.3	-	-	10.5	10.6
Rethrins II	9.7	10.0	10.1	9.8	-	-	8.0	6.1
Total Rethrins	23.0	21.5	21.5	21.1	20.1	19.3	18.5	16.7

	% of rethrins I			% of rethrins II		
	cinerin I	jasmolin I	pyrethrin I	cinerin II	jasmolin II	pyrethrin II
Proposed Method	21.0	8.3	70.7	28.9	9.9	61.2
Head[175]	23.1	9.3	68.6	29.4	10.5	60.1

Analysis of the World Standard Extract allowed direct comparison to be made with the results by several other methods which have been published by Head[175]. These are shown in Table 12. It was immediately apparent that the rethrin II content, and hence the total rethrin content, as determined by the proposed method, were low compared to those values returned by the other methods. The rethrin I value was found to be 80 per cent of the P.E.K.[78] value and about 93 per cent of those returned by the A.O.A.C. variants[77] but equal to the D.N.P.[100] value. The rethrin II value was only about 65 per cent of the results obtained by the mercury-reduction methods[77,78]. The results were therefore in reasonable agreement with the estimate of the true rethrin content put forward by Brierley and Brown[123,124] (see.1.4.8.), the rethrin II result being slightly lower.

The possibility that this could be attributed to loss of pyrethrin II during analysis and not fully accounted for by the inclusion of the response of thermally degraded material in the area response of this component (see 2.1.2.6.) was discounted on the grounds that the relative proportion of pyrethrin II to the other rethrins II would need to be abnormally high to yield rethrin II values comparable with those returned by other methods. Further, the determined values of the relative proportions of the rethrins in the World Standard Extract were in good agreement with those published by Head[175] (Table 12).

The possibility of error in the relative response factor of rethrins II to rethrins I cannot be ruled out in the present investigation since the theoretical value (see 2.1.2.1.) is 9 per cent greater than that found experimentally (see 2.1.2.5.).

However, use of the theoretical value would only yield a value of 6.7 per cent for the rethrin II content of the World Standard Extract, still considerably less than the results returned by the other methods of analysis (8 - 10 per cent, see Table 12).

The rethrin II values therefore, although lower than indicated by previous workers (see 1.4.8.), would certainly appear to be more accurate than the results returned by other methods. It was therefore concluded that the proposed method provided a more accurate assessment of the true rethrin content of pyrethrum extracts than any of the current methods of analysis.

2.2 Quantitative evaluation of gas-chromatographic data

2.2.1. Limitations of methods of chromatogram interpretation

All quantitative gas chromatographic results inherently contain errors, the extent of which depends upon the equipment, the methods of analysis, calibration and calculation and sample handling. The choice of stationary phase, its loading on the support, the dimensions of the column and the operating conditions and their effect on chromatographic performance have already been exemplified (see 1.4.7.). The limitations of using external standards requiring quantitative injection, as a method of calibration, have also been critically assessed (see 2.1.2.1.). There remains the need to choose a method of evaluating the chromatographic output.

The detector response is normally plotted on a strip recorder to yield a chromatogram from which the concentrations of the sample components or their relative proportions, have to be manually calculated. The only chromatogram parameter that is directly proportional to the amount of sample component is peak area although the use of linear peak parameters (such as peak height, peak width, retention time) often provides an adequate

substitute and simplifies chromatogram evaluation. Methods based on these have been thoroughly investigated.

Evans and Scott[177] obtained high precision using peak heights and Deans[171] advocates their use in preference to peak areas for better repeatability. It is acknowledged however that the relationship between peak height and component concentration is very sensitive to flow rate and column temperature. Flow controllers, for example, are inadequate due to their temperature sensitivity[178] and pressure control is necessary. It is concluded that only peak-area measurement is generally applicable for chromatogram interpretation except perhaps under control - laboratory conditions or where the accuracy required does not justify more rigorous methods.

Other methods requiring only the measurement of linear peak parameters are quadrature, which is the product of peak height and peak width at half peak height, triangulation, which is the area of the triangle formed by the peak base and tangents through the points of inflection, and the product of peak height and retention time, all of which are used successfully under certain conditions. The first two methods attempt to approximate the determined areas to the true area of the peak.

Methods of absolute area determination are planimetry, cutting out the area and weighing, the disc integrator and, in recent years, the electronic digital integrator. Johnson[179] and Gough and Walker[180] have reviewed the numerous studies that have been undertaken to establish the merit of each of the methods of peak area determination. Superior precision is claimed by different workers for most of the methods but since the precision is dependent upon manual evaluation for all but electronic integrators the results probably indicate the limits of precision

to be reached by individual workers. The conclusions must therefore be that precision of the same order can be achieved by any of the methods although in general use, those involving linear parameters may show slight superiority.

With assymmetric peaks, methods utilising linear peak parameters may maintain their precision but lose their accuracy. Scott and Grant[181] reversed their preference for quadrature and triangulation over planimetry for distorted peaks.

The advent of electronic integrators has undoubtedly improved on the precision of peak area measurement although the requisite apparatus is generally expensive which introduces an additional consideration in assessing the merits of this method of chromatogram evaluation. An increase in accuracy and precision is achieved by the removal of reliance on human evaluation and the elimination of recorder characteristics, such as rate of chart drive, pen speed, electrical zero stability, as a source of error by directly using the detector signal and in no way requiring a recorder trace. The results are usually printed out in the required format removing a further source of operator error. The only source of error in the use of electronic integrators appears to be in the method of peak sensing and the correction for baseline drift. This led Gough and Walker[180] to conclude that accuracy was worse than the conventional methods of chromatogram evaluation. The limitations of analogue and digital peak sensing methods have been reviewed by Johnson[179], the conclusions being that for wide applicability a certain amount of human judgement is necessary. Under certain conditions even the most sophisticated systems may make interpretation errors and an analyst can substantially improve accuracy by making arbitrary decisions. The vast majority of available systems

however do not provide any information on the analytical report so that key decisions that have been made may be checked.

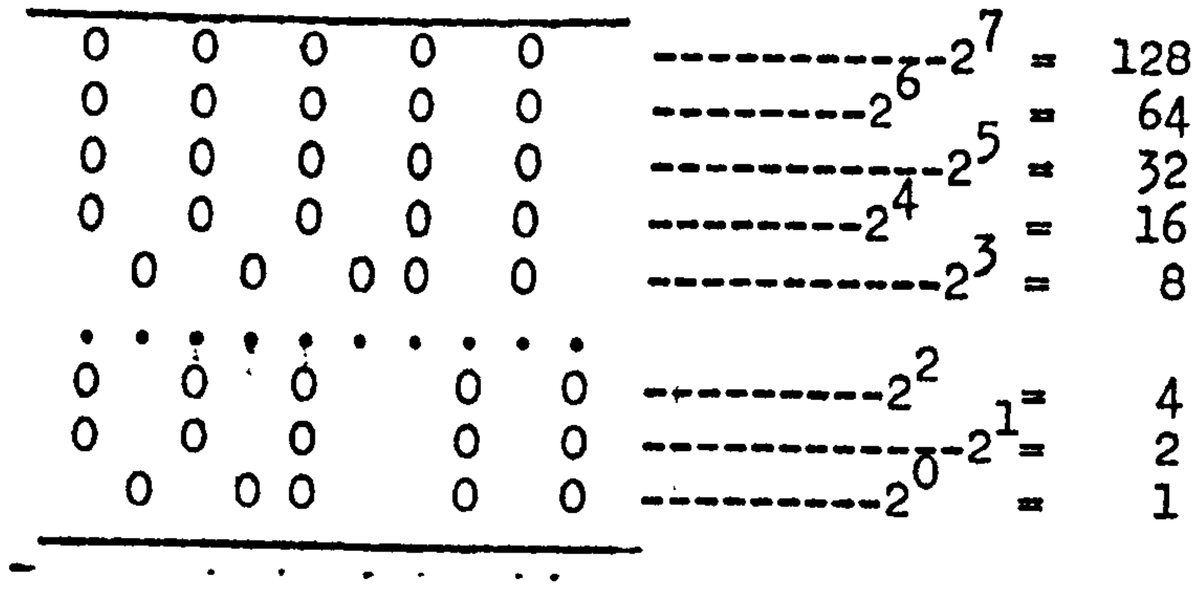
2.2.2. Digitisation of the gas-chromatographic output and its evaluation by a programmed digital computer

2.2.2.1. Hardware

During the early part of this work chromatogram evaluation was achieved by quadrature, where chromatographic peaks were reasonably symmetrical, or by planimetry, where peaks were assymmetric, e.g. rethrin analyses. The availability of an analogue-to-digital converter (A.D.C.), produced at a relatively moderate cost by the School of Electrical Engineering, offered a potentially more accurate and precise method of evaluating the chromatographic output than the tedious and time-consuming planimetry method. This was used in conjunction with a paper-tape punch to directly record the detector response in a form suitable for processing by a digital computer.

Detector response is sampled at fixed intervals (a choice of five sampling rates is available rangeing from approximately two samples per second to one sample every three seconds) and recorded by an 8 bit paper-tape punch. The A.D.C. was calibrated so that maximum detector output (amplified to 100 mV) gave zero binary signal and zero detector output gave maximum 8 bit output ($\sum_{n=0}^{n=7} 2^n = 255$). Each recorded value was followed by its complement as a check on satisfactory punch operation and the complement is therefore proportional to the detector output. A typical paper-tape output is shown in Figure 10.

Figure 10 Typical binary paper-tape output

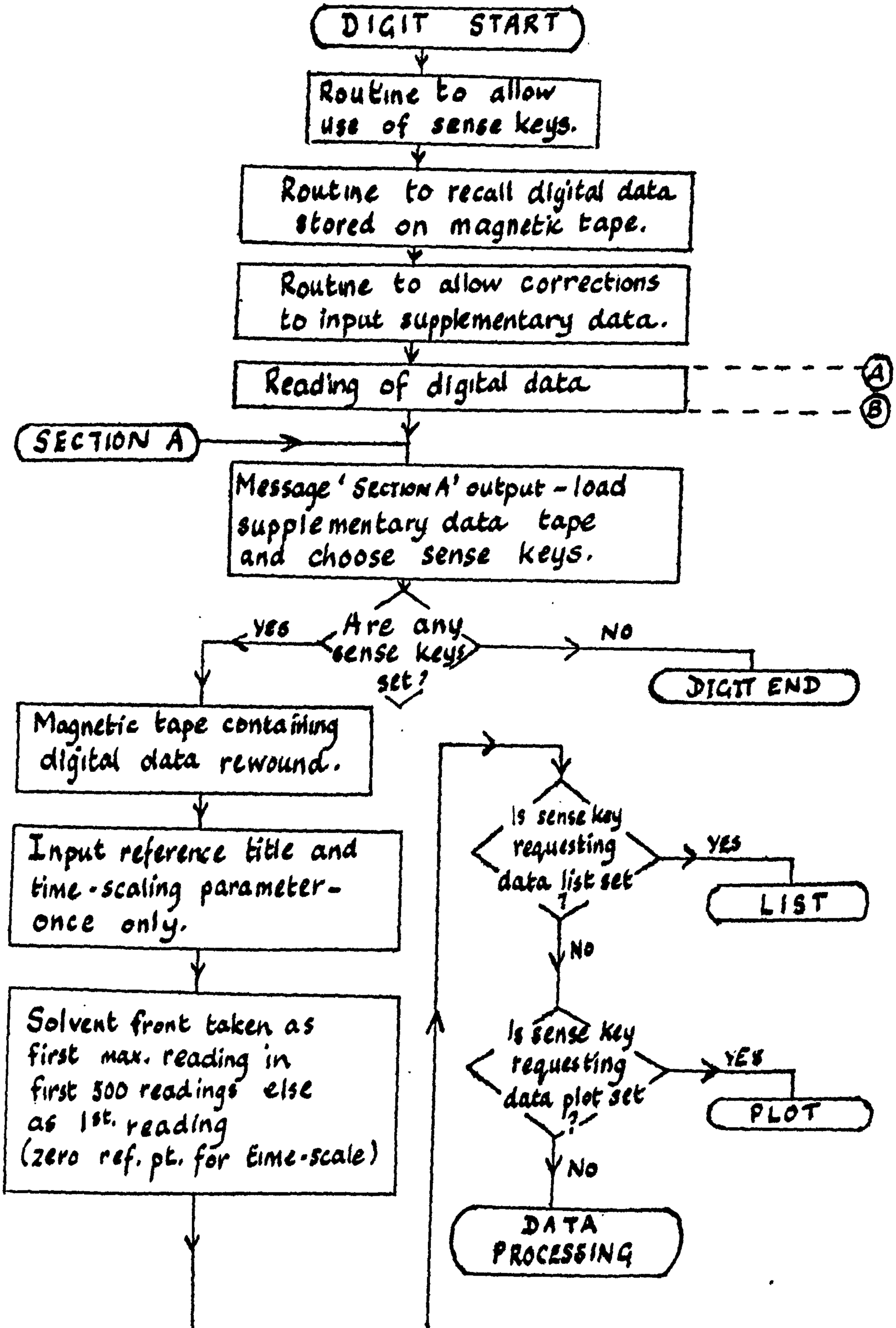


Using this type of process, evaluation of the chromatographic output is confined entirely to software interpretation of the digitised output. A computer program was therefore designed and written to carry out this operation.

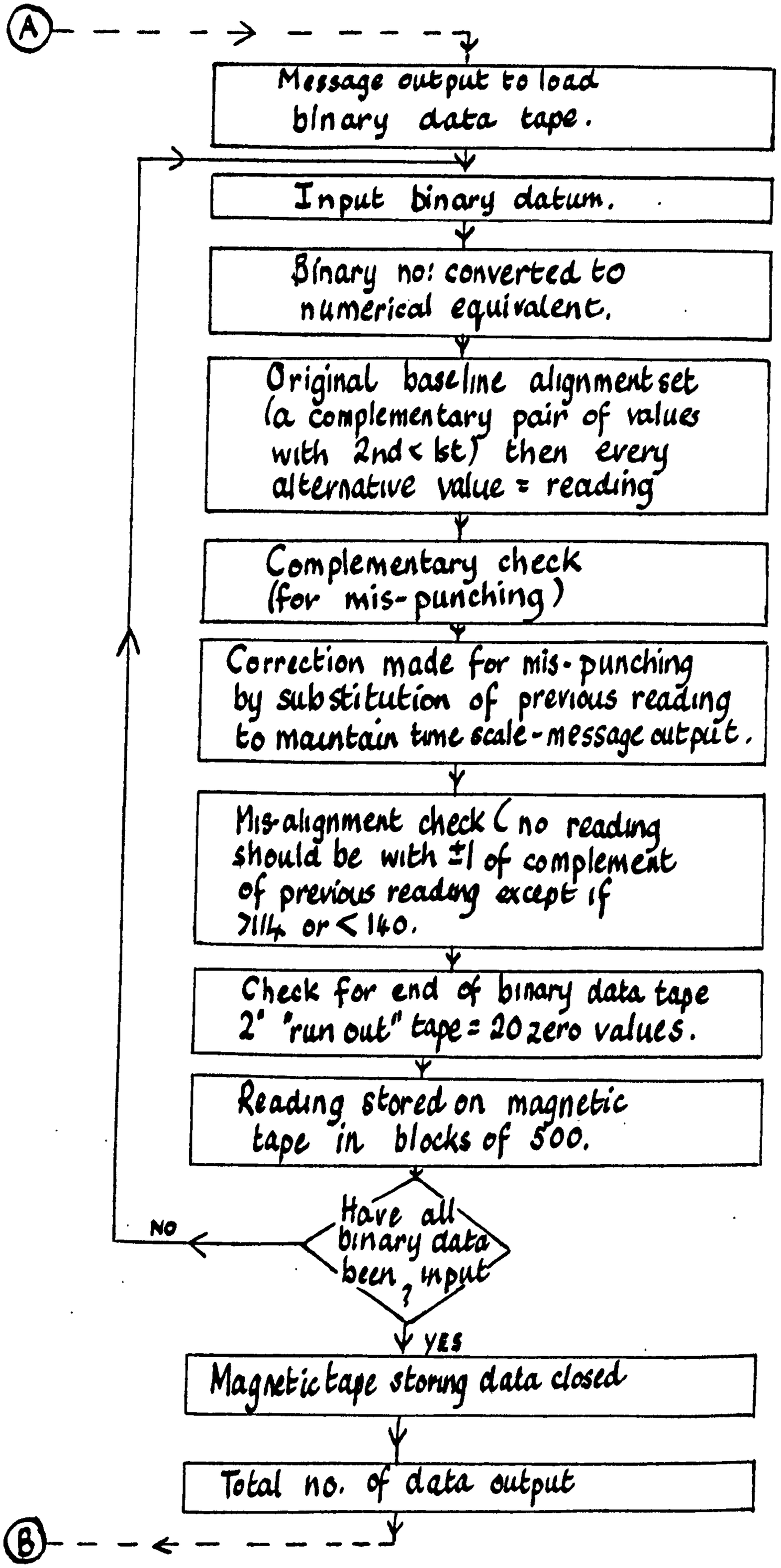
2.2.2.2. Software

The program, entitled DIGIT, presented in flow-chart form in Figure 11, embodies four sections which may be termed (a) reading of digital data, (b) processing of digital data, (c) plot of digital data, and (d) list of digital data. The program is segmented by the computer on compilation into these four parts (to save on core storage) and stored individually on magnetic tape. For evaluation of the chromatographic output only sections (a) and (b) are required. Sections (c) and (d) supply a supplementary service enabling the digital data to be displayed for closer scrutinisation if required. The selection of (b), (c) or (d) is made by utilising the sense keys on the control panel of the computer.

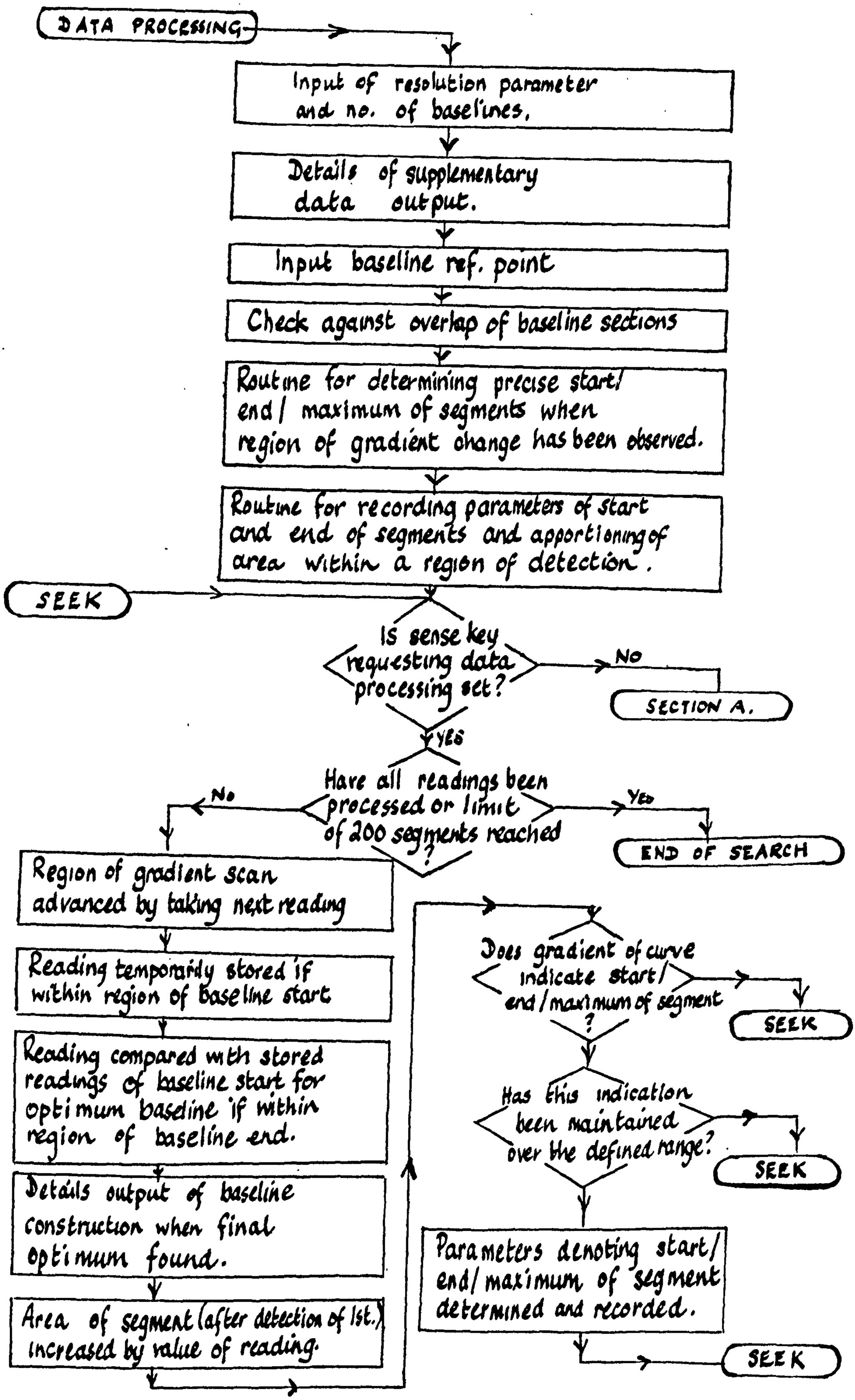
Figure 11 Flow chart form of computer program DIGIT.



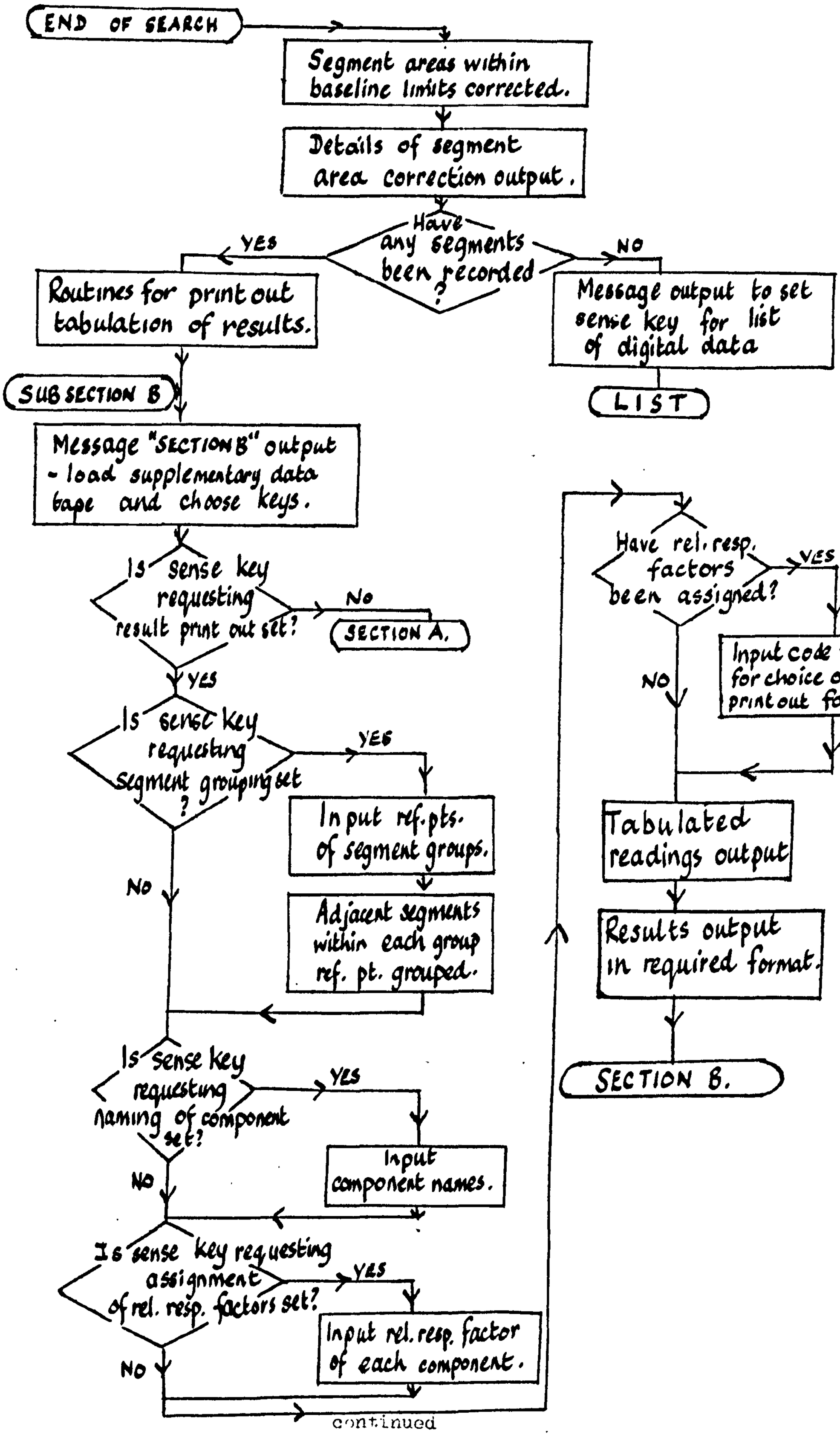
continued


A
Message output to load binary data tape.
Input binary datum.
Binary no: converted to numerical equivalent.
Original baseline alignment set (a complementary pair of values with 2nd < 1st) then every alternative value = reading
Complementary check (for mis-punching)
Correction made for mis-punching by substitution of previous reading to maintain time scale - message output.
Mis-alignment check (no reading should be with ±1 of complement of previous reading except if >114 or <140.
Check for end of binary data tape 2" "run out" tape = 20 zero values.
Reading stored on magnetic tape in blocks of 500.
Have all binary data been input ?
NO
YES
Magnetic tape storing data closed
Total no. of data output
B

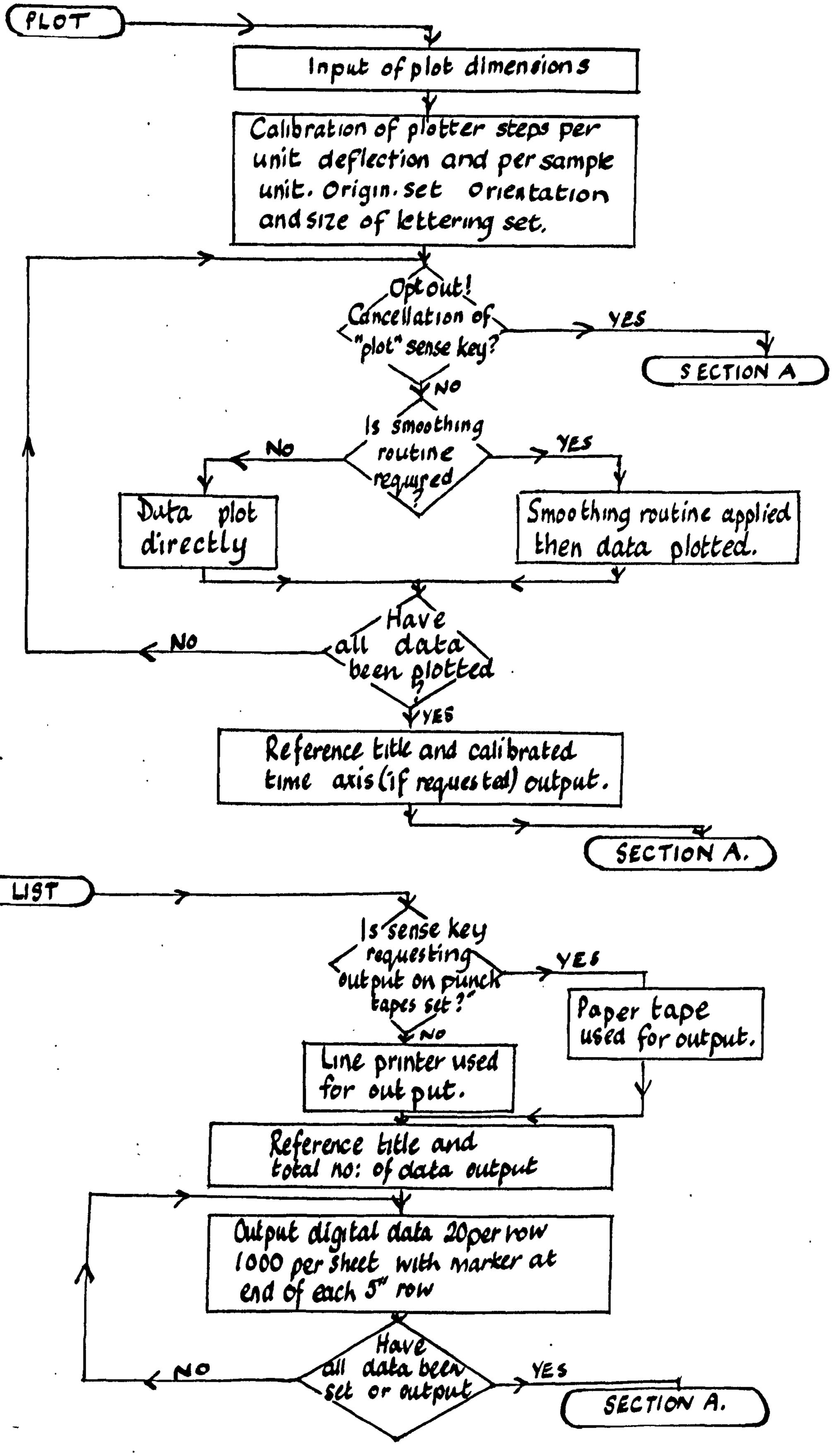
continued


DATA PROCESSING
Input of resolution parameter and no. of baselines.
Details of supplementary data output.
Input baseline ref. point
Check against overlap of baseline sections
Routine for determining precise start/ end/ maximum of segments when region of gradient change has been observed.
Routine for recording parameters of start and end of segments and apportioning of area within a region of detection.
SEEK
Is sense key requesting data processing set?
NO
SECTION A.
YES
Have all readings been processed or limit of 200 segments reached ?
NO
YES
END OF SEARCH
Region of gradient scan advanced by taking next reading
Reading temporarily stored if within region of baseline start
Reading compared with stored readings of baseline start for optimum baseline if within region of baseline end.
Details output of baseline construction when final optimum found.
Area of segment (after detection of 1st.) increased by value of reading.
Does gradient of curve indicate start/ end/ maximum of segment ?
SEEK
Has this indication been maintained over the defined range?
SEEK
Parameters denoting start/ end/ maximum of segment determined and recorded.
SEEK

continued


END OF SEARCH
Segment areas within baseline limits corrected.
Details of segment area correction output.
Have any segments been recorded?
YES
NO
Routines for print out tabulation of results.
Message output to set sense key for list of digital data
SUB SECTION B
LIST
Message "SECTION B" output - load supplementary data tape and choose keys.
Is sense key requesting result print out set?
No
SECTION A.
YES
Is sense key requesting segment grouping set?
YES
Input ref.pts. of segment groups.
No
Adjacent segments within each group ref. pt. grouped.
Is sense key requesting naming of component set?
YES
Input component names.
NO
Is sense key requesting assignment of rel. resp. factors set?
YES
Input rel. resp. factor of each component.
NO
Have rel. resp. factors been assigned?
YES
Input code value for choice of print out format
NO
Tabulated readings output
Results output in required format.
SECTION B.

continued


PLOT
Input of plot dimensions
Calibration of plotter steps per unit deflection and per sample unit. Origin. set Orientation and size of lettering set.
Opt out! Cancellation of "plot" sense key?
YES
SECTION A
NO
Is smoothing routine required?
No
YES
Data plot directly
Smoothing routine applied then data plotted.
Have all data been plotted?
No
YES
Reference title and calibrated time axis (if requested) output.
SECTION A.
LIST
Is sense key requesting output on punch tapes set?
YES
NO
Paper tape used for output.
Line printer used for output.
Reference title and total no: of data output
Output digital data 20 per row 1000 per sheet with marker at end of each 5" row
Have all data been set or output
NO
YES
SECTION A.

(a) Reading of digital data

Each punched value is read into the computer and converted to its numerical equivalent so that the data is in a suitable form for operating instructions written in a high level programming language (Algol 60). Initial alignment of a pair of values, which together constitute a reading, is made by advancing the binary data tape in the reader until the complementary value is found which is less than the signal value. This reading corresponds to the pre-solvent baseline and the second value, being proportional to the detector output (see 2.2.2.1.), is the one recorded.

The complementary nature of subsequent pairs of values is checked before recording the reading. Where the complementary check proves false and advancing the tape one value fails to find a complementary pair, the previous reading is re-recorded. The time-scale is therefore maintained throughout any anomalous punching and a message reporting this correction is supplied to the operator.

Misalignment is assumed to have occurred, and is corrected, if a reading is within plus-or-minus one of the complement of the previous reading except if it is between 114 and 140, equivalent to approximately 50 per cent full scale deflection, when such a situation may be correctly encountered.

Blocks of 500 readings are transferred to magnetic tape store and the end of the binary data is recognised by twenty zero values, equivalent to two inches of "run-out" tape.

(b) Processing of digital data

The processing entails scanning the results by peak-sensing and baseline construction procedures, recording the relevant parameters, followed by baseline construction to peak areas and printing out of the results in the required format. The readings stored on magnetic tape are transferred into the computer store in blocks of five hundred, although this is for the convenience of computer operation only since each result is processed in turn which in fact simulates on-line processing. However, on transference of the readings a record of them is retained on the magnetic tape until over-written with a different set of readings.

Certain parameters are required as supplementary data for the processing to take place in the manner of choice and these are input at the control-teletype or on punched paper-tape (see Table 13). In addition to the provision for a reference title and time-scaling parameter, only parameters for peak detection, baseline construction and peak area correction are essential. The values of these parameters are arbitrarily assessed from the chromatogram which is normally output from the gas chromatograph at the same time as the digitised response data and in this way a certain degree of manual control may be maintained over the processing of the data (see 2.2.1.). In the absence of a chromatogram, one may be simulated by the use of the plotting section (d), of the program (see below). Additional parameters, which are optional, allow for grouping of adjacent segments or peaks, naming of peaks, relative response factor assignment and a choice of printout format.

Table 13 Parameters for digital data processing

	No. of parameters	Comments
Reference title	'TITLE'	-
Time-scaling parameter	1	-
Resolution value	1	-
Baseline construction	1	no. of baselines
	2	range of baseline start
	2	range of baseline end
Range of baseline	2	range of area correction
	T/F	choice of bending baseline
Baseline 'bending'	2*+ 1*	range of extension if bent and of area correction
Peak/segment grouping	1*	no. to be grouped
	2*	range of each group
Peak naming	1*	length of longest name
	'NAME' *	for each grouped peak
Rel. resp. factor assignment	1*	for each grouped peak
Format choice	1*	format code value
Others	n*	dependent on format choice (e.g. int. stand. peak no., sample wts., concns.)

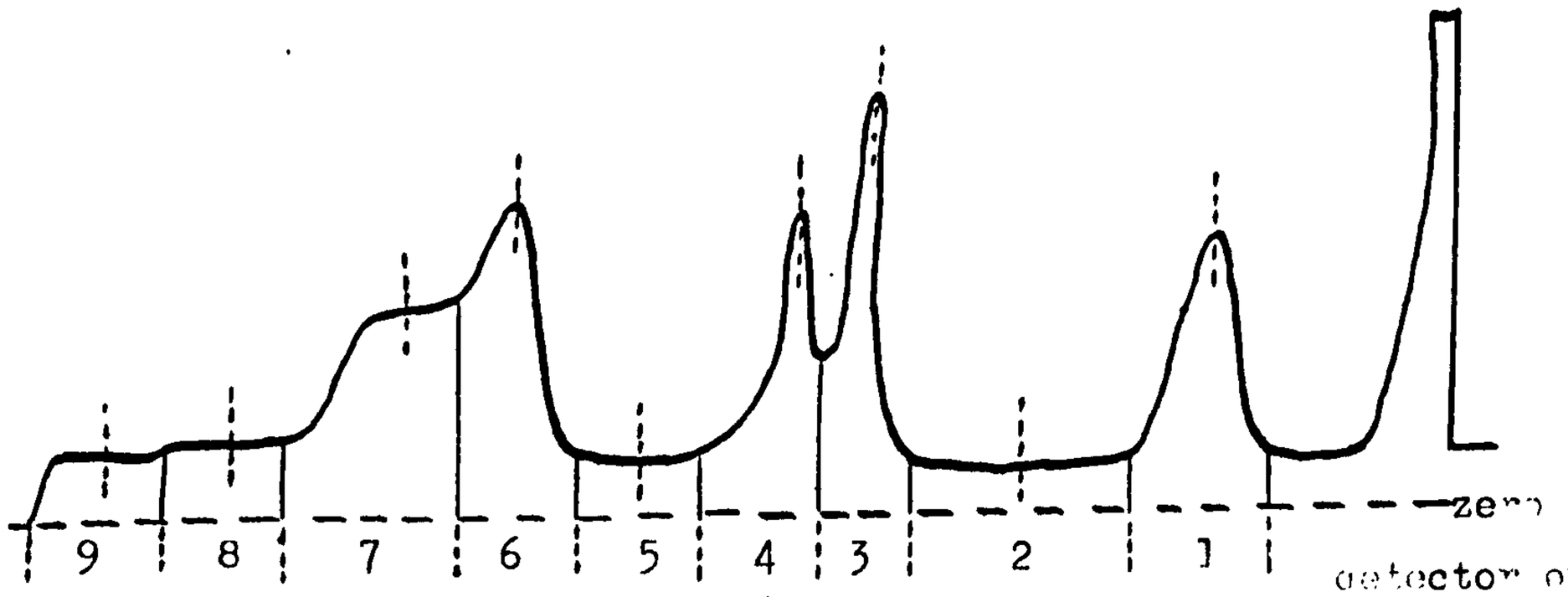
* optional extra parameters

Time-scaling parameters Input of the time-length of the chromatogram or a code value corresponding to the sampling rate allows a time scale to be allotted to the digital data. Subsequent parameters, input in units of time, may then be interpreted in terms of the number of sample units.

Peak-sensing or resolution parameter The peak-sensing procedure relies upon a suitable change in the rate of change of detector response, i.e. in the gradient of the chromatographic curve. 'Peak' maxima are recognised when the gradient ceases to be positive and the end of 'peaks' when the gradient ceases to be negative. The start of the first 'peak' is detected by a positive gradient but subsequent 'peaks' are taken as starting at the end of the previous 'peak'. A maintained zero gradient value corresponds to baseline sections or shoulders. In this way the chromatogram is divided into segments (see Figure 12).

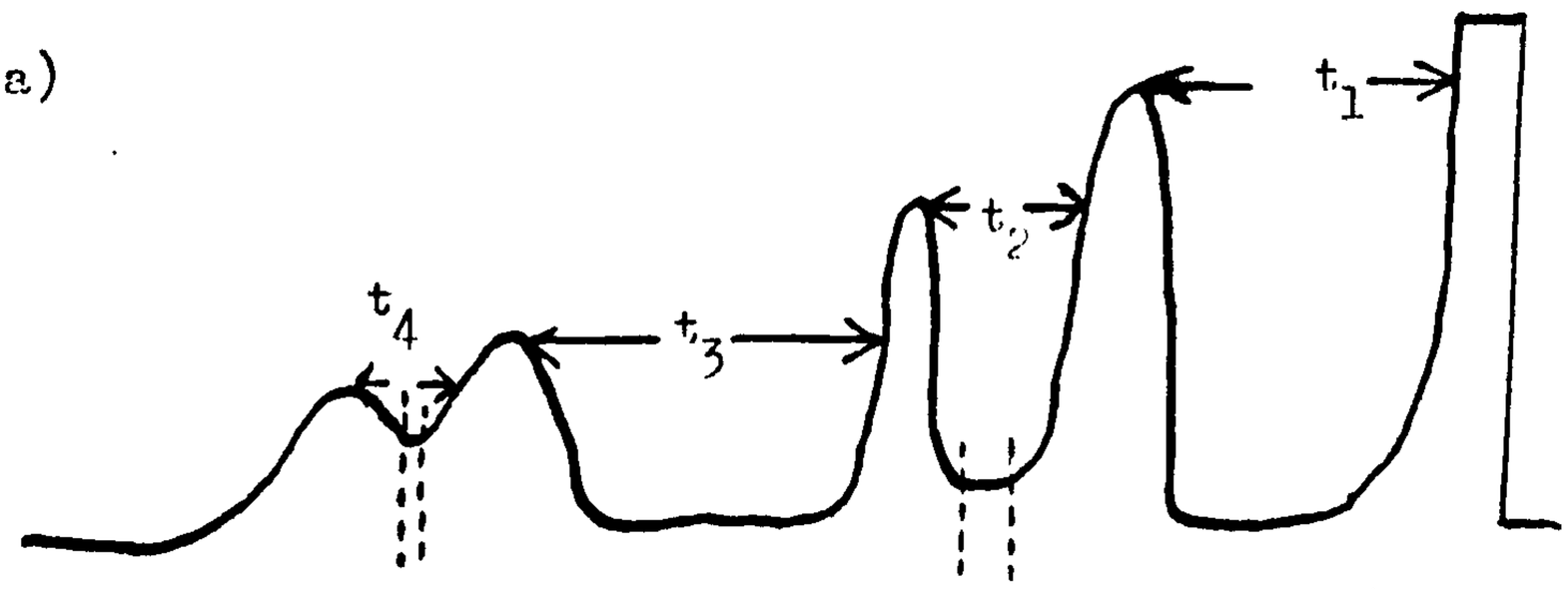
To protect against anomalous changes in gradient sign produced by occassional incorrect readings, the change is required to be maintained over a number of successive readings (proportional to the size of the gradient region) before it is confirmed. The readings contained within a gradient region are stored temporarily, each 'new' reading substituting for the 'oldest' reading which is no longer required. When a change in gradient sign is noted however the replaced reading is retained in store so that if the change is confirmed the temporary stored readings may be rescanned to determine the precise maximum/minimum position (sampler-unit number), and reading at this position. In the case of shoulders the midpoint of the plateau is taken as the position of the maximum. These values which adequately define each segment are stored by the computer.

Figure 12 Division of chromatogram by peak-sensing process.



| - segment maximum positions

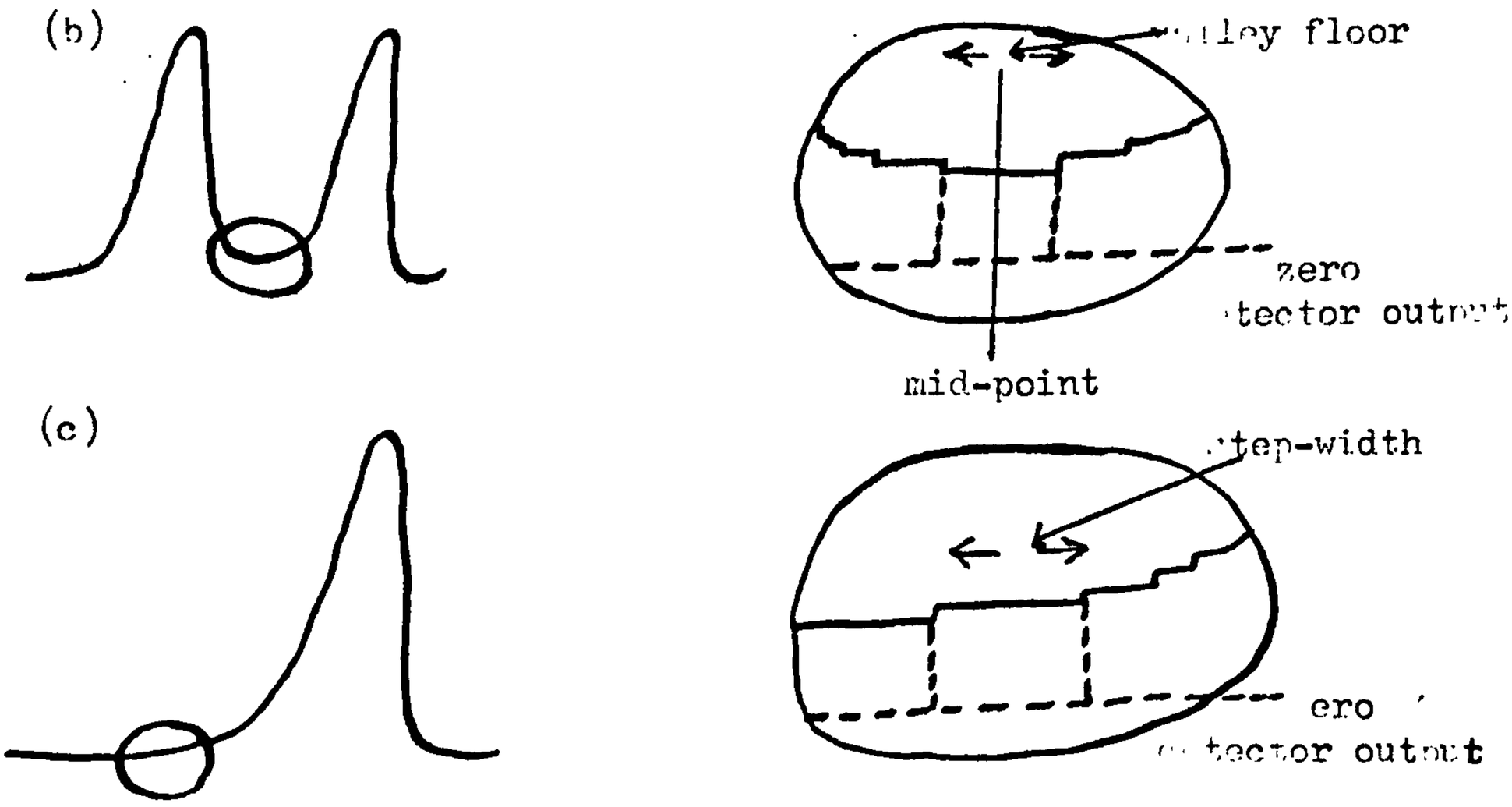
Figure 13 Criteria for choosing value of resolution parameters.



t_1 = max. time difference between peaks

v_2 = time-widths of valley floor between incompletely resolved peaks

$$v_1 < \text{resolution value} < t_4$$



The size of the range over which the gradient is determined is defined by the value afforded to the peak-sensing parameter. The choice of this value is determined by two considerations. The value should not exceed the time difference between the two closest peaks (as defined by Figure 13a) which are to be resolved but should be greater than the time-width of the largest valley 'floor' (see Figure 13a). The latter criterion ensures that the midpoint of the valley 'floor' (see Figure 13b) is taken as the division between peaks not separated by a section of baseline; alternatively the 'floor' is recorded as a segment.

An additional outcome of a low resolution value would be the detection of 'false' shoulders (Figure 13c). This arises out of the digital nature of the data so that, for example, the tail of a peak is in fact a stepwise decline and where the width of the 'step' exceeds the resolution value the 'step' is resolved from the peak, as one would require a true shoulder to be recorded, because it becomes indistinguishable from a true shoulder (see Figure 12, c.f. segments 8 and 9 with 7). The resolution of 'false' shoulders can be corrected by using the facility of peak-grouping (see below) to combine the 'shoulder(s)' with the main peak.

Restriction limits are placed on the resolution parameter, by the program, of two hundred seconds for the upper limit and ten sample units for the lower limit to prevent inputting extreme values by mistake and yielding totally meaningless results.

The area of each segment is determined by continuous summation of the readings as they are processed, the summed area at the end of each segment being stored and the parameter reset to zero. Provision is made for the detection of up to two hundred segments since this approached the store capacity

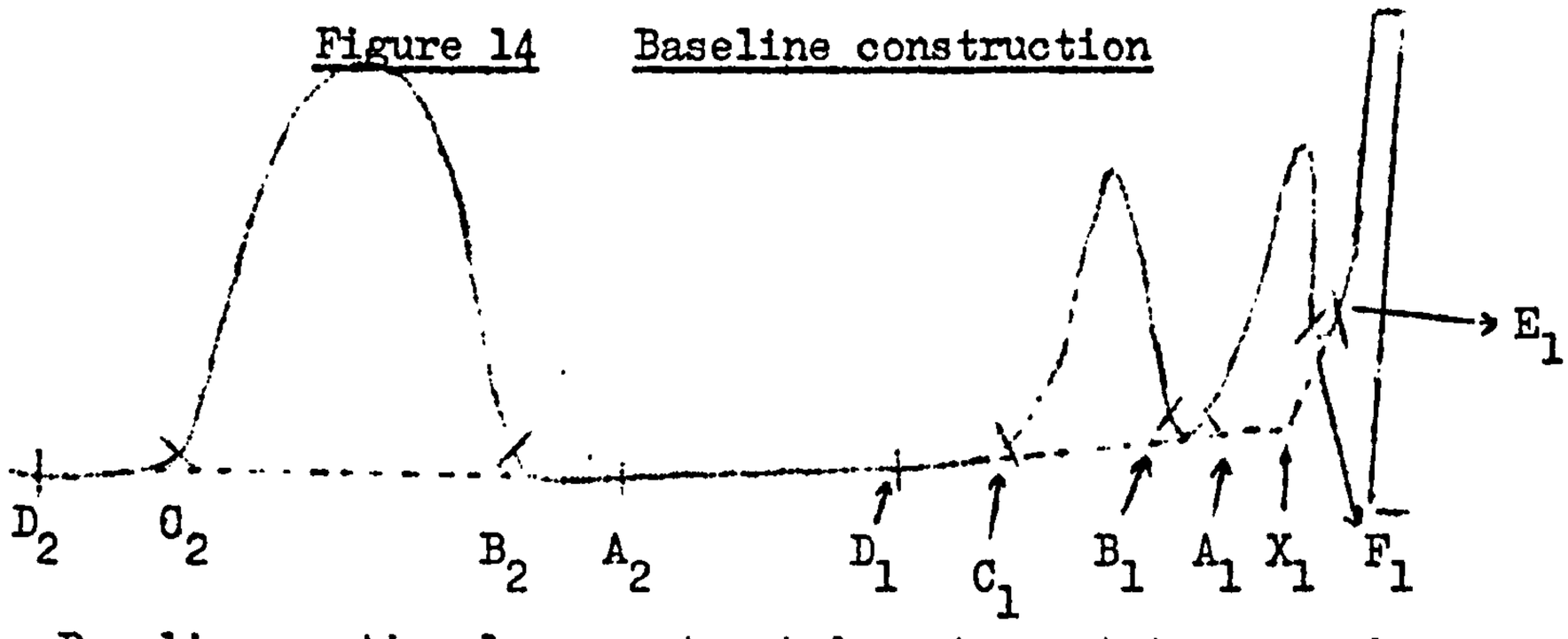
of the computer although provision could be made for a greater number by using magnetic tape storage facilities.

Baseline construction parameters These parameters enable the optimum baseline to be assigned to the chromatogram, defined as being the tangent to the curve at two separate points which are in a given region of the chromatogram (see Figure 14). This may be compared to the manual procedure of constructing a straight baseline with a rule. The start and end of each baseline constructed (any number of baseline sections may be assigned) therefore requires two reference points to denote the range in which they may be found. Each reference point is input in units of time. The baseline sections are constructed during the processing of the digital data by temporarily storing each reading within the range of the start of the section, to be compared with each reading in the end-range as they are encountered.

The points of the tangent to the curve do not however define the limit of the baseline section. Two additional parameters define this and enable the baseline to be linearly extrapolated in either direction. Segment areas within the baseline 'limits', to the nearest start/end of a segment, are corrected for the baseline when all the digital data have been processed and the corrected areas stored.

An additional optional feature of the baseline construction procedure is the 'bending' of the baseline by a further extension at either 'limit'. This allows solvent tailing to be taken into account (to a first approximation) (see Figure 14). It is achieved by constructing the optimum tangent to the curve within a desired range (defined by two parameters) from the baseline 'limit'. This may also be linearly extrapolated, if required, to a new baseline limit (defined by a further parameter).

Figure 14 Baseline construction



Baseline section 1 : constructed as tangent to curve in range A_1B_1 and C_1D_1
extrapolated to limit X_1
bent as tangent to curve in range E_1F_1

Baseline section 2 : constructed as tangent to curve in range A_2B_2 and C_2D_2

Details of the construction of each baseline section are output for a manual check by the analyst.

Peak or segment grouping parameters These are optional supplementary data which enable the combining of adjacent peaks for the desired result format (see below) or of adjacent segments into a single peak where the tail of a peak has been resolved into one or more shoulders (see 'resolution parameter').

The total number of groups and two parameters per group are required. The latter, input in units of time, indicate the approximate range of each group. Segment/peak areas within this range, to the nearest start/end of a segment/peak, are combined and the peak height (maximum reading) and retention time (sampler unit number of maximum reading) of the group are taken as the values in store for the highest segment/peak within the group.

Wherever a grouping parameter is required to coincide with a baseline construction point (see above) it is preferable for greater precision, to input the value in its negative form so that it is identified with the baseline construction point.

Identification and/or relative response factor parameters

The assignment of a name and/or relative response factor to each segment, peak or group may be nade for the identification of the components on the result printout and for further calculations of the areas determined for them.

Format parameters If relative response factors have not been assigned the choice of printout format is restricted to the output of absolute areas and no format parameter values are required. Alternatively the results may be expressed with (1) normalised areas, (2) areas standardised relative to an internal standard, or (3) standardised areas with the calculated weight andconcentration of each component based on known weights and concentrations of sample and standard. The required format is selected by input of the appropriate code value and further supplementary data is input if required (see Table 13 and Appendix)

(c) Plot of digital data

By the use of the digital plotter, a peripheral output device on most computer systems, the conventional chromatogram may be reproduced. This section of the program provides a routine for performing this operation. The only parameters required are the dimensions of the plot. The digital data may be plotted directly but an optional smoothing routine is provided.

Since full-scale deflection is 255 unit steps, a unit deflection for a time reproduction of the chromatogram is approximately one-twentyfifth of an inch. Therefore a 'stepping' effect is produced in the deflection-direction. Since a sampler-unit is equivalent to approximately one-twohundredth of an inch this effect is not observed in the time-direction. The smoothing routine completely removes the observed effect by averaging a unit change in deflection over a number of sampler

units, namely, from the midpoint of one step to the midpoint of the other. Additional smoothing is performed where oscillation between values occurs, usually the outcome of a 'noisey' baseline.

The value of the plotting procedure is the production of the chromatogram on a reduced scale and the majority of chromatograms presented in this work were reconstructed from their digitised output in this way.

(d) List of digital data

This section of the program is merely an output routine for the digital data in a suitably tabulated form. The data is listed twenty readings per row, one thousand readings per sheet and an identifying mark is placed at the end of every fifth row, equivalent to one hundred readings, for ease of tracing a particular reading. Although any error in the processing of the data, due to incompatible supplementary data, will be highlighted by the supplementary output of the data processing routine, the availability of a visual display of the digital data may facilitate the discovery of the source of error if this is not immediately apparent.

2.2.3. Accuracy of chromatogram evaluation by the proposed method

In order to confirm the accuracy of chromatogram evaluation by computer analysis of the digital data the method was used to determine the relative concentration of the components in an ideal two-component system. The two compounds chosen for this investigation were n-decane and n-undecane since these are readily attainable standards of high purity and their relative response factor may be taken as unity (see 2.1.2.1.). (The relative response factor of undecane relative to decane, as defined by equation (iii) (p. 50) is 0.999). Further, since both the compounds are liquid at room temperature their purity could be demonstrated by direct

g.l.c. analysis without recourse to dissolution in a solvent. This indicated the presence of no other components in concentration greater than 0.1 per cent.

Two samples were prepared containing mixtures of decane and undecane in a known proportion by weight (accuracy > 0.1 per cent) and subjected to several gas-chromatographic analyses (Table 14). Different volumes of sample solution were injected to yield chromatograms of a variety of peak sizes. Under the same chromatographic conditions the retention times of the sample components are consistant between chromatograms and therefore the peak area reflects the height of the peak.

One sample was analysed under different chromatographic conditions so that for a given peak area the peak height was reduced at longer retention times. This allowed comparison of the results of chromatogram evaluation of different shaped peaks for the same component mixture.

Statistical treatment of the results (Table 15) showed that chromatogram evaluation by the developed computerised method was more precise than that using the planimetry method of peak area measurement. Calculation of the relative response factor from the determined relative area response further demonstrated the precision of the computerised method and, on the assumption that the true value is unity, a greater degree of accuracy.

One chromatogram (A(iii)) yielded results which were anomalous by comparison with the other results. This exemplified the necessity of replicate chromatographic analyses to obtain accurate results. This result was rejected in statistical treatment.

Table 14 Precision test of chromatogram evaluation

Sample		Area response (digital units)		Computer undecane:decane		Planimeter* undecane:decane	
		decane	undecane	area ratio	rel. resp. factor	area ratio	rel. resp. factor
A	(i)	2596	3046	1.173	0.993	1.18	0.987
	(ii)	8303	9691	1.167	0.998	1.21	0.963
	(iii)	719	1055	1.467	0.794	1.49	0.781
	(iv)	4398	5069	1.153	1.010	1.14	1.022
B1	(i)	2428	4046	1.666	0.948	1.68	0.940
	(ii)	936	1456	1.566	1.008	1.44	1.100
	(iii)	2304	3413	1.481	1.066	1.56	1.012
	(iv)	4082	6370	1.561	1.012	1.62	0.975
	(v)	3432	5409	1.576	1.002	1.51	1.046
	(vi)	3753	6007	1.601	0.986	1.55	1.019
B2	(i)	5984	9369	1.566	1.008	1.54	1.025
	(ii)	8263	12842	1.554	1.016	1.56	1.012
	(iii)	8902	13481	1.514	1.042	1.46	1.082

* average of 5 determinations

undecane - decane ratio by weight in sample A = 1.165

undecane - decane ratio by weight in sample B = 1.579

B2 - chromatographed under different chromatographic conditions

(retention times increase B (i) - B (iii))

Table 15 Statistical treatment of results on precision test of chromatogram evaluation

	Area ratio						Relative response factor	
	computer			planimeter				
Sample	A	B1	B2	A	B1	B2	Com-puter	Plan-imeter
No. of results	3##	6	3	3##	6	3	12##	12##
Mean	1.164	1.575	1.545	1.18	1.56	1.52	1.007	1.015
Standard deviation	0.010	0.060	0.027	0.035	0.084	0.053	0.029	0.046
Coefficient of variation	0.009	0.038	0.018	0.030	0.054	0.035	0.028	0.046
95% confidence limits	0.025	0.063	0.067	0.087	0.088	0.132	0.018	0.029

the calculations exclude A (iii)

Where the chromatographic conditions yielded relatively longer retention times (analyses of sample B2) the calculated relative response factor appeared to be in error. This was noted for both methods of chromatogram evaluation. This is probably due to a lower value for the area response of undecane relative to decane in accordance with the fact that a small but greater proportion of the area becomes indistinguishable from the baseline at longer retention times. This supports the use of experimental rather than theoretical relative response factors and of consistant operating conditions.

2.3. Stability studies

The true rethrin stability in pyrethrum extracts may well be masked by the presence of extraneous matter. Head has recently reviewed present knowledge concerning the non-rethrin composition of pyrethrum oleoresin[12]. This is seen to contain many compounds which could promote autoxidative deterioration of extracts. Chlorophylls are efficient photosensitisers[183,184] and although carotenoids quench excited oxygen molecules[185] both may act as catalysts by decomposition of peroxides[183,184] (see 2.3.1.). The proportion of unsaturated, non-hydroxy fatty acids, amounting to more than 10 per cent of the extract[126], provides an adequate quantity of labile substrate for autoxidation (see 2.3.4.2.).

During the production of Pale Extract the refining processes remove much of the fatty acid content and decolourise the extracts; refined extracts are consequently reported to be more stable[132,145]. However the majority of work on rethrin stability studies has been performed on thin films of extracts and the inadvisability of correlating such information to bulk extract studies has already been discussed (see 1.6.2.).

2.3.1. Autoxidation and Inhibitive processes

Naturally occurring fats are acknowledged as being prone to autoxidative deterioration. This has been attributed to the presence of unsaturated compounds which have been shown to be relatively unstable in the presence of air or oxygen[183,186]. The oxidation processes are considered to involve a free-radical chain mechanism[183,184,186-188] which may be described by the following series of reactions:

Initiation:	inactive material $\xrightarrow{\text{activation}}$	free radicals (X•)	(iv)
	$X^{\bullet} + RH \longrightarrow XH + R^{\bullet}$		(v)
Propagation:	$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$		(vi)
	$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$		(vii)
Hydroperoxide decomposition:	$ROOH \longrightarrow RO^{\bullet} + {}^{\bullet}OH$		(viii)
	$2ROOH \longrightarrow RO^{\bullet} + ROO^{\bullet} + H_2O$		(ix)
Self-termination:	$2ROO^{\bullet} \longrightarrow$	inactive products	(x)
Inhibition: chain breaking:	$ROO^{\bullet} + IH \longrightarrow ROOH + I^{\bullet}$		(xi)
peroxide destruction:	$ROOH + Y \longrightarrow ROH + YO$		(xii)
Inhibitor product stabilisation	$I^{\bullet} + RH \longrightarrow IH + R^{\bullet}$		(xiii)
	$I^{\bullet} + O_2 \longrightarrow IOO^{\bullet}$		(xiv)
	$I^{\bullet} + ROO^{\bullet} \longrightarrow IOOR$		(xv)
	$I^{\bullet} \xrightarrow{-H^{\bullet}}$	stable product	(xvi)
	$2I^{\bullet}$ / $2IOO^{\bullet} \longrightarrow$	dimerised stable products	(xvii)

RH = organic substrate, IH = inhibitor, Y = preventive

Reaction (iv) is promoted by heat or light. Absorption of light by the substrate or impurities containing chromophoric groups (e.g. ketones, conjugated dienes) may lead to dissociation of a chemical bond to produce free radicals and thermally induced dissociation of bonds will lead to similar results. Whether direct reaction between oxygen and the organic substrate can occur, as depicted by equations (xviii) and (xix) appears to be the subject of some dispute. Uri[189] claims that the energy requirements are

$$RH + O_2 \longrightarrow R^{\bullet} + HO_2^{\bullet} \qquad \text{(xviii)}$$

$$R_2C = CR_2 + O_2 \longrightarrow R_2C(OO^{\bullet})-CR_2 \qquad \text{(xix)}$$

prohibitive but the majority of workers[190-192] appear to conclude that reaction is possible and may initiate the chain reaction in the absence of initiators or initiating agents. If the oxygen molecule has been activated by radiation the reaction is much more probable and the presence of photosensitizers in the substrate will enhance this mode of initiation[185]. Addition of radicals or a radical source, e.g. thermally unstable peroxides, to the substrate will of course initiate reaction (v) directly. In the absence of initiators a substrate may exhibit a relatively long induction period, i.e. no noticable oxidation rate.

Except at low partial pressures of oxygen, reaction (vi) is rapid, that is it has a low activation energy, and therefore only peroxy radicals are generally of importance in subsequent reactions. In the absence of steric factors the rate of reaction (vii) generally depends upon the stability of the radical being formed and the point of oxidative attack is related to the structure of the substrate.

Thermal homolysis of hydroperoxides gives free radicals which lead to chain branching and accounts for the autocatalysis encountered in many oxidations. The overall decomposition is said to be complex[193], sometimes being first order in hydroperoxide as characterised by reaction (viii) and sometimes being second order (ix). Hydroperoxide decomposition is catalysed by metals that can undergo a valency transition[194,195]. The overall effect is a reduction in the apparent activation energy of the uncatalysed

$$ROOH + M^{n+} \longrightarrow RO\cdot + OH^- + M^{(n+1)+} \qquad (xx)$$

$$ROOH + M^{(n+1)+} \rightleftharpoons ROO\cdot + H^+ + M^{n+} \qquad (xxi)$$

bimolecular reaction (ix) but a reversal of reaction (xxi) may in some cases prevent regeneration of the catalyst and result in inhibition by removal of peroxy radicals.

In the absence of inhibitors the chain reaction terminates by mutual reaction of radicals (x). Products are of numerous types dependent on the substrate; aldehydes, ketones, acids and epoxides having been reported[183]. Inhibitors, or antioxidants as they are usually termed where the chain reaction is autoxidative, may be classified into two mechanistically distinct groups[186] whereby one interferes with the initiation processes and the other the propagation reactions.

The chain-breaking inhibition process which interferes with the propagation reactions by removing radicals from the medium was generally considered to involve a hydrogen abstraction reaction (xi)[196]. The radical formed from the inhibitor is stabilised by resonance and therefore insufficiently reactive to start a new chain (xiii) (xiv) particularly where the active site is sterically protected[197] (e.g. 2,6-butylated phenols). The success of reaction (xi) in competition with reaction (vii) is a measure of the efficiency of the inhibitor.

The fate of inhibitor molecules appears to be complex depending to some degree upon the reaction conditions as well as the efficiency and structure of the inhibitor. In some cases reaction with a secondperoxy radical certainly occurs to form an addition product (xv) while in others a further hydrogen radical is abstracted and then rearrangement occurs to form stable products (xvi). Dimeric products have also been isolated (xvii).

A different theory of chain-breaking inhibition which involves the reversible formation of a poroxide radical - inhibitor complex, which may be a transition state or a definite entity (xxii), has been suggested[198]. This mechanism has been confirmed

$$\left.\begin{aligned} IH + ROO\cdot &\rightleftharpoons [ROO - IH] \\ [ROO - IH] + ROO\cdot &\longrightarrow \text{products} \end{aligned}\right\} \quad \text{(xxii)}$$

for only a few weak antoxidants and it is feasible[199] that this complex formation is of importance only when reaction (xi) is relatively slow.

Destruction of peroxides to form non-radical products removes the main source of free radicals from the system and prevents the initiation of fresh chains. Many sulphides and phosphides are effective antioxidants of this type[200] and may be characterised by reaction (xii). Some phenols and amines have been reported as exerting their antioxidant effect by this process[201]. Antioxidants of this type may also be taken to include any compound which removes the source or cause of radical formation[186]; such as ultra-violet stabilisers and metal deactivators.

It is therefore possible that any rethrin instability in pyrethrum extracts is promoted by autoxidation of the unsaturated fat content and possibly catalysed by trace metal contamination.

2.3.2. Long-term trials on the stability of rethrins in refined extracts

At the commencement of this work there was a limited amount of data from controlled studies on concentrated bulk extracts. The early work of Gnadinger et al[202], in an experiment designed to study the stabilising effect of antioxidants, used extracts which they termed 'highly concentrated' but which in fact contained only 15 per cent rethrins. These were neither dewaxed

nor decolourised and the reported losses of 10 to 20 per cent of the total rethrin content over 3 to 4 months are not relevant to the present work. Moore[96] however reported that a single undiluted sample of nitromethane concentrate (see 1.1.2.) was reduced to about 85 per cent of its original rethrin content, as determined by the official method of analysis (see 1.4.1.), after storage for 6 months at 4°C. This appeared to support the work of Campbell and Mitchell[141] who found similar losses in such extracts exposed to sunlight for 3 months while maintained at 30°C. There was therefore a need to study the rethrin stability of refined pyrethrum extracts over long periods of time.

Laboratory prepared samples of Pale Extract and Nitromethane Extract (see 1.1.2.) were chosen for this study. The former, in a concentrated form, was the sample type of primary interest but the stabilising effect, or lack of it, of greater purification could be illustrated by a study of the latter type of extract. To study the stabilising effect of B.H.T. normally present in commercially produced extracts (see 2.3.3.), samples of refined extracts prepared from B.H.T.-free oleoresin were also studied.

Throughout this course of study rethrin analyses were performed by the developed g.l.c. method (see 2.1.) unless otherwise stated. An approximate correlation with A.O.A.C. results may be obtained (see 1.4.1. and 2.1.2.11.) by multiplying the total rethrin concentration by a factor of 1.3 (or the rethrin I content and rethrin II content by 1.1 and 1.5 respectively).

2.3.2.1. Nitromethane Extract

A method based on the procedure of Barthel, Haller and La Forge[16] was used to prepare a Nitromethane Extract from a commercially produced pyrethrum oleoresin containing B.H.T. To illustrate the dilution effect on sample stability portions of the extract were suitably diluted with nitromethane to provide samples of a range of rethrin concentrations. Commercially prepared Pale Extract concentrates are reported to contain approximately 60 per cent rethrins[14], as determined by the A.O.A.C. procedure but samples containing approximately 50 per cent rethrins are of interest for marketing purposes[14]. The most concentrated nitromethane sample was therefore chosen to contain the latter quantity of rethrins.

The samples were stored at ambient temperatures in filled and half-filled, screw-top, glass sample bottles in an attempt to restrict the availability of air to the former and highlight the relative instability of the latter. The results of rethrin analyses on the samples over a period of several months are shown in Table 16.

Analysis of the most concentrated sample (NJ), stored in a half-filled bottle, indicated no significant loss of rethrins over a period of 4 months. Subsequent analysis over 8 months storage however indicated serious loss (25 per cent of the original rethrin content). This sudden rapid deterioration was not confirmed by analysis of a duplicate sample (NK) for which an equivalent 3.7 per cent loss was recorded, hardly significant in terms of experimental error.

Analysis results of a third sample (NG) of the same dilution as samples NJ and NK, were more in accord with those of the latter sample. This sample, stored in a filled sample bottle, exhibited a loss of 11 per cent of its original rethrin concentration over 11 months. This, in conjunction with the results of sample NK, inferred that significant rethrin degradation had only just commenced and after allowance had been made for the additional 2 months storage of sample NG the results were within the limits of experimental error. It appeared therefore that the rapid deterioration of sample NJ was an anomalous result. It was additionally noted that sample NG, stored with no air space above the level of the solution, did not exhibit greater stability than sample NK but this probably inferred that air had not been totally excluded, or that sufficient air was present for the degree of deterioration observed.

Sample NF, a more dilute sample initially containing 28.5 per cent rethrins, showed no significant loss over 9 months.

Both sample NG and sample NF were analysed again after two and a half years storage and found to have incurred losses of 30 per cent and 15 per cent of their initial rethrin concentration respectively. The more concentrated form of the extract did show greater instability therefore over a long period of storage but over the first 9 months there was no detectable difference.

The results of the stability trials with Nitromethane Extract solutions provided incontrovertible evidence of a higher degree of stability of highly refined bulk extracts than

had been inferred by earlier workers.

Although rethrin degradation was pronounced over the full period of the trial there was relatively little over the first 9 to 12 months. This was confirmed by a further study of a sample of a different Nitromethane Extract (NE). Initially containing 34.6 per cent rethrins its rethrin concentration was intermediate between samples NF and NG although its involatile concentration was similar to that of the latter (i.e. contained more extraneous matter due to less efficient refining). This sample exhibited a loss of 18 per cent of its rethrin content over a 22 month period but no significant detectable loss over the first 7 months.

The rapid deterioration of sample NJ may therefore be considered to have not been typical. The observations may be rationalised in view of the fact that the sample had been repeatedly opened to the air in the course of extracting samples for analysis. The volume of this sample had thus been reduced to approximately one quarter of its original size and therefore by comparison with other samples its surface area to volume ratio had been considerably increased. This, and possible contamination by pro-oxidants were considered the probable reason for this rogue result.

2.3.2.2. Pale Extract

A similar study was made of a Pale Extract for comparison with the study of the Nitromethane Extract. A hexane solution of this extract was suitably diluted to provide two samples (PC and PB) of approximately the same dilution, weight per volume, as the Nitromethane Extract samples. The rethrin concentration of these samples was found to be 46.0 and 27.1 per cent respectively. Sample PC was analysed five times over

a period of 2 years. No significant rethrin loss was detected by the analysis after 5 months storage but a consistant rate of deterioration was indicated by subsequent analyses (Table 17). After 2 years the rethrin content had been reduced by 30 per cent of its original value.

The more dilute sample, PB, was only examined at the end of the 2 year period and was found to have lost 19 per cent of its original rethrin content.

A comparison of the rethrin stability in Pale and Nitromethane Extract can be seen in Figure 15. The more dilute forms of both types of extract had greater stability over long periods of storage but there was no significant difference between the stability of these samples and the stability of more concentrated extracts over the first few months. The effect of dilution was however to reduce the overall extract instability. This is a general phenomenon of substrates labile to autoxidation. An induction period of about 6 months was observed with the Pale Extract concentrate sample, comparable with that observed for Nitromethane Extract samples but the rate of degradation, after this induction period, was markedly greater in the former case. The absence of extraneous matter in nitromethane samples therefore conferred a significant improvement on extract stability with respect to the rethrins. Improved stabilisation of commercial extracts could therefore be achieved by a more efficient refining process.

2.3.2.3. Nitromethane Extract containing no added antioxidant

The stabilising effect of the antioxidant B.H.T. is well established (see 1.5.3. and 2.3.3.) but no comparison has been made of its stabilising properties on Nitromethane and Pale Extracts stored in bulk. Solutions of extracts prepared from antioxidant-free pyrethrum oleoresins were therefore used to provide further information on the true stability of the rethrins in refined pyrethrum extracts.

A Nitromethane Extract solution free of B.H.T. (sample AF/NA) which initially contained 53.0 per cent rethrins (g.l.c. analysis) exhibited immediate deterioration (Table 18 and Figure 15) when stored in glass sample bottles at ambient temperatures under comparable conditions to samples containing B.H.T. (see 2.3.2.1.). The rate of rethrin loss appeared to be constant and culminated in the loss of one quarter of the rethrin content over the 18 month period of study. The rate was also comparable with that of sample NG, the Nitromethane Extract containing antioxidant, after the latter's initial 5 to 6 month induction period. This inferred that the presence of B.H.T. in Nitromethane Extract solutions only delays the onset of sample deterioration rather than retards it.

The rethrin content of a more dilute sample (AF/NB) determined at the end of the 18 month period of study was found to have decreased from 21.45 per cent to 18.5 per cent (Table 18).

2.3.2.4. Pale Extract containing no added antioxidant

A Pale Extract solution free of antioxidant (sample AF/PF) of rethrin concentration comparable with both the dilute Pale Extract sample containing B.H.T. (PB) and the antioxidant-free nitromethane sample (AF/NB) was also used in the stability trials.

This sample exhibited the greatest rate of deterioration encountered in these studies (Table 19 and Figure 15). In 10 weeks the rethrin content decreased from 24.5 per cent to 19.8 per cent and although the rate of loss subsequently decreased only 37 per cent of the initial rethrin content remained by the end of the 19 month period of study. The sample was therefore much less stable than either a Pale Extract containing B.H.T. or an antioxidant-free Nitromethane Extract of equivalent initial rethrin concentration.

These studies illustrate the instability of B.H.T.-free Pale Extract even when stored in a relatively dilute form and confirmed the stabilising effect of added antioxidant. Comparison with more highly refined extract samples (Nitromethane Extract) indicated that the greater instability of Pale Extracts is due primarily to the presence of extraneous matter in the latter. The inclusion of B.H.T. however, at present added to milled flowers and pyrethrum oleoresin, appeared to provide adequate stabilisation of Pale Extract samples, even in a concentrated form, for several months (see 2.3.2.2.).

2.3.2.5. Relative stability of the individual rethrins

The use of g.l.c. for the analysis of rethrins in pyrethrum extracts allowed the determination of the individual rethrins and it was therefore possible to study their respective stabilities during the stability trials. Resolution of the cinerin and jasmolin components was often found to be poor on old columns (see 2.1.2.8.) but it was considered adequate to group these components together rather than estimate their respective concentrations since a greater difference in stability may be expected to occur between the pyrethrins and the cinerin-plus-

Table 16 Rethrin loss from Nitromethane Extract samples

Sample	% involatiles	Storage Period	% PI	% CJI	% RI	% PII	%CJII	% RII	% R	PI/CJI PII/CJII	RI/RII	% loss
NJ	63.8 w/w (68.5 w/v)	Nil	18.5	7.9	26.4	10.1	6.5	16.6	43.0	1.935	1.59	-
		6 weeks	18.45	8.3	26.75	10.45	6.5	16.95	43.7	1.955	1.58	(-1.6)
		4 months	18.2	7.9	26.1	9.45	6.15	15.6	41.7	1.97	1.67	3.0
		8 months	14.5	6.4	20.9	6.3	5.0	11.3	32.2	1.82	1.85	25
NK	"	Nil	18.5	7.9	26.4	10.1	6.5	16.6	43.0	1.935	1.59	-
		9 months	18.1	7.4	25.5	9.6	6.3	15.9	41.4	2.02	1.60	3.7
NG	"	Nil	18.5	7.9	26.4	10.1	6.5	16.6	43.0	1.935	1.59	-
		11 months	15.9	7.8	23.7	9.1	5.5	14.6	38.3	1.88	1.62	11
		30 months	11.95	5.55	17.5	8.1	4.35	12.45	29.95	2.025	1.40	30
NF	42.3 w/w (45.5 w/v)	Nil	12.2	5.3	17.5	6.7	4.3	11.0	28.5	1.935	1.59	-
		9 months	12.4	5.2	17.6	5.9	4.0	9.9	27.5	1.935	1.77	3.5
		30 months	10.2	4.7	14.9	5.45	3.85	9.3	24.2	1.83	1.60	15
NE	62.8 w/w (67.5 w/v)	Nil	15.9	6.1	22.0	7.4	5.2	12.6	34.6	2.06	1.74	-
		7 months	14.55	6.45	21.0	7.4	5.4	12.8	33.8	1.85	1.64	(2.3)
		22 months	11.95	4.85	16.8	6.5	5.05	11.55	28.35	1.86	1.455	18

P = pyrethrin, CJ = cinerin + jasmolin, R = rethrins

Table 17 Rethrin loss from Pale Extract samples

Sample	% involatiles	Storage Period	% PI	%CJI	% RI	%PII	%CJII	% RII	% R	P/CJ	RI/RII	% loss
PC	76.3 w/w (68.8 w/v)	Nil	21.0	8.8	29.8	9.7	6.5	16.2	46.0	2.00	1.84	-
		5months	20.6	9.0	29.6	9.5	6.3	15.8	45.4	1.97	1.88	(1.3)
		9months	19.45	9.05	28.9	8.0	5.6	13.6	42.1	1.875	2.10	8.5
		17months	16.4	7.2	23.6	8.3	5.1	13.4	37.0	2.00	1.75	20
		24months	14.3	6.4	20.7	6.7	4.45	11.15	31.85	1.935	1.85	31
PB	44.9 w/w (33.8 w/v)	Nil	12.4	5.15	17.55	5.7	3.85	9.55	27.1	2.00	1.84	-
		24months	10.6	4.0	14.6	4.45	2.95	7.4	22.0	2.16	1.98	19

p = pyrethrin, CJ = cinerin + jasmolin, R = rethrins

Table 18 Rethrin loss from B.H.T.-free Nitromethane Extract samples

Sample	% involatiles	Storage period	% PI	% CJI	% RI	%PII	%CJII	% RII	% R	P/CJ	RI/RII	% loss
AF/NA	79.0 w/w	Nil	26.4	9.9	36.3	10.1	6.6	16.7	53.0	2.205	2.17	-
	(84.5 w/v)	3½months	24.05	10.25	34.3	9.5	6.05	16.35	50.65	1.96	2.10	4.4
		11months	18.9	9.9	28.8	9.05	6.45	15.5	44.3	1.71	1.86	16
		18months	16.8	8.9	25.7	8.55	6.05	14.6	40.3	1.695	1.76	24
AF/NB	32.0 w/w	Nil	10.5	4.2	14.7	4.1	2.65	6.75	21.45	2.205	2.17	-
	(34.2 w/v)	18months	8.7	3.6	12.3	3.7	2.5	6.2	18.5	2.03	1.98	14

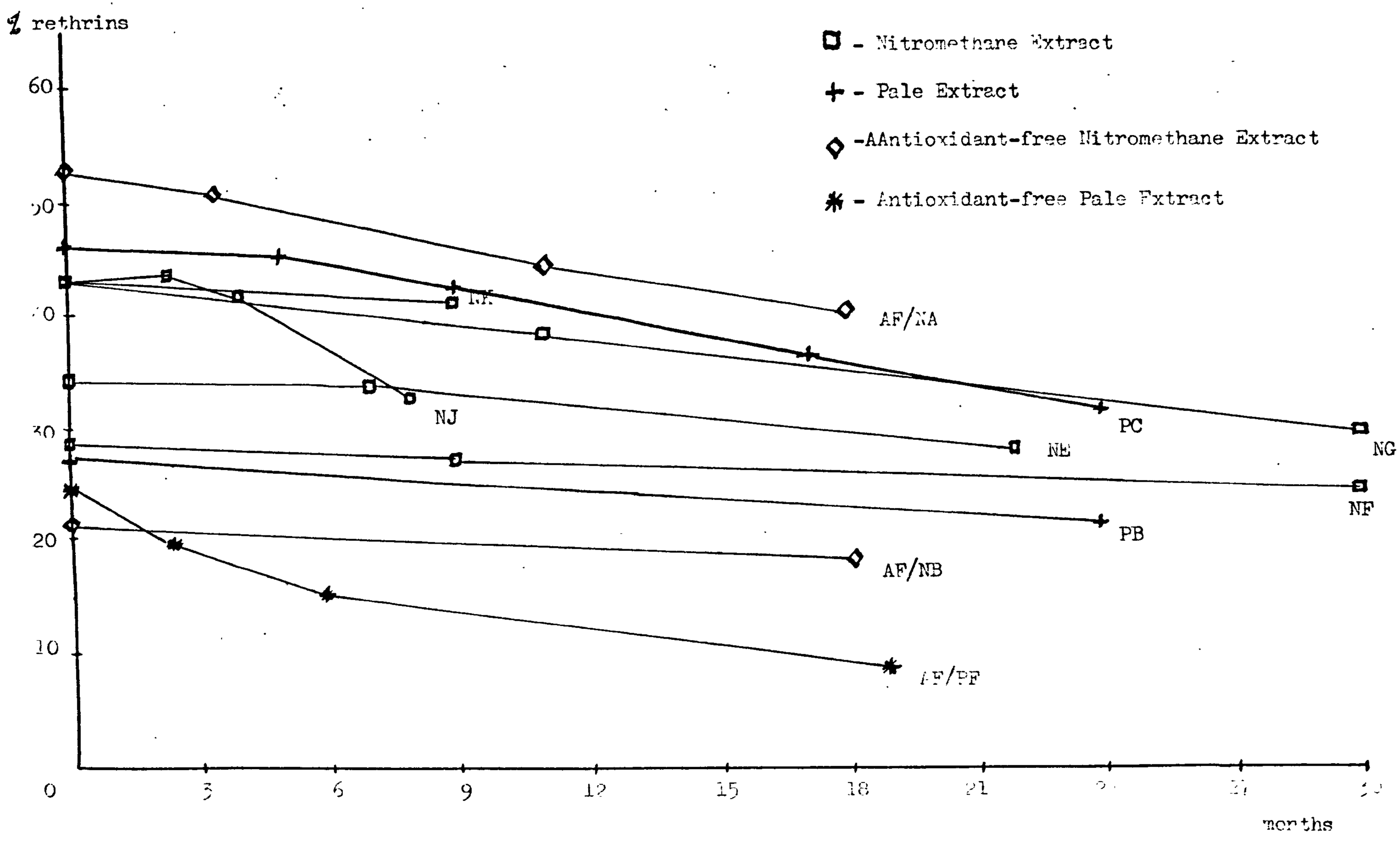
p = pyrethrin, CJ = cinerin + jasmolin, R = rethrins

Table 19 Rethrin loss from B.H.T.-free Pale Extract sample

Sample	% involatiles	Storage Period	%PI	%CJI	%RI	%PII	%CJII	%RII	%R	P/CJ	RI/RII	% loss
AF/PF	52.7 w/w	Nil	11.35	3.95	15.3	6.1	3.1	9.2	24.5	2.475	1.67	-
	(47.2 w/v)	10 weeks	9.0	3.9	12.9	4.35	2.55	6.9	19.8	2.07	1.87	19
		6 months	7.2	3.2	10.4	3.3	2.2	5.5	15.9	1.945	1.89	35
		19 months	3.05	2.8	5.85	1.5	1.7	3.2	9.05	1.01	1.85	63

p = pyrethrin; CJ = cinerin + jasmolin; R = rethrins

Figure 15 Rethrin loss from refined pyr thrum extracts



jasmolin components (see 1.5.2.).

In the non-stabilised extract samples the trend of the pyrethrin to cinerin-plus-jasmolin ratio indicated preferential degradation of pyrethrins (see Table 18 and 19). The rethrin I to rethrin II ratio in the non-stabilised Nitromethane Extract sample (AF/NA) indicated greater instability of the rethrins I. The pyrethrin I concentration of the involatiles of sample AF/NA and sample AF/PF fell from 49.8 to 41.6 per cent and 46.4 to 33.7 per cent respectively.

The only notable exception to the trend indicated by the non-stabilised samples was given by sample NJ where serious loss of pyrethrin II resulted in greater loss of rethrins II (see Table 16). Since the rapid deterioration of this latter sample was considered to be anomalous (see 2.3.2.1.) the instability of pyrethrin II in this sample may not be comparable with data from other samples.

In most of the other trials the rate of loss of individual rethrins was insufficient for definite conclusions to be made about their relative stabilities. This particularly applied where the total rethrin degradation was slight.

2.3.2.6. Chromatographic observation of a probable rethrin degradation product

The gas-liquid chromatograms on 1 per cent N.P.G.S. packed columns of stored extracts generally contained only the characteristic peaks found in those of the originally prepared samples. The chromatograms of some of the stored samples however indicated the presence of a new component which was found to elute between pyrethrin I and cinerin II. Its retention time relative to cinerin I was determined as 4.6.

A component with a similar relative retention time (4.55) has been reported by Head et al during the analysis of degraded thin films and claimed to be an artefact of pyrethrin I[145]. Similar compounds were found to be formed from cinerin I, jasmolin I and allethrin I (allethronyl chrysanthemate) but no corresponding rethrin II homologues could be found. No absolute identification of these compounds was made but based on their relative retention times they were postulated to be formed by modifications in the acid moiety of the chrysanthemates and therefore were designated rethrins IA.

The new component found in the present work was therefore tentatively assumed to be the component designated by Head as pyrethrin IA[145]. Its concentration in degraded samples was only small and the peak corresponding to this component was only distinguishable from the baseline under gas-chromatographic conditions equivalent to those of rethrin II analysis. Under such conditions some thermal degradation of the pyrethrins occurs on the column (see 2.1.2.6.) and therefore tailing of the 'pyrethrin I' peak often made it difficult to ascertain the position of the baseline in this region of the chromatogram.

Sample NJ however, after 8 months storage, was found to contain pyrethrin IA determined as approximately 13 per cent of the cinerin II-plus-jasmolin II fraction, assuming a relative response factor of unity. Sample NK, the duplicate of sample NJ, was found to contain approximately 7 per cent of this component after a similar period of storage but neither the chromatogram of sample NG nor sample NF were found to indicate its presence.

Table 20 Relative proportion of a decomposition product (designated pyrethrin IA) noted in some chromatograms of degraded extracts

Samples	NJ	NK	AF/PF	AF/PF
Storage Period	8 months	9 months	2½ months	6 months
Initial rethrin concentration	43.0%	43.0%	24.5%	24.5%
Rethrin loss	10.8%	2.6%	4.7%	8.6%
% rethrin loss	25	37	19	35
Pyrethrin I loss	4.0% ± 0.7	0.4% ± 0.7	2.35% ± 0.4	4.15% ± 0.4
'Pyrethrin IA' as % of CJ II	13 ± 3	7 ± 3	5 ± 3	10 ± 3
% pyrethrin IA in sample based on concentration of CJ II	0.65 ± 0.2	0.44 ± 0.2	0.13 ± 0.1	0.22 ± 0.1

CJ II = cinerin II - jasmolin II fraction

The only other sample to yield this component was sample AF/PF. The relatively rapid decomposition of the rethrins in this sample was accompanied by its formation in amounts of approximately 5 and 10 per cent (relative to cinerin II-plus-jasmolin II fraction) over a 10 weeks and 6 month storage period respectively.

Calculated as a percentage of the sample, the proportion of pyrethrin IA was very small (see Table 20) and, except for sample NK, accounted for only a minor part of the pyrethrin I loss.

By comparison with the work of Head et al[145] the formation of pyrethrin IA in degraded extracts may also be expected to be accompanied by the formation of cinerin IA and jasmolin IA. Such compounds have been reported to have retention times relative to cinerin I of 2.50 and 3.33 respectively[145]. They would therefore elute within the tail of pyrethrin I and be unnoticed in chromatograms of extracts containing this component. Although this would increase the apparent proportion of pyrethrin I it would be insignificant in view of the proportion of cinerin I and jasmolin I relative to pyrethrin I.

Analysis after a long period of storage did not indicate the formation of pyrethrin IA in other samples or an increase in its relative proportion despite observed pyrethrin I loss. It was, in fact, not noted in later chromatograms.

It was therefore concluded that pyrethrin IA was formed in significant amounts only under specific conditions of degradation. Its presence in samples undergoing rapid deterioration would indicate that it is the product of a

rapid rethrin degradation process. Further, since it was found only in extracts stored for a comparatively short time it is probable that over a long period of storage a slower rate of rethrin degradation is predominant and degradation of the primary product itself is at a rate sufficient to prevent its concentration in the sample.

2.3.3. Changes in butylated hydroxytoluene (B.H.T.) content during long term stability studies

2.3.3.1. Theory of the stabilising action of B.H.T.

B.H.T. (XV, 1.5.3.) has been found to be one of the most effective stabilisers of pyrethrum powders[151] and approximately 0.5 per cent is added to the flowers during milling. For the stabilisation of extracts a further quantity is added but the final level of B.H.T. concentration is apparently not controlled andthe amount present in pyrethrum oleoresin may vary considerably[203] and the level in refined extracts has not been reported. In practice however adequate stabilisation of extracts containing 20 to 25 per cent rethrins is achieved for several months.

Stabilisation of the pyrethrum extracts is almost certainly due to the antioxidant properties of B.H.T. The role played by this compound in the inhibition of autoxidative processes has been extensively studied (for reviews see references 183, 184, 186-188) and the mechanism of its antioxidant action has been shown to be a chain-breaking inhibition process (see 2.3.1.) (Figure 16). In its initial reaction with a peroxy radical a hydrogen radical is transferred thus temporarily retarding the chain process but the

B.H.T.-radical product (XVIII) stabilises itself by reaction with a second peroxy radical to form a diperoxide (XIX) which is a fairly stable entity at temperatures not greatly in excess of room temperature. Other possible stabilisation products, e.g. dimers, are reported not to be formed under normal conditions of autoxidation[204].

The B.H.T. concentration therefore falls during its inhibition of autoxidation and it would appear possible to follow the early stages of pyrethrum extract deterioration by the decline in B.H.T. content.

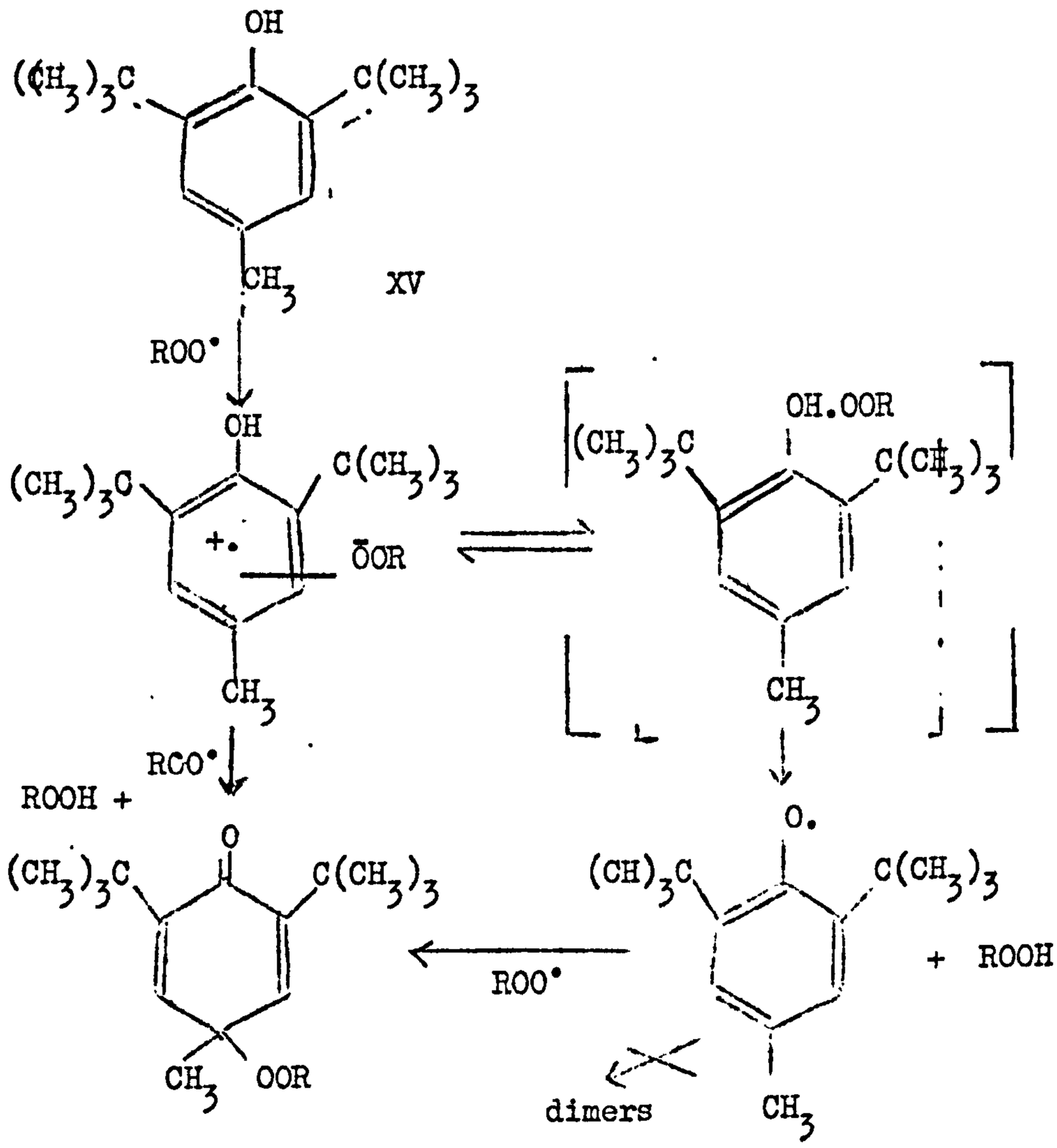


Figure 16 Reaction scheme for the antioxidant action of B.H.T.

2.3.3.2. Analysis of B.H.T. in refined extracts

General methods of B.H.T. analysis have been developed based on colorimetric determinations the most favoured of which is one based on the method of Anglin et al[205]. This estimates the B.H.T. content by its reaction with ferric chloride-2,2'-dipyridyl reagent but the reaction is slow[205] in non-aqueous media and other reducing compounds interfere with the method, e.g. the naturally occurring antioxidants, tocopherols (see 2.3.4.1.). Furthermore extraction of B.H.T. is usually necessary primarily to concentrate the component but also because the analysis generally cannot be carried out in the anhydrous fat solution. Solvent extraction techniques have generally been found to be unsatisfactory[205,206] since B.H.T. is more soluble in fat and fat solvents than in extracting solvents although acetonitrile is reported to have been used successfully[207]. B.H.T. is therefore generally extracted from the fat by steam distillation[205,206]. Separation by adsorption chromatography, both column[205,208] and thin-layer[207,209,210] has had varying success. In recent years, g.l.c. analysis of antioxidants has been widely investigated and direct B.H.T. analysis has been achieved by this method[211-213] although prior partial separation is sometimes still preferred[214,215].

Samples of refined pyrethrum extract were therefore examined by g.l.c. on both polar (PEGA) and non-polar (Apiezon L) columns to investigate the potential of direct g.l.c. analysis for B.H.T. The chromatograms of Nitromethane Extract and of Pale Extract (Figures 17 and 18) showed the presence of a component which eluted with a similar retention time on both stationary phases to that of B.H.T. Comparison with B.H.T.-free extracts confirmed the assignment of B.H.T. to this component.

Figure 17 Gas-liquid chromatogram from B.H.T. analysis of refined pyrethrum extracts on Apiezon L stationary phase

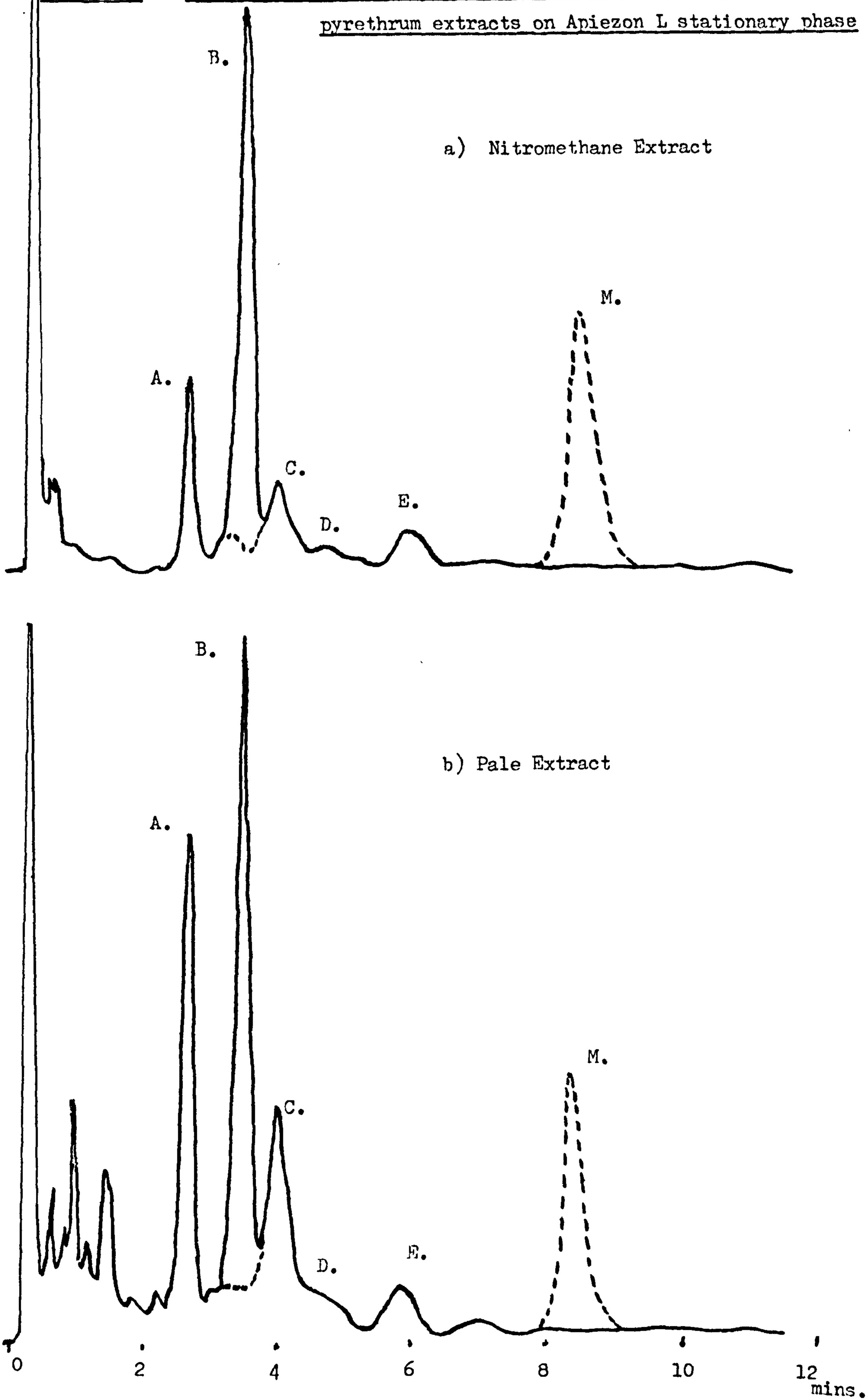
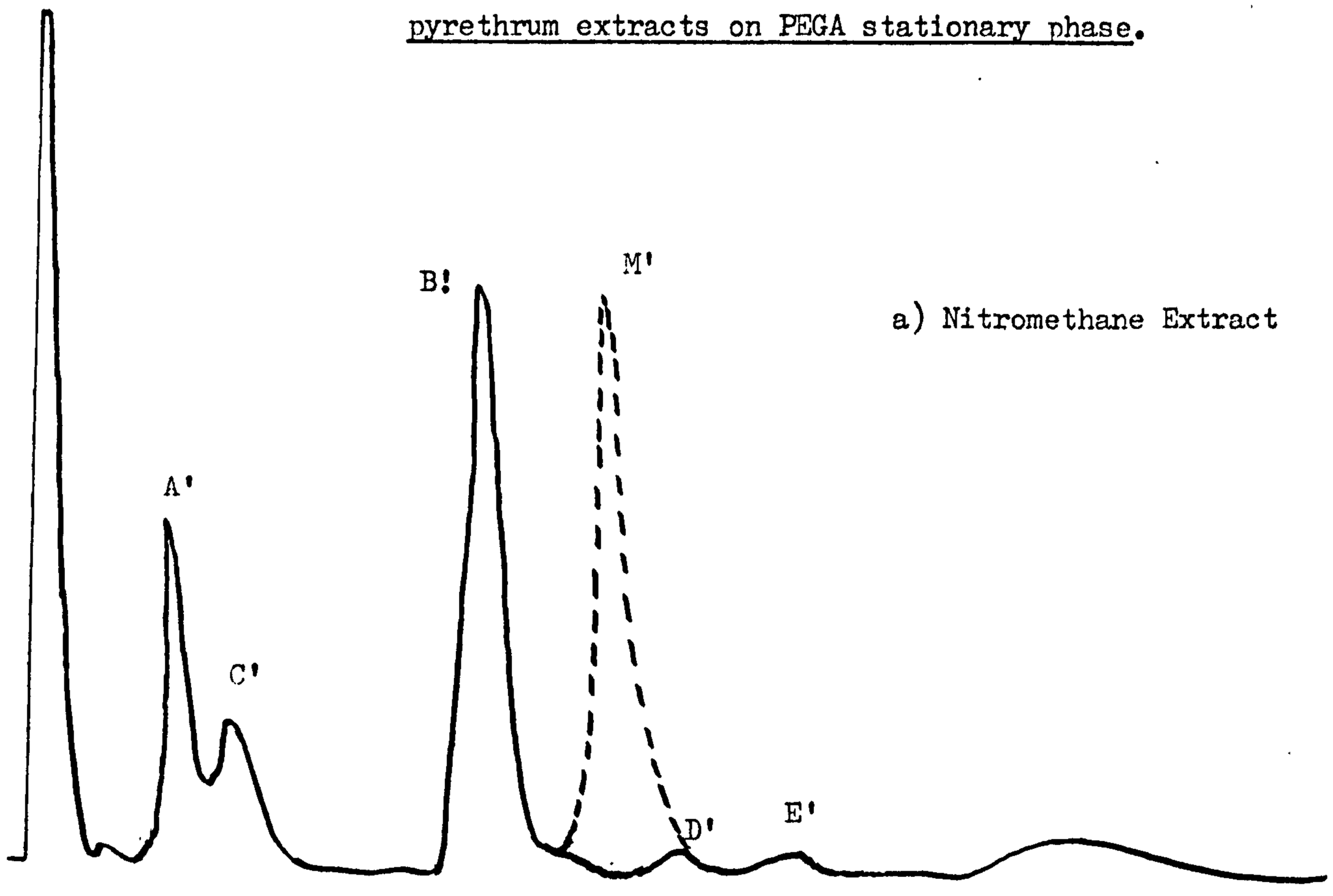
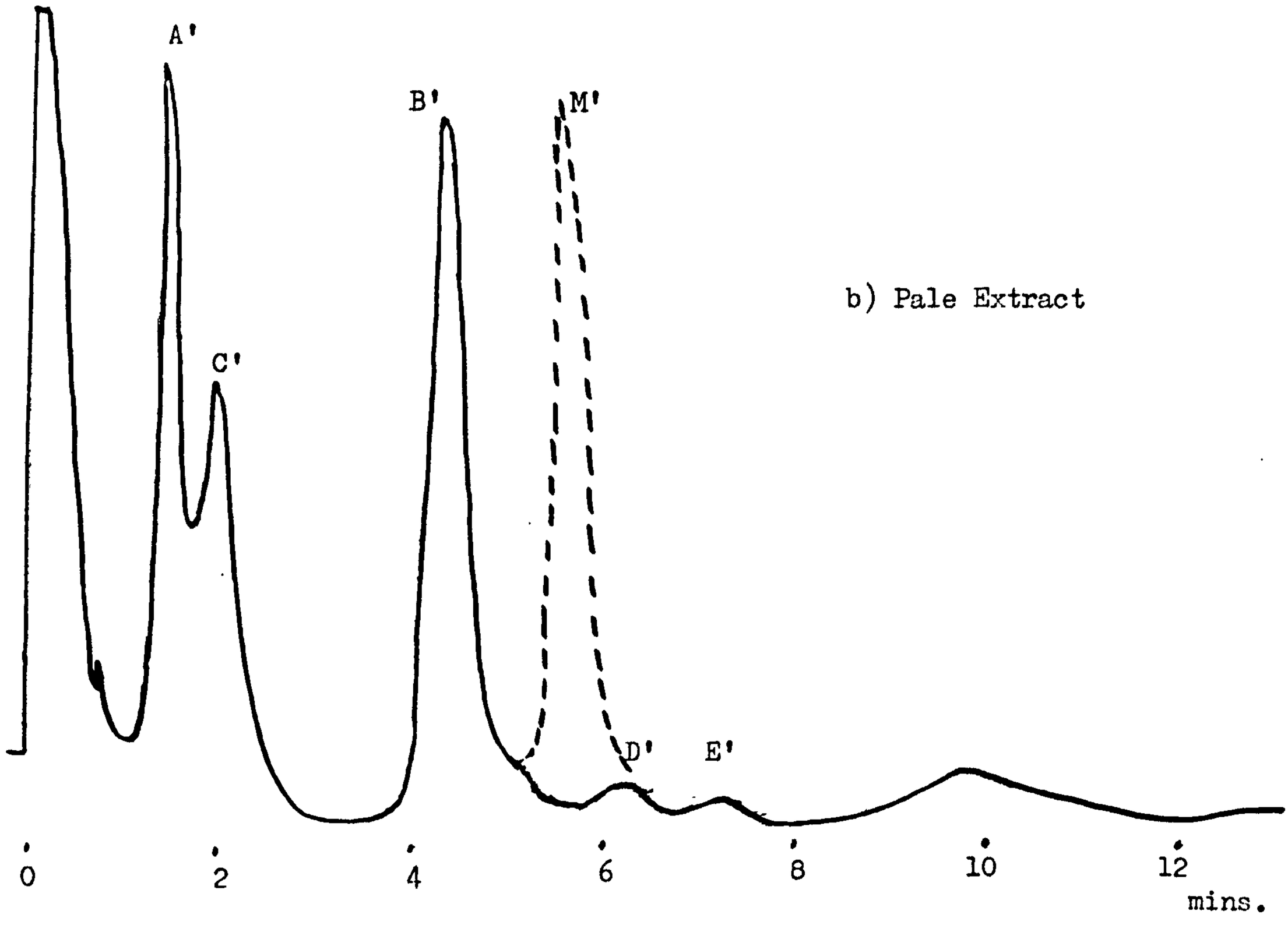


Figure 18 Gas-liqid chromatogram from B.H.T. analysis of refined pyrethrum extracts on PEGA stationary phase.



a) Nitromethane Extract



b) Pale Extract

The equivalent chromatograms of Nitromethane Extract and Pale Extract did not exhibit any great differences. On Apiezon L Pale Extract was shown to contain some relatively volatile material which was absent from the Nitromethane Extract and five components were noted as being characteristic of both extracts in this region of the chromatograms (Figure 17; A - E). Similarly five characteristic components were noted in the chromatograms from PEGA (Figure 18; A' - E'). The equivalent chain lengths of these components with respect to the methyl esters of fatty acids are shown in Table 21. The relative proportion of these components however was slightly different in each extract. By comparison with the B.H.T. component (B, B') the other components were more predominant in Pale Extract compared to Nitromethane Extract which reflects their relative ease of extraction by hydrocarbon solvents compared to nitromethane. In particular component C was found to be present in varying amounts in different Pale Extracts.

For quantitative analysis of the B.H.T. content in these extracts by g.l.c. on both stationary phases methyl myristate (M, M') was chosen as internal standard because of its solubility in nitromethane (c.f. alkanes) and it was noted during the determination of equivalent chain lengths that no component present in theextracts eluted in the region of methyl myristate elution on Apiezon L (E.C.L. = 14.0) and only a relatively minor component (D') would produce any interference with the area assessment on PEGA chromatograms (E.C.L. = 14.0) for which a small correction could be made.

Table 21 Equivalent chain lengths* of major components in chromatograms for B.H.T. analysis (see Figures 17 and 18)

10% Apiezon L		12.5% PEGA	
Component	E.C.L.	Component	E.C.L.
A	11.4	A'	10.5
B (B.H.T.)	12.1	B' (B.H.T.)	13.3
C	12.3	C'	11.45
D	12.6	D'	14.2
E	13.0	E'	15.35

* with respect to methyl esters of fatty acids

M,M' = methyl myristate (E.C.L. = 14.0)

The B.H.T. - methyl myristate relative response factor was determined over a range of B.H.T. concentrations and of chromatographic conditions and found to be constant within the limits of experimental error associated with chromatographic analyses (~ 2 per cent) but a slight variation was found between different chromatographic detectors (see 3.6.4.)

Apiezon L columns were generally used for B.H.T. analyses as a lower limit of detection, which is determined by interference by other components, could be achieved but results were often confirmed by analysis on PEGA stationary phase (see Table 23). The limit of detection was estimated to be in the region of 0.08 per cent and 0.2 per cent of the solvent-free extracts in Apiezon L and PEGA stationary phase respectively.

Table 22 B.H.T. analysis of refined extracts

Sample	% of solvent-free extract
Nitromethane Extract 1	4.20
Nitromethane Extract 2	3.01
Pale Extract 1	6.13
Pale Extract 2	5.07
World Standard Extract	4.5 - 5*

* the World Standard Extract contained 1.58% B.H.T.

Expressed as a percentage of the solvent-free extract, Pale Extract generally contained a greater proportion of B.H.T. The results of analyses on two Nitromethane Extracts and two Pale Extracts are shown in Table 22. A sample of the World Standard Extract[175], a blend of Pale Extracts, was found to contain 1.58 per cent B.H.T.; approximately 4.5 to 5 per cent in the solvent-free sample. The lower concentration in Nitromethane Extracts reflects the lower solubility of B.H.T. in this solvent compared to hydrocarbon solvents as reported by earlier workers[205,206].

2.3.3.3. Correlation of B.H.T. and rethrin loss from refined extracts

The B.H.T. content of the refined pyrethrum extract samples undergoing long term stability trials was determined at various stages of extract deterioration. The results of this investigation on the Nitromethane Extract concentrate samples are shown in Table 23 and on the Pale Extract concentrate sample in Table 24. The results are correlated with rethrin analyses (see 2.3.2.) in Figure 19.

Table 23 B.H.T. loss from Nitromethane Extract concentrate sample

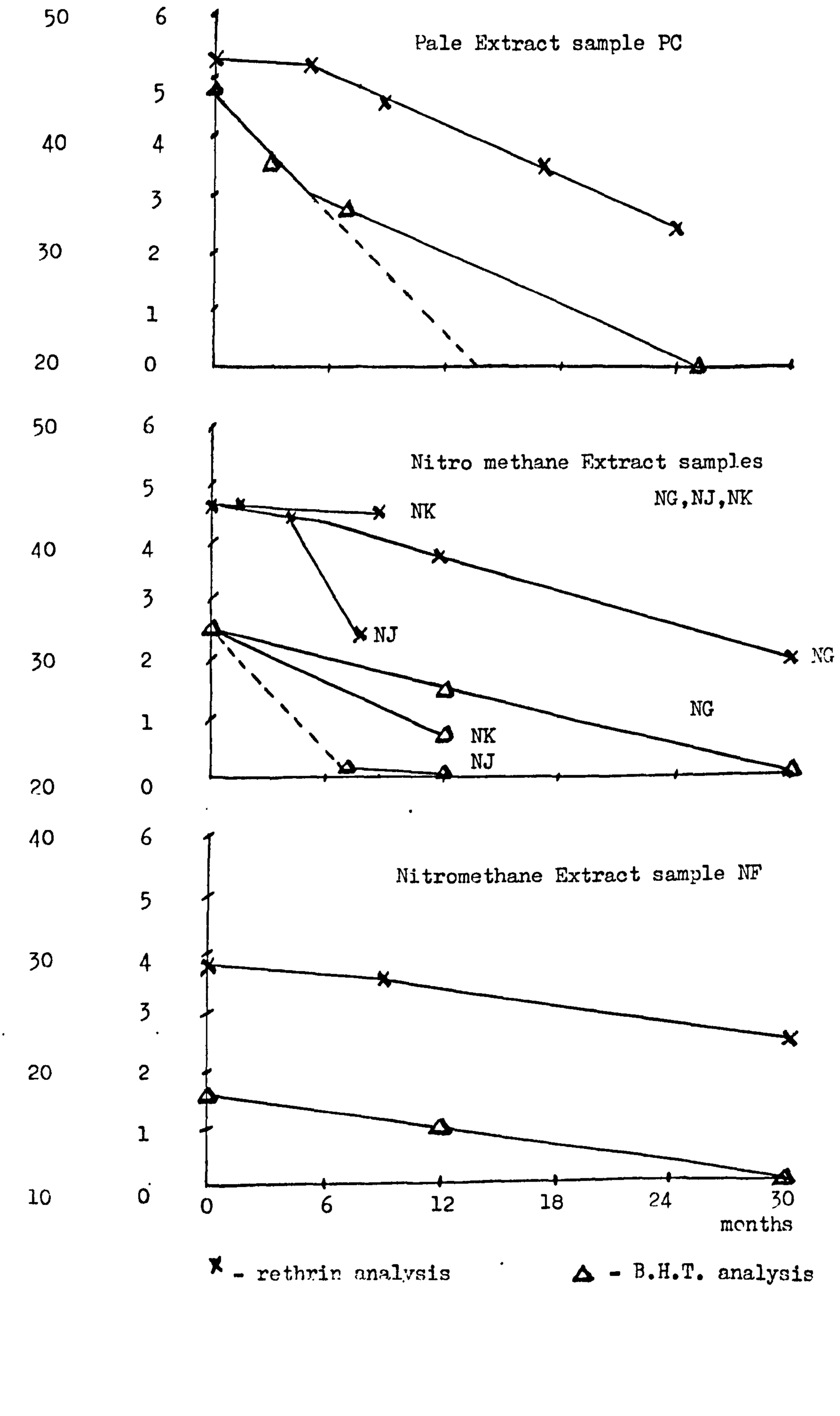
Sample	% involatiles	Storage Period	% B.H.T. in sample		i. Equivalent B.H.T. in involatiles	
			on Apiezon L	on PEGA	on Apiezon L	on PEGA
NJ	63.0 w/w (60.5 w/v)	Nil*	2.60	2.37	4.20	3.71
		7 months	0.15	-	0.23	-
		12 months	~~>~~ 0.057	~~>~~ 0.15	~~>~~ 0.077	~~>~~ 0.19
NK	"	Nil*	2.60	2.37	4.20	3.71
		12 months	0.70	0.65	1.10	1.02
NG	"	Nil*	2.60	2.37	4.20	3.71
		12 months	1.61	1.40	2.54	2.20
		30 months	~~>~~ 0.054	-	~~>~~ 0.085	-
NF	42.3 w/w (45.5 w/v)	Nil*	1.77	1.57	4.20	3.71
		12 months	0.97	-	2.29	-
		30 months	0.055	-	0.13	-

* determined from analysis of a very dilute sample stored at -5°C for 3 months

Table 24 B.H.T. loss from Pale Extract concentrate sample

Sample	% involatiles	Storage Period	% B.H.T. in sample	(Apiezon L) in involatiles
PC	76.3 w/w	Nil	4.68	6.13
	(68.8 w/v)	3 months	3.52	4.61
		7 months	2.62	3.50
		25 months	≯ 0.061	≯ 0.080

Figure 19 Correlation of B.H.T. and rethrin loss from refined pyrethrum extracts.



The initial B.H.T. content of the Nitromethane Extract sample was not determined at the start of the stability trials because the method of analysis had not been developed but was determined 3 months later by analysis of a very dilute sample of the same extract which had been stored at -5°C. It was therefore assumed that B.H.T. degradation in this sample would be negligible and would contain the equivalent concentration of B.H.T. of the more concentrated samples at the start of the trials.

The Pale Extract sample exhibited a greater rate of loss of B.H.T. than the Nitromethane Extract samples NG and NK, complementary to a faster rate of rethrin loss (after the induction period associated with the latter degradation). This is further support for the view that non-rethrin material, which is in greater concentration in Pale Extract, leads to greater extract instability.

The initial lower rate of B.H.T. loss in sample NG compared to sample NK, which were similar samples prepared from the same extract, may possibly be explained as being due to the absence of sufficient air available to the former since this sample was stored in a full container. The slight difference in rate of rethrin degradation was considered to be insignificant (see 2.3.2.1.) and the observed rethrin loss in sample NG may have been to some extent due to a different degradation process (e.g. photodecomposition).

Sample NJ, the nitromethane sample which exhibited an anomalous rapid rate of rethrin loss (see 2.3.2.1.) also showed virtual complete loss of B.H.T. after 7 months but this may have occurred in the later stages of storage and probably part of the sudden change in stability.

No initial period of B.H.T. stability was observed in any of the samples and the end of the induction period in respect of rethrin degradation did not coincide with the complete destruction of the B.H.T. This is in accordance with the concept of the antioxidant being gradually destroyed while inhibiting the autoxidation of the substrate (see 2.3.1.). However, by virtue of its mode of action (see Figure 16) a fall in the concentration of B.H.T. necessarily means that peroxide radicals are concurrently being formed by oxidation of the substrate. The antioxidant only inhibits the formation of hydroperoxides (2.3.1; equation (vii)) and thus the chain-branching reactions (equation (viii) or (ix)). Complete inhibition however is unlikely (that is, efficiency of antioxidant < 100 per cent) and therefore the induction period of rethrin degradation may be taken to correspond to a period of free radical concentration. This would lead to the eventual degradation of the rethrins. This would explain why rethrin losses were observed while antioxidant was still in the system.

It is probable however that the rate of increase of degradation rate remains low while B.H.T. is present and when the B.H.T. has been completely removed competition of degradation products as labile substrate, enhanced as the concentration of rethrins falls, would prevent a continued increase in the rate of rethrin loss and could, in fact, lead to a decrease (see degradation of B.H.T.-free Pale Extract, 2.3.2.4.).

2.3.4. Examination of some natural antioxidant and pro-oxidant compounds likely to affect pyrethrum extract stability

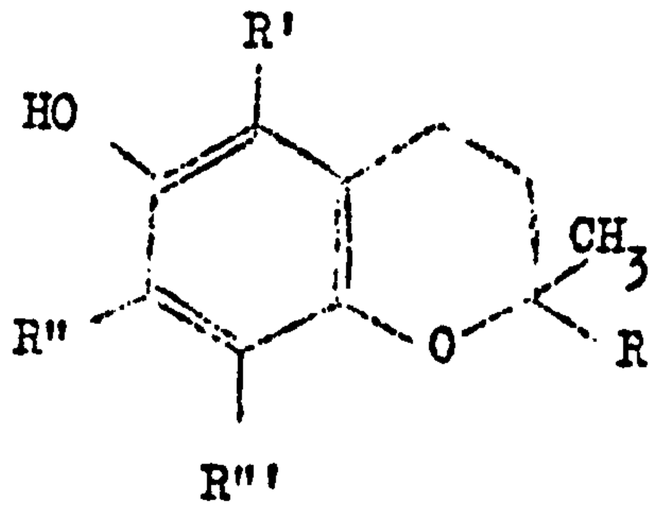
2.3.4.1. Tocopherols

The early work of Olcott and Mattill[216] showed that vegetable oils contained appreciable proportions of naturally occurring antioxidants which have since been shown to be tocol[217] derivatives (Figure 20). Four tocopherols and four tocotrienols are reported to exist in nature[218]; previously reported tocopherol isomers[219,220] having had their structures reassigned[218] (see Figure 20). α - and γ-tocopherols appear to be the most abundant in nature[220,221] and α-tocopherol is an important vitamin (Vitamin E). The tocopherols occur to only a minor extent however in animal fats (1 - 50 ppm) by comparison with vegetable oils (30 - 1000 ppm)[220,221] since animals, unlike plants, are unable to synthesise these compounds but they may be accumulated in the fat by ingestion.

The stabilisation of lipids by the tocopherols has been demonstrated by comparison of natural oils with those reconstituted from purified components[222] and by the marked improvement of the stability of animal fats containing added tocopherols or incorporated with 5 to 10 per cent vegetable oils[223]. There appears however to be a limiting concentration of tocopherols (about 500 ppm) above which no further increase in stabilising effect is observed and may in fact be detrimental[224-226]. This accounts for the relatively minor effect of addition of tocopherols to many vegetable oils[227].

The mechanism of their antioxidant activity appears to be typically phenolic[186,220,227]. They inhibit the chain mechanism by hydrogen transfer to the peroxy radicals (2.3.1. equation (xi)) and are steadily destroyed during the induction

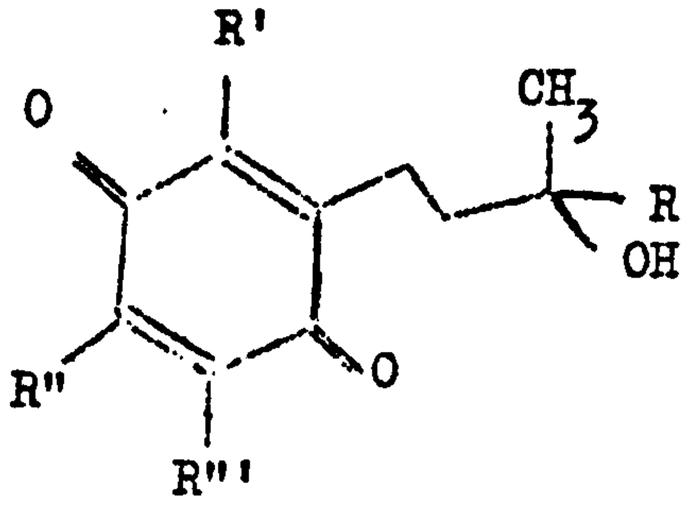
Figure 20 Structural relationship of the tocopherols[218]



R'	R''	R'''		
			$(CH_2-CH_2-CH_2-\underset{CH_3}{CH})_3CH_3$	R $(CH_2-CH_2-CH=\underset{CH_3}{C})_3CH_3$
Me	Me	Me	∝ -tocopherol	∝ -tocotrienol (ξ -tocopherol)
Me	H	Me	β -tocopherol	β -tocotrienol (ε -tocopherol)
H	Me	Me	γ -tocopherol	γ -tocotrienol (η -tocopherol)
H	H	Me	δ -tocopherol	δ -tocotrienol
Me	Me	H	ξ -tocopherol	wrongly assigned structures
Me	H	H	ε -tocopherol	wrongly assigned structures
H	Me	H	η -tocopherol	wrongly assigned structures
H	H	H	tocol[217]	
			2-methyl-2-(4',8',12'-trimethyl -tridecyl)-6-hydroxychromane	

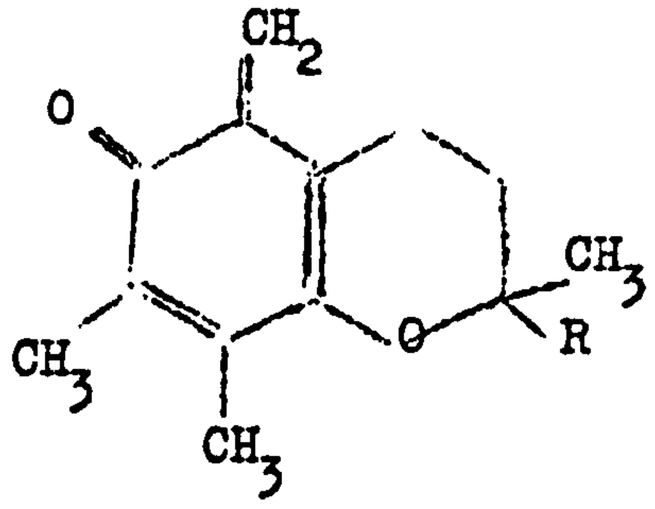
period, the end of which coincides with its complete destruction[228].

With reference to work on ∝-tocopherol the major product appears to be tocopheryl quinone (XX) which is reported to be formed, in the case of ∝-tocopherol, via a methine intermediate (XXI)[229].



tocopheryl quinone

XX



methine intermediate

XXI

Other quinone products and dimers have also been reported to be produced[230,231] often, under certain reaction conditions, in high proportions[232]. Radical addition products (2.3.1.; equation (xv)) do not appear to be formed[233].

Pyrethrum extracts, being of vegetable origin, may be expected to contain appreciable amounts of tocopherols but it is possible that some may be removed during the refining processes. Their relative tocopherol content may therefore have an important bearing on their stability.

The method of tocopherol analysis mostly used is that proposed by Emmerie and Engel[234] in which the sample is treated with a mixture of ferric chloride and 2,2'-dipyridyl in ethanolic solution. Tocopherols reduce the iron to the ferrous state;

this then combines with the dipyridyl to form a red ferrous-dipyridyl complex (Fe dipy.$_3^{2+}$) which is measured spectrophotometrically at about 520 nm. Under standardised conditions the method is claimed to give a high degree of accuracy and precision with pure tocopherols. Any reducing compounds will also reduce the iron thus giving rise to complex formation and Emmerie and Engel have reported that carotene produces interference[234]. Extraction of the tocopherols or the removal of interfering substances is therefore often necessary before estimation of the tocopherol content.

Recently however there have been several reports in the literature of g.l.c. being used in the detection of tocopherols[235-237]. They have been confined almost entirely however to the analysis of pharmaceutical products. Applied to food substrates the extraction and partial purification were still necessary[238].

In the present study a hexane solution of commercially prepared refined pyrethrum extract was qualitatively examined by g.l.c. using the chromatographic conditions described in the literature. By comparison with the chromatogram of a solution of pure α-tocopherol similarly chromatographed it was apparent that the response of any tocopherol present in concentrations of less than 0.1 per cent would not be detected due to the presence of other components eluting with similar retention times. A direct g.l.c. method of tocopherol analysis was therefore considered inapplicable to the present problem without considerable development. Prior purification appeared inevitable.

The use of adsorption chromatography as a purification procedure has been reported[239] and in the present investigation an attempt was made to separate the tocopherol fraction from the greater proportion of other material in pyrethrum extract using a column packed with silica. The resolution achieved however was inadequate. By comparison with pure ∝-tocopherol samples the eluate portion presumed to contain the tocopherol fraction was coloured, probably with carotenoids which would lead to interference in the Emmerie-Engel method of estimation, and g.l.c. examination indicated the presence of several components of similar retention time equivalent to that of ∝-tocopherol. Further, t.l.c. examination showed that a discrete band was not being eluted from the silica column.

A saponification procedure was therefore chosen as an effective way of removing much of the interfering material and also serving to concentrate the tocopherol fraction[239]. Many methods of saponification described in the literature do not give satisfactory recovery of the tocopherols but provided suitable precautions are taken[240] nearly quantitative recovery can be achieved.

A paper chromatographic method for the final separation of the tocopherols from the unsaponifiable matter and partial separation of the individual tocopherols[239] may be considered to have been superceded by the t.l.c. procedure proposed by Whittle and Pennock[241]; both use two-dimensional chromatography. A one-dimensional t.l.c. procedure which achieves separation of the tocopherols into two zones consisting chiefly of ∝- and ɣ-tocopherol has been described by Shone[242].

Table 25 T.l.c. of unsaponifiables of pyrethrum oleoresin

	Rf	Visible	Response to short UV	Emmerie-Engel reagent
Extract unsaponifiables	0.63	Yellow	-	-
	0.57	-	strong	faint
	0.44	-	medium	-
	0.41	-	strong	v. strong
	0.38	-	strong	v. strong
	0.35	-	medium	-
	0.23	-	medium	-
	0.20	-	medium	-
	0.17	-	medium	-
	0.13	Yellow	-	-
	0.09	-	medium	faint
	0.05	-	medium	medium
	0.01	Br. Orange	-	-
α-tocopherol solution	0.42	-	strong	v. strong

Adsorbent = Kieselgel HF; Eluent = 5% Ether-benzene

Samples of pyrethrum oleoresin were saponified by a procedure based on those reported in the literature. The unsaponifiable fractions constituted about 30 per cent of the extract and these were examined qualitatively by t.l.c. using the conditions described by Shone[242]. The results are shown in Table 25. An α-tocopherol standard had a R_f value of 0.42 and two components in the extract unsaponifiable fraction

responded strongly to Emmerie-Engel reagent in this region of the chromatogram with R_f values 0.41 and 0.38. These were tentatively assumed to be α - and β -(including β' -) or δ -tocopherol respectively by reference to Whittle and Pennock[241]. Shone[242] recorded R_f values of 0.56 and 0.38 for α - and β-tocopherol respectively but these were attained on non-commercial grade adsorbent.

Although the individual tocopherol isomers do not exhibit the same degree of antioxidant activity[226,243] their relative concentration in different pyrethrum extracts may be reasonably taken as being constant. The total tocopherol content may therefore be taken to reflect their stabilising effect on the extracts. For comparative purposes therefore it was adequate to determine the total tocopherol concentration in pyrethrum extracts. To this purpose, components eluting with R_f values between 0.36 and 0.43 were grouped together for the quantitative determinations of total tocopherol content.

After removal of the assigned tocopherol zones from several plates the material was eluted from the adsorbent and examined by g.l.c. and infra-red spectrometry. The gas-liquid chromatogram showed several components to be present but a major component eluted with a retention time corresponding to that of α -tocopherol. The infra-red spectrum (Figure 21) was consistant with that of pure α -tocopherol (Figure 22); the peak assignments are shown in Table 26.

Since refined extracts will not contain much of the material removed by the saponification procedure direct t.l.c.-spectrophotometric analysis of the refined extracts was investigated.

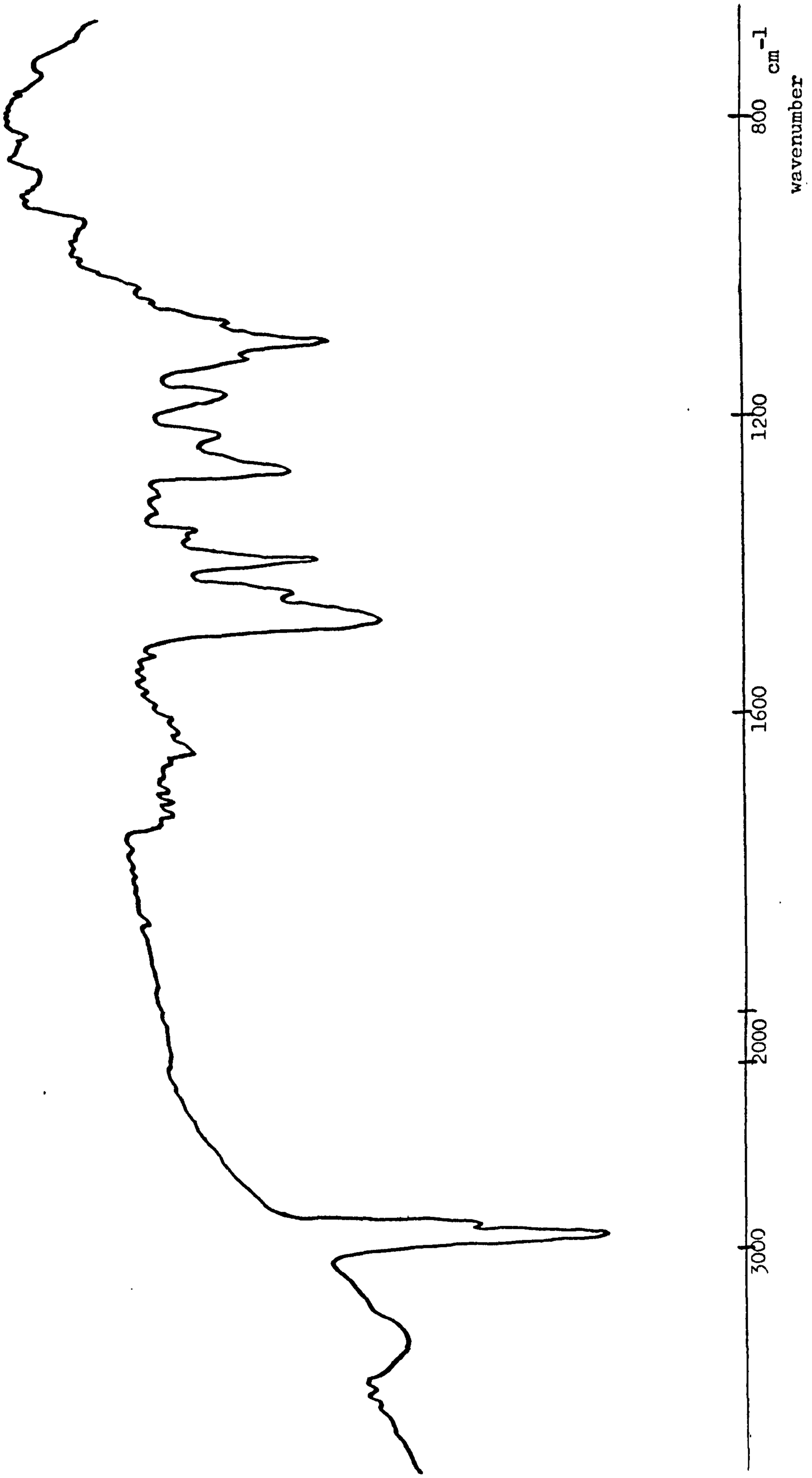


Figure 21 Infra-red spectrum of the 'tocopherol' fraction of a Pale Extract.

Figure 22 Infra-red spectrum of α-tocopherol.

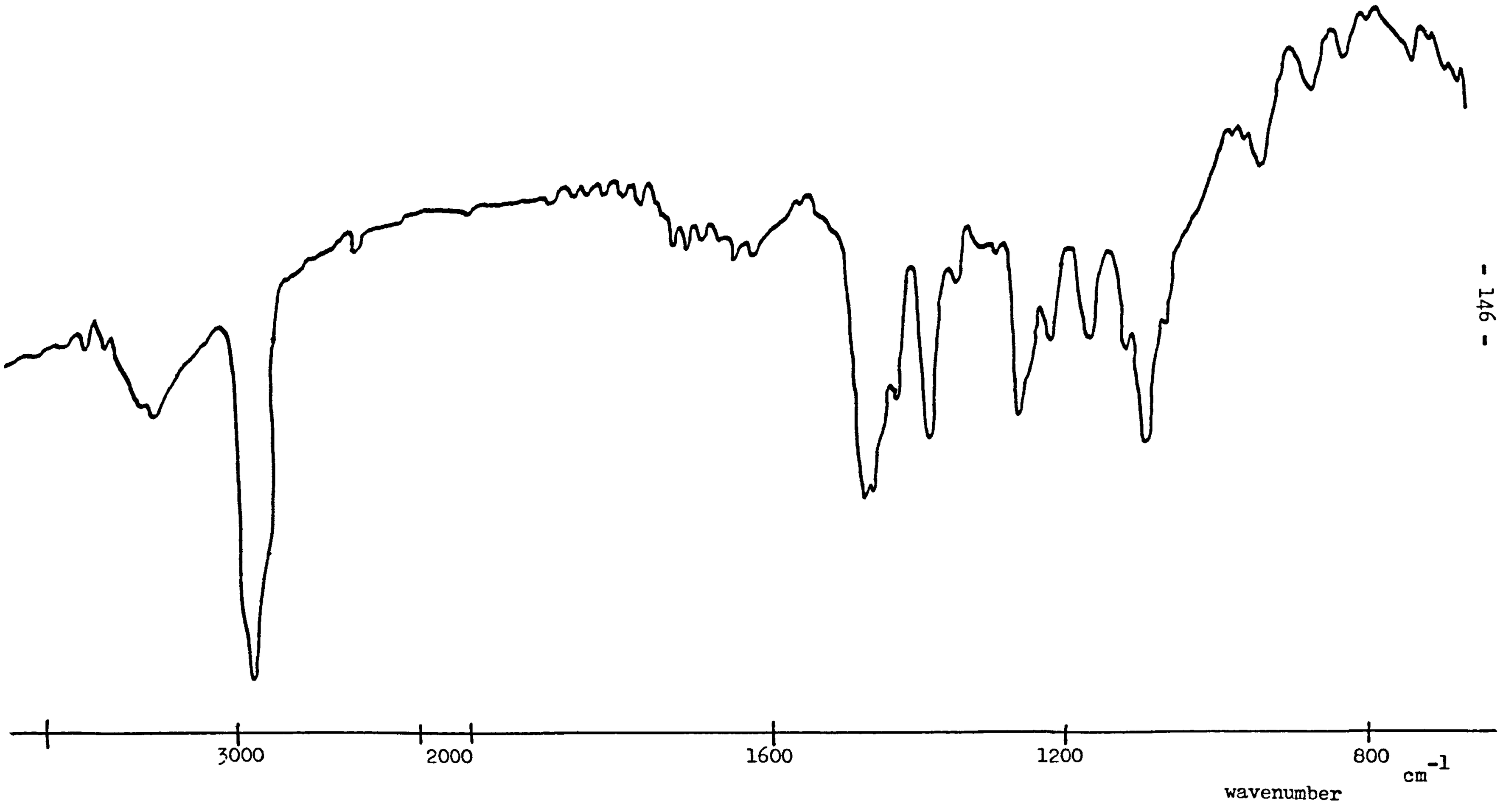


Table 26 Peak assignments of I.R. spectrum of 'tocopherol' fraction

Absorption wavelength	Assignment
3450 nm.	-OH stretch
1470 nm.	$-CH_2$ bend
1430 nm.	$-CH_3$ bend
1385 nm.	$-C-CH_3$
1265 nm.	phenolic C-O
1210 nm.	-OH bend
1090 nm.	C-O-C (chroman ring)

c.f. Knapp and Tappel[233]

A commercially refined Pale Extract concentrate was found to contain 560 p.p.m. of tocopherol by analysis including the saponification procedure and 574 p.p.m. when saponification of the sample was omitted. These results were not significantly different and omission of the saponification procedure in the tocopherol analysis of refined extracts was therefore possible.

Analysis of α-tocopherol solutions (0.05% in hexane) by both procedures indicated recoveries of about 88 per cent when saponification was included, and 93 per cent when omitted (see Table 27). Some loss of tocopherol appeared to have occurred therefore during both procedures, greater loss having occurred in the method which included the saponification of the sample but losses appeared to be constant. These results are in accordance with the reports of earlier workers[239-241,244] and therefore for comparison of results the method appeared suitable.

Table 27 Tocopherol analyses

Sample	With saponification procedure	Omitting saponification procedure
Oleoresin	600*, 580* 880, 893	-
Commercially refined Pale Extract concentrate (63.5% involatiles)	540, 580	524*, 574
Pale Extract (B.H.T.-free) (55.0% involatiles)	-	150, 164
-tocopherol (> 98% purity)	0.87×10^6 0.90×10^6	0.93×10^6 0.94×10^6

* trial experiment; values in p.p.m.

Results of tocopherol analyses on several samples of pyrethrum extracts are shown in Table 27. The commercially refined Pale Extract concentrate sample was found to retain a considerable proportion of the tocopherol content found in crude pyrethrum oleoresin. The B.H.T.-free Pale Extract contained markedly less tocopherol and it is possible that some had been consumed in stabilising this extract.

2.3.4.2. Unsaturated fatty acid derivatives

Vegetable fats are generally characterised by a high degree of unsaturation[186] and the autoxidation of organic substrates is recognised as being substantially enhanced by the presence of unsaturated compounds[183,184,188,245].

The presence of unsaturated fatty acid derivatives in pyrethrum oleoresin has been demonstrated by Head[126] whereby this type of extract was shown to contain about 40 per cent fatty acid material of which half were non-hydroxy fatty acids. Of these, the unsaturated acids, oleic (XXII, x = 7, y = 1, z = 6), linoleic (XXII, x = 4, y = 2, z = 6) and linolenic (XXII, x = 1, y = 3, z = 6) constituted 57 per cent (equivalent to 11.6 per cent of the extract). Autoxidation of these compounds will undoubtedly occur during the autoxidative degradation of pyrethrum extracts.

$$CH_3(CH_2)_x(CH = CH - CH_2)_y(CH_2)_zCOOH$$

XXII

The unsaturated fatty acid content of refined pyrethrum extracts has not previously been reported. Undoubtedly some of the lipid material is removed in the dewaxing process[14] although low temperatures will lead to the preferential precipitation of saturated material.

The fatty acid composition of a commercial Pale Extract concentrate was therefore examined by g.l.c. (as the methyl esters) of the fatty acids derived from the extract by saponification. Examination on non-polar (Apiezon L) and polar (PEGA) stationary phases indicated the presence of relatively large quantities of the unsaturated esters, methyl oleate, methyl linoleate and methyl linolenate (see Figures 23 and 24 respectively). Methyl palmitate (methyl ester of XXII, x = 14, y = z = 0) was also present as a major constituent and methyl myristate (methyl ester of XXII, x = 12, y = z = 0) and methyl stearate (methyl ester of XXII, x = 16, y = z = 0) as minor

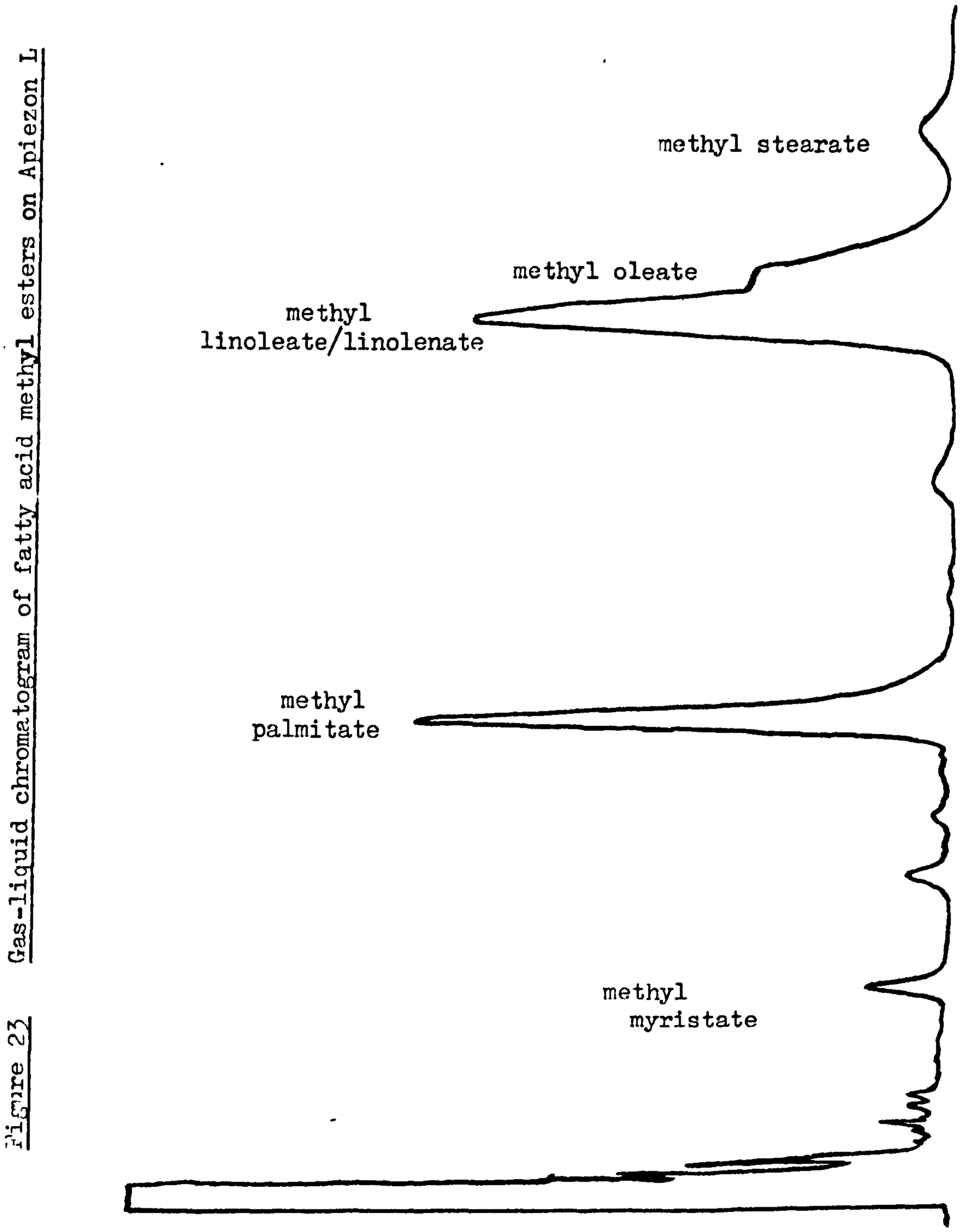


Figure 23 Gas-liquid chromatogram of fatty acid methyl esters on Apiezon L

Figure 24 Gas-liquid chromatogram of fatty acid methyl esters on PEGA

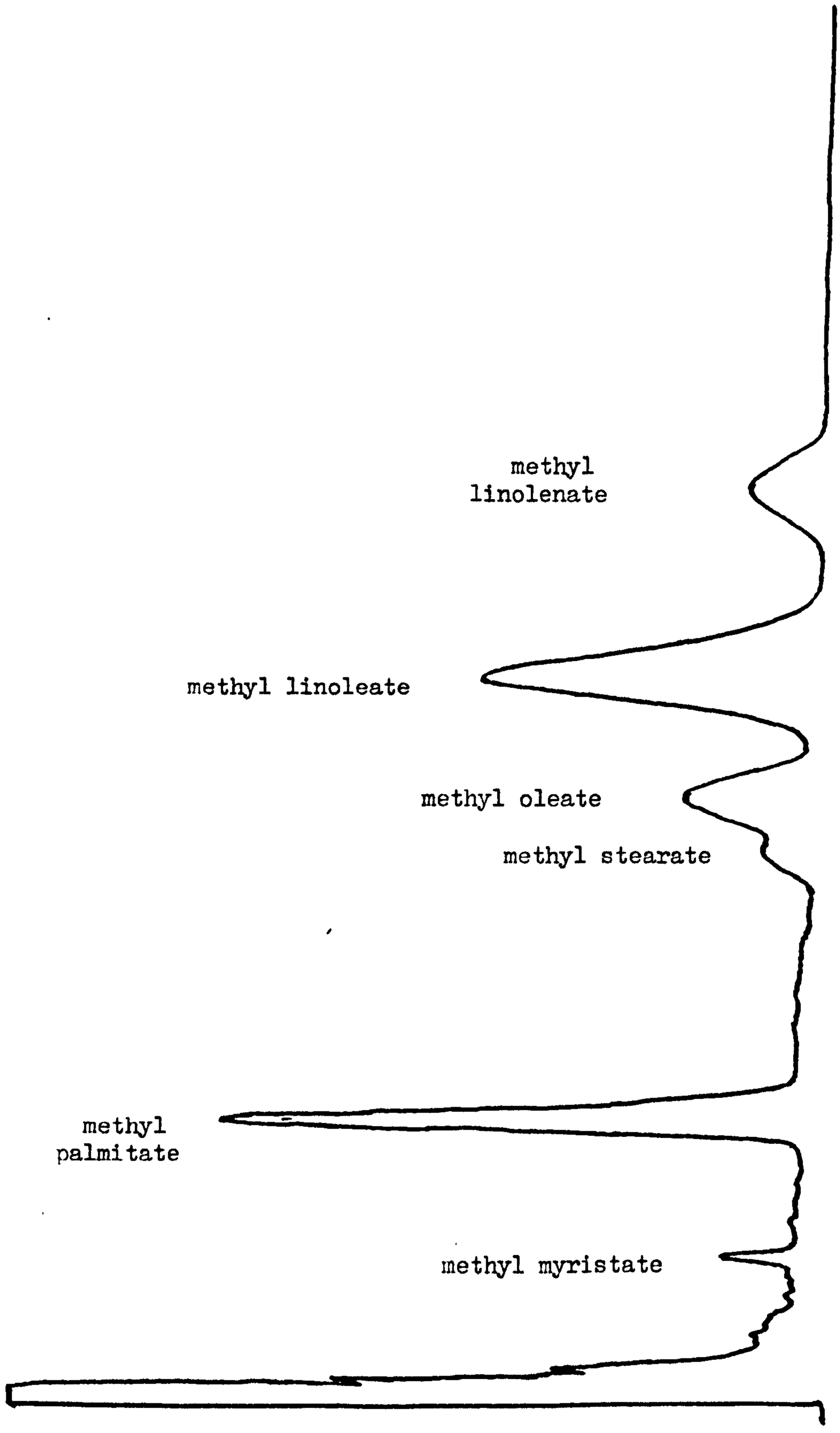


Table 28 Fatty acid analysis of a commercial Pale Extract concentrate

Common name	E.C.L. of ester on Apiezon L observed	pubd.	E.C.L. of ester on P.E.G.A. observed	pubd.	Normalised concn: %	% concn. in extract
Myristic	14.0	14.0	14.0	14.0	2.7	0.17
Palmitic	16.0	16.0	16.0	16.0	30.1	1.88
Stearic	18.0	18.0	18.0	18.0	4.8	0.30
Oleic	17.7	17.7	18.2	18.2	12.7	0.79
Linoleic	17.55	17.6	18.8	18.8	38.5	2.40
Linolenic	17.55	17.6	19.5	19.5	11.2	0.7

constituents. Identification of the components was based on a comparison with the retention times of the methyl esters of saturated acids and the published equivalent chain lengths (E.C.L.) for the unsaturated esters[246] (see Table 28).

Quantitative assessment of the major components was achieved by using methyl myristate (pure by g.l.c. and infra-red spectrometry) as internal standard; a correction being made for the presence of this compound in the sample. As an approximation an equal weight response factor for all the fatty acid esters was assumed (see 2.1.2.1.).

The unsaturated fatty acids constituted 3.9 per cent of the extract sample thus confirming the presence of compounds in Pale Extract which are known to be susceptible to autoxidation. In particular, the relative abundance of linoleic acid is important since methylene-interrupted dienes (i.e. $RCH=CH-CH_2-CH=CH-CHR'$) are more susceptible to autoxidation than mono-enes or conjugated dienes[183,184,186,188,245] and the

presence of small quantities (< 5 per cent) of the more labile compounds has been shown to have a marked effect upon the stability of more stable substrates[247-249]. Their presence in Pale Extracts therefore may catalyse rethrin degradation in extracts by being the primary source of reactive free radicals which may then react with the rethrins causing their degradation and thereby leading to a shortening of the rethrin degradation induction period. The presence of antioxidants (natural and added) would inhibit the autoxidation of fatty acid derivatives and thus appear to stabilise the rethrins.

In addition to the unsaturated fatty acid content (free and combined) of pyrethrum extracts, the free fatty acid concentration could also play an important role in their stability. Acids are known to autoxidise faster than their corresponding esters[245], probably resulting from the participation of the acid group in the decomposition of the peroxides. Further the effect of acidic organic material on metallic parts of the extraction and refining plants may be to lead to metal contamination which catalyses autoxidation (see 2.3.1. and 2.3.4.3.).

The organic acidity[250] was therefore determined by potentiometric titration of a solution of extract samples in aqueous methanol and found to be equivalent to 6.9 per cent stearic acid in pyrethrum oleoresin and 5.6 per cent in a commercial Pale Extract concentrate. These are relatively high values for vegetable fats and this may be important in either direct or indirect (metal contamination) catalysis of

the autoxidative deterioration of extracts.

2.3.4.3. Peroxides

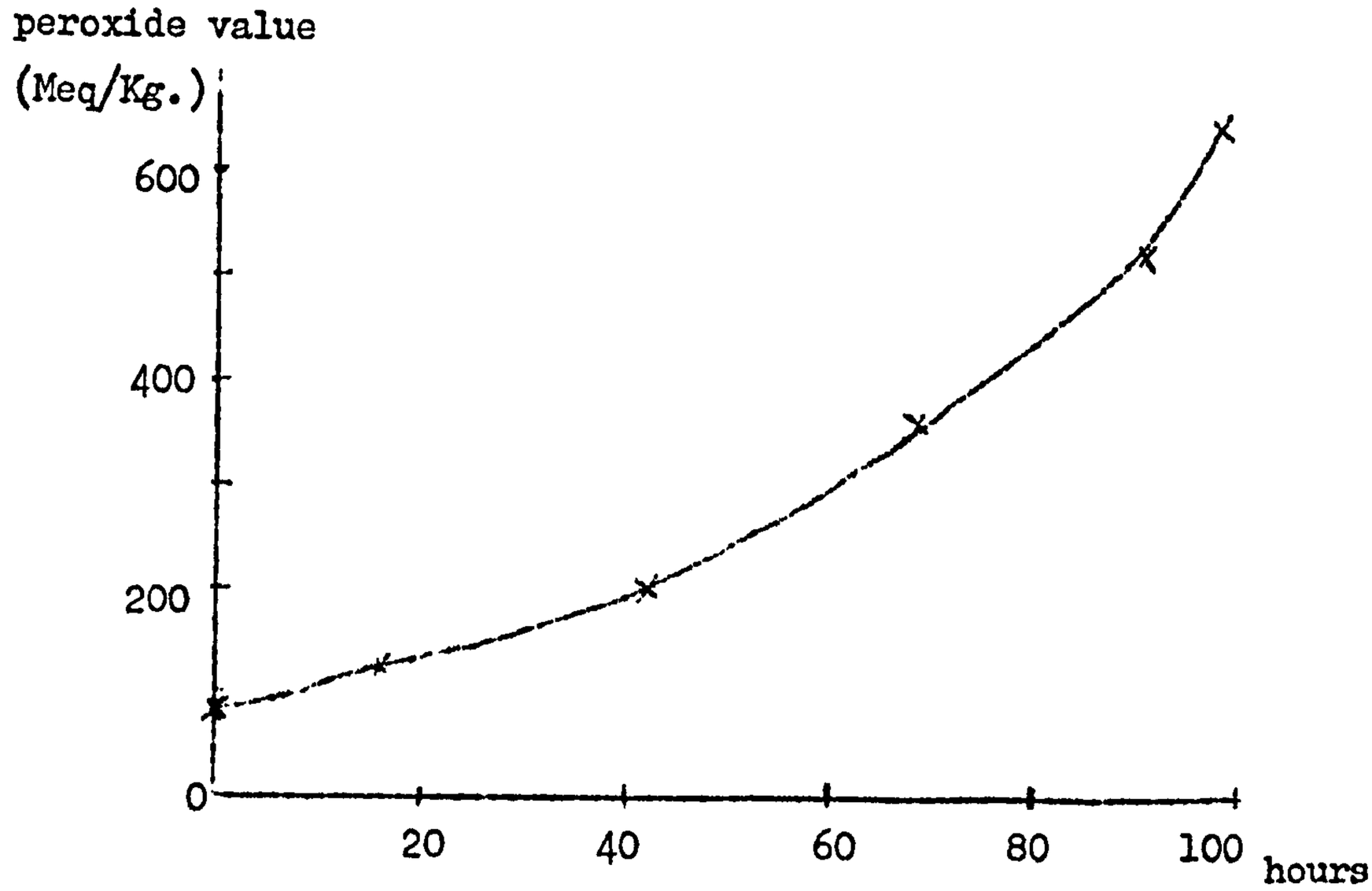
The autocatalytic nature of autoxidation reactions leads to an increase in the concentration of hydroperoxides in unhibited systems (see 2.3.1.) although a point will be reached where the rate of decay exceeds the rate of formation. Other peroxides are formed as secondary products of autoxidation but several workers have demonstrated that virtually all the peroxides present in the early stages of autoxidation are hydroperoxides[250-254]. Quantitative determination of the peroxides has therefore become the most commonly used analytical method for assessing the stability of a substrate, its degree of deterioration or for following the early stages of autoxidation.

The most common analytical methods of peroxide determination are based on the reduction of the peroxide link, for example by I^-, Fe^{2+}, Sn^{2+}, As^{2+}, followed by the determination of excess reagent or reaction product[255,256]. The iodometric method, involving the determination of iodine liberated from potassium iodide (equation xxiii), is the procedure most commonly used.

$$ROOR' + 2KI + 2H^+ \longrightarrow I_2 + ROH + R'OH \qquad \text{(xxiii)}$$

The choice of solvent has been the subject of many investigations[257]. An ionising solvent is necessary but is often mixed with a solvent of low polarity in order to solubilise fatty material. Mixtures of acetic acid and chloroform have been proposed but the amount of chloroform should not exceed that necessary to dissolve the substrate[258] and Sulley concluded[259]

Figure 25 Autoxidation of a highly unsaturated fat (linseed oil)



that discrepancies observed when this solvent system was used were caused by incomplete reaction due to too high a concentration of chloroform leading to precipitation of the potassium iodide.

The method chosen for the present work was that reported by Sulley[259], based on the method of Lea[260], in which the potassium iodide and substrate are added down a column of refluxing 50 per cent chloroform - acetic acid solvent. This excludes the presence of air from the reaction, a constant source of error in most methods which generally necessitates working in an inert atmosphere. The success of the method was demonstrated by the fact that blank determinations returned zero values unless solvents were impure and that consistant results were obtained over a range of sample sizes (often there is a tendency for the apparent peroxide value to increase significantly as the size of sample is reduced)[259].

Table 29 Peroxide values of various pyrethrum extracts

Sample	Peroxide value Mq./kg		
+ Oleoresin	1.8	1.6	2.1
* Oleoresin, B.H.T.-free	1.0	1.3	1.1
+ Pale Extract Concentrate	0.8	0.7	0.9
* Pale Extract, B.H.T.-free	0.5	0.4	0.5
// Pale Extract sample	0.6	0.5	0.3
// Nitromethane Extract	0.4	0.2	

+ industrially prepared; * laboratory produced;

// laboratory refined from commercial oleoresin.

The applicability and precision of the method was demonstrated by the analysis of a standard benzoyl peroxide sample (when three consecutive analyses gave 97.0, 97.8 and 97.2 per cent purity) and by following the autoxidation of a sample of linseed oil, a vegetable fat containing a high proportion of unsaturated lipid material (see Figure 25). Blank determinations invariably returned zero values.

Pyrethrum extracts analysed for peroxide content showed that both crude and refined extracts contained negligible concentrations of peroxides when freshly prepared and stored at sub-zero temperatures (Table 29).

2.3.4.4. Trace iron contamination

The catalytic effect of transition metals on autoxidation processes is well known[183,184,186-188] although the overall effect of metals appears to be quite complex. There is a good deal of evidence to suggest that metal ions exert their pro-oxidant effect by forming unstable coordination complexes with alkyl hydroperoxides followed by election transfer to give free radicals (see 2.3.1.)[184]. Metals are therefore claimed to be effective only if peroxides are present[261] but direct initiation by metallic ions has been reported to occur in some cases[262,263].

Many heavy metal ions which possess two or more valency states with a suitable oxidation-reduction potential between them react with hydroperoxides but where the metal may act as both oxidant and reductant (2.3.1.; Equations xx and xxi) only a small quantity of the metal ion need be present to have a pronounced pro-oxidant effect since it is rejuvenated in the course of further action. Trace metal contamination (~ 1 p.p.m.) may therefore lead to an increase in the rate of substrate deterioration while only a few p.p.m. of metallic impurities will cause rapid autoxidative degradation in highly unsaturated systems[183].

The degree of pro-oxidant activity varies with the metal but attempts have been made to establish sequences of activity[264,265]. Copper and iron invariably appear foremost in the lists and the latter, because of its prevalence in materials used for industrial plant construction and storage containers, has been

found to be an important source of metal ion catalysis of autoxidative deterioration of natural products; examples being the production of soyabean oil[265] and of palm oil[266].

The moderately high acidity of commercial pyrethrum extracts (see 2.3.4.2.) raises the probability of iron contamination by contact with metallic surfaces although the dissolution of metals by organic acids in hydrocarbon media has been shown to be dependent on the presence of oxygen or peroxides[268,269]. The iron content of pyrethrum extracts were therefore examined.

The determination of iron in oils and food stuffs by a variety of methods has been described in the literature[270-272]. One of the most favoured method utilises o-phenonthroline as a complexing agent with iron in the chemically reduced state to form a coloured complex whose intensity may be determined spectrophotometrically at 510 nm and is proportional to the concentration over a wide range[271].

Interference by many organic anions has been reported and by other metal ions, but in general this only occurs when their concentration greatly exceeds that of iron[271]. To obliviate the effect of organic material samples are generally 'ashed' to destroy all such matter. This may be achieved by 'dry ashing', (heating at a temperature of about 500°C for several hours) or 'wet ashing' (heating with concentrated sulphuric and nitric acids)[272]. For the determination of iron wet ashing has been shown to give satisfactory recovery over a wide range of concentrations (0.2 to 1000 p.p.m.) provided standard precautions are taken, whilst dry ashing would appear to give erratic results[273].

Table 30 Iron content of pyrethrum oleoresin determined by the o-phenanthroline method using reagents of various degrees of contamination

Reagents	Sample	Blank	Concentration in oleoresin
1. G.P.R. reagents	161 µg	117 µg	100 ppm
2. Analar reagents	88 µg	64 µg	36 ppm
3. Reagents of v. low iron content	23 µg	10 µg	8.5 ppm
	35 µg	16 µg	14 ppm

Utilising a wet ashing procedure and the o-phenanthroline method of iron determination unsatisfactory results were obtained on a sample of pyrethrum oleoresin (see Table 30). The results were inconsistant due to high blank values which presumably were caused by iron contamination of the reagents, in particular the mineral acids. Even reagents relatively free of iron returned values in excess of the corrected iron content of the sample. Therefore although the accuracy of the method may undoubtedly have been improved, as well as the precision, by procedural improvements and replicate analyses, it was concluded that this method was limited to demonstrating that the iron content of this sample of oleoresin was not in excess of a few parts per million. From a catalytic viewpoint however, this would still be a considerable iron concentration and therefore an alternative method of analysis was considered.

A method which does not suffer from the vagaries of contaminated solvents is neutron activation analysis[274,275]. In this method the element to be analysed is converted to a radioactive isotope which then decays by emission of a characteristic range of energies.

This method of analysis is particularly suitable for the determination of iron despite the relative low abundance of the naturally occurring isotope of iron (iron - 58) with the correct nuclear characteristics (see Table 31) since the sensitivity required (~ 0.1 p.p.m.) is moderate in neutron activation studies[275]. The half-life of decay of the irradiation product (iron - 59) is moderately long (45 days). Although this decreases the sensitivity of iron analysis to some extent it allows adequate time for sample preparation after irradiation (for irradiation measurements) and for radioactive material of a shorter life to degrade thus simplifying the energy spectrum. Simplification of the spectrum by purification is likely to be necessary only when used in conjunction with pulse height analysers of moderate resolving power and since the iron to be analysed has been converted to iron - 59, iron-contaminated solvents are then of no consequence.

The scheme shown in Figure 26 indicates the radioactive processes involved for iron. The most prominent peaks in the emission spectrum occur at 1.292 and 1.095 MeV (see Figure 27).

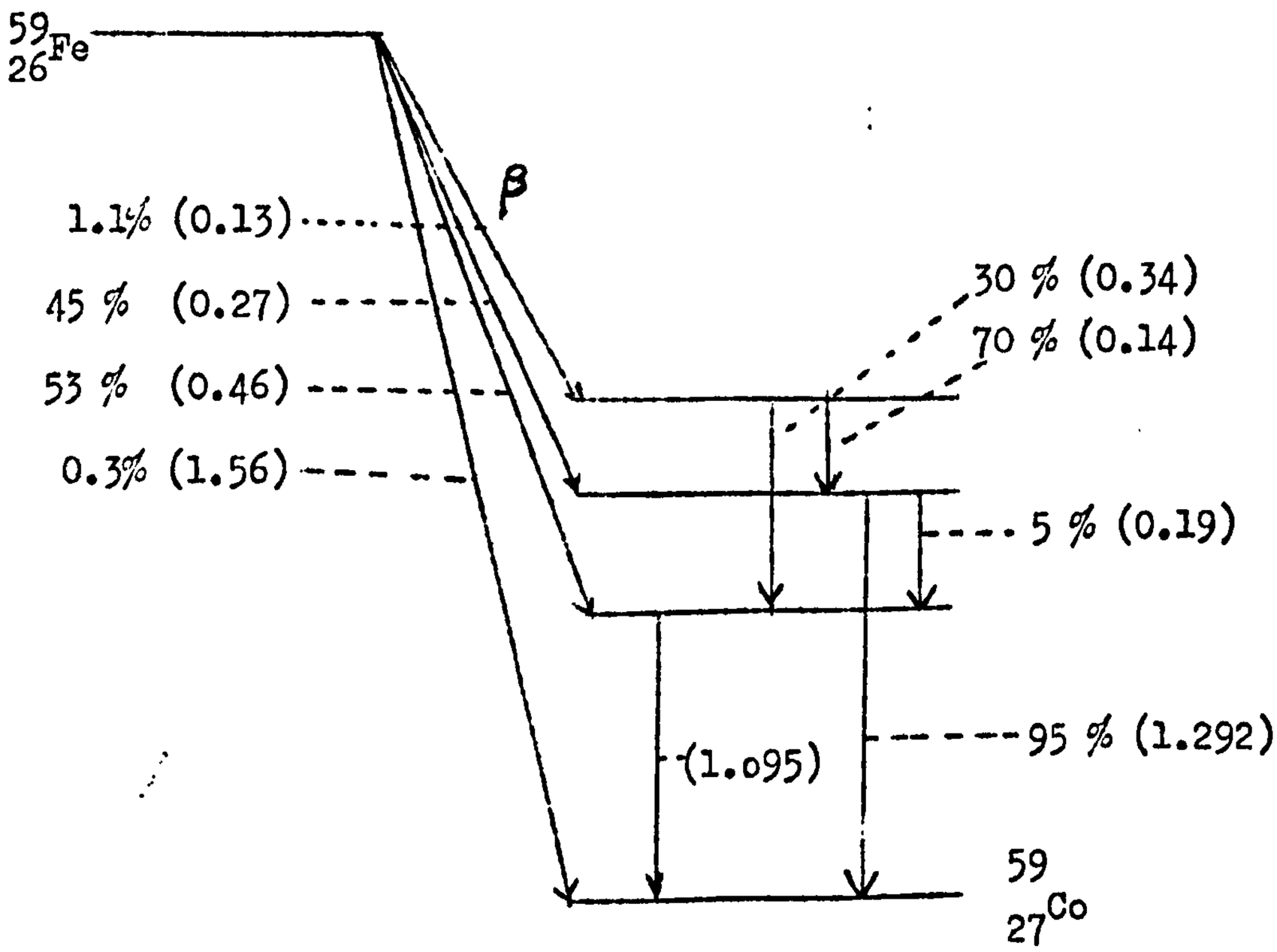
The main source of error in this method of iron analysis is the possibility of interference arising from the formation of iron - 59 from a different source. Cobalt - 59 and nickel-62

Table 31 Naturally occurring isotopes of iron.

Isotope	Relative abundance	Activation cross-section to thermal neutrons	Irradiation product and half-life
iron-54	5.84	-	-
iron-56	91.68	-	-
iron-57	2.17	-	-
iron-58	0.31	0.98 ± 0.10	iron-59 , 45 days

Figure 26 Decay scheme of iron-59

$$^{58}_{26}Fe(n,\gamma) \longrightarrow {}^{59}_{26}Fe \xrightarrow{\beta^-,\gamma} {}^{59}_{27}Co$$



( ) - energy emission, MeV

Figure 27 Emission spectra of iron-59.

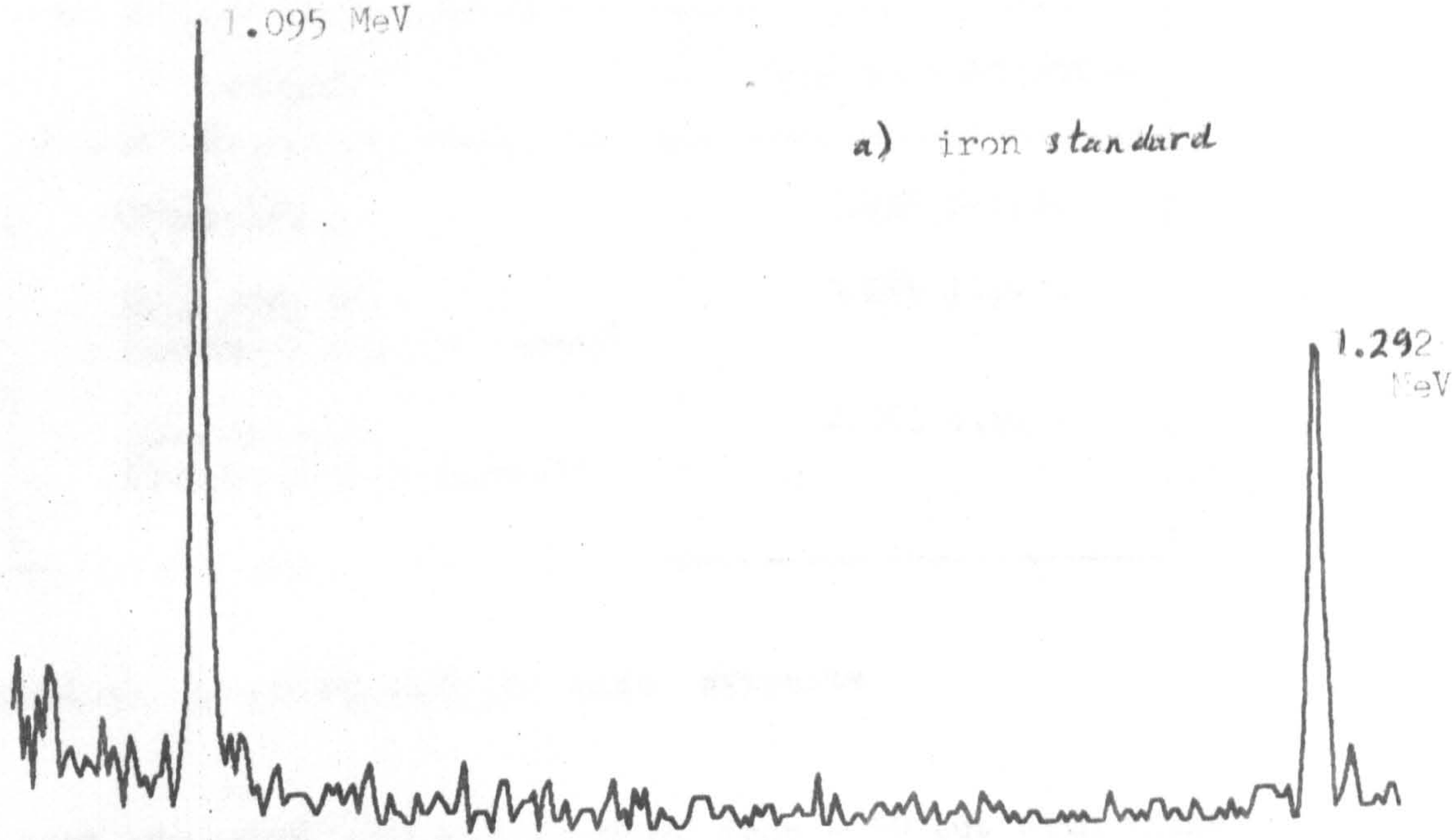


Table 32 Iron content of pyrethrum extracts determined by neutron activation analysis

Sample	Iron concentration
Oleoresin	0.215 p.p.m.
Pale Extract (commercially produced)*	0.126 p.p.m.
Pale Extract (laboratory produced)*	0.051 p.p.m.

* prepared from different oleoresin extracts

interact with fast neutrons to yield iron - 59 but both these isotopes have low activation cross-sections[274] and relatively few neutrons in the reactor will be of sufficient energy to bring about such reactions.

Pyrethrum extract samples analysed by this method yielded results which were substantially lower than could have been determined by a chemical method of analysis (Table 32). The laboratory prepared Pale Extract contained the lowest proportion of iron but industrially prepared extracts, both crude and refined, did not contain substantial amounts.

Catalysis of pyrethrum extract degradation due to the presence of iron would therefore appear not to be a problem since the natural low iron content of the extracts was not found to be increased by contamination arising from the extraction and refining processes. However, the history of the samples examined was largely unknown and it is improbable that the containers used to transport these samples (aluminium for the commercially produced extracts; glass for the

laboratory prepared one) were similar to those used commercially. The possibility of iron contamination as a source of instability in other samples should therefore not be disregarded. The ease of contamination was emphasised by the results of recent work[276] on other samples of pyrethrum extracts which yielded values for the iron content ranging from 3 to 17 p.p.m. (a totally laboratory prepared refined extract being the most contaminated).

A recent publication by Marshall[277] has inferred that iron contents in the region of 300 p.p.m. may be encountered and that a concentration of 20 p.p.m. is the normal level. It is unlikely that iron contents of this degree are of natural origin and the results are more probably due to the use of highly contaminated extracts and/or the use of unsuitable chemical methods of analysis.

2.3.4.5. <u>Changes in concentration of rethrins, peroxides and antioxidants (natural and added) during induced extract deterioration</u>

Natural antioxidant and pro-oxidant concentration levels were not monitored during the long-term stability studies on pyrethrum extracts because appropriate methods of analysis had not been developed. However, examination of a sample of the B.H.T.-free Pale Extract (2.3.2.4.), which had been stored at sub-zero temperatures, showed that it contained negligible quantities of peroxides and of iron (see 2.3.4.3. and 2.3.4.4.). The other extracts (2.3.2.1., 2.3.2.2., 2.3.2.3.) were prepared from oleoresin extracts in which their concentration was also found to be low and the determination of these constituents in similar, freshly prepared extracts indicated that the refining process did not increase their concentration (see 2.3.4.3. and 2.3.4.4.).

As part of this present work the effect was studied of the addition of these pro-oxidants to refined extracts. The addition of peroxides (as autoxidised linseed oil) shortens the induction period since they have sufficient thermal decomposition rates to yield a high proportion of new free radicals. Iron was added in the form of ferric stearate to catalyse the decomposition of hydroperoxides (see 2.3.1.). To further enhance the rate of extract deterioration the samples were subjected to constant agitation by a stream of air.

The samples used were from two extract sources; one a commercial Pale Extract concentrate containing B.H.T., the other a Pale Extract containing no B.H.T. The iron content of both these extracts had been shown to be less than 1 p.p.m. and the peroxide values to be less than 1 milliequivalent per kilogram (see 2.3.4.4. and 2.3.4.3.). To one sample of each extract autoxidised linseed oil was added so that the initial peroxide concentration was approximately 20 milliequivalents per kilogram of substrate. Ferric stearate was added to another sample of each extract so that the samples contained 2 p.p.m. of iron. A further sample of each extract was used as a control.

Analyses over a period exceeding one month (see Tables 33 and 34 and Figures 28 and 29) recorded a rising peroxide content and a decreasing natural antioxidant concentration but only slight losses of B.H.T. (where present) and of rethrins.

The samples containing B.H.T. exhibited a markedly slower rate of peroxide accumulation compared to those samples prepared from B.H.T.-free extract. Of the B.H.T.-containing samples

Table 33 Effects of induced oxidation on a commercial Pale Extract sample containing B.H.T.

Storage Period (days)	Peroxide Value (Meq/Kg)			Tocopherol content (p.p.m.)			% B.H.T. concentration			% rethrin concentration by g.l.c.			% rethrin concentration by U.V.		
	N	F	L	N	F	L	N	F	L	N	F	L	N	F	L
Nil	0.8	0.8	10.9	549	549	549	2.13	2.13	2.13	36.85	36.85	36.85	40.2	40.2	40.2
9	10	16	27	541	541	417	-	-	-	-	-	-	38.6	37.9	36.1
21	-	20	32	-	409	233	-	-	-	-	-	-	-	39.2	36.6
27	17	23	30	-	-	-	-	-	-	-	-	-	-	-	-
42	23	25	33	262	183	47	2.02	2.01	1.63	36.8	37.1	34.55	38.6	39.9	35.0

N = no additive

F = Ferric stearate added 2 p.p.m. of Fe^{III}

L = Peroxidised linseed oil added

Table 34 Effects of induced oxidation on Pale Extract containing no B.H.T.

Storage Period (days)	Peroxide value (meq/Kg)			Tocopherol content (by p.p.m.)			% Rethrin concentration by g.l.c.			by U.V.		
	N	F	L	N	F	L	N	F	L	N	F	L
Nil	0.5	0.5	18.6	157	157	157	25.1	25.1	25.1	30.2	30.2	30.2
12	12	19	30	124	110	60	-	-	-	-	31.8	30.6
20	-	30	50	-	60	24	-	-	-	-	30.0	28.7
25	19	50	76	-	-	-	-	-	-	-	-	-
30	46	90	120	54	17	14	22.95	21.6	19.2	30.9	27.0	27.6

N = no additive

F = Ferric stearate added 2 p.p.m. of Fe^{III}

L = Peroxidised linseed oil added

Figure 28 Induced deterioration of Pale Extract concentrate containing B.H.T.

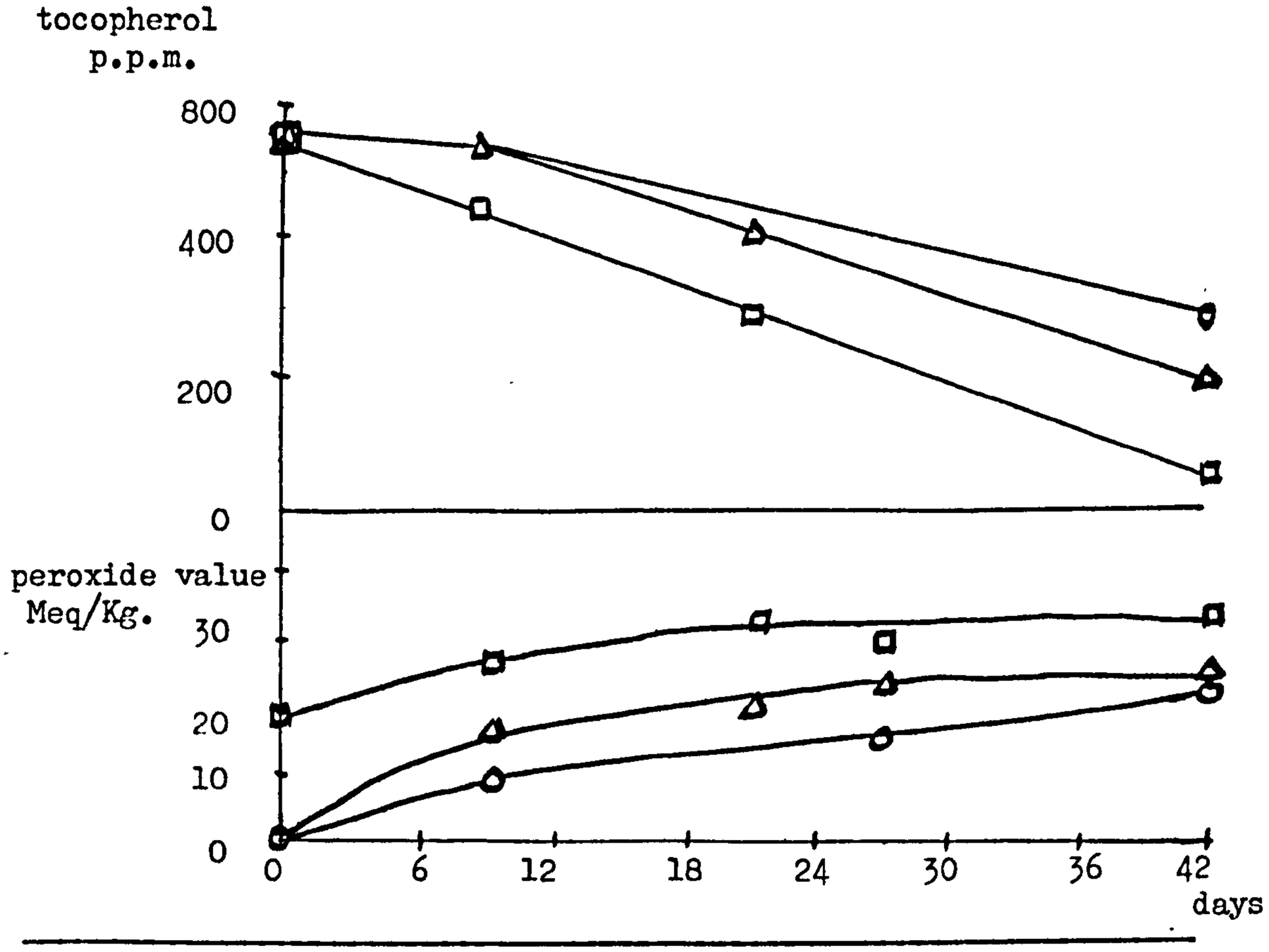
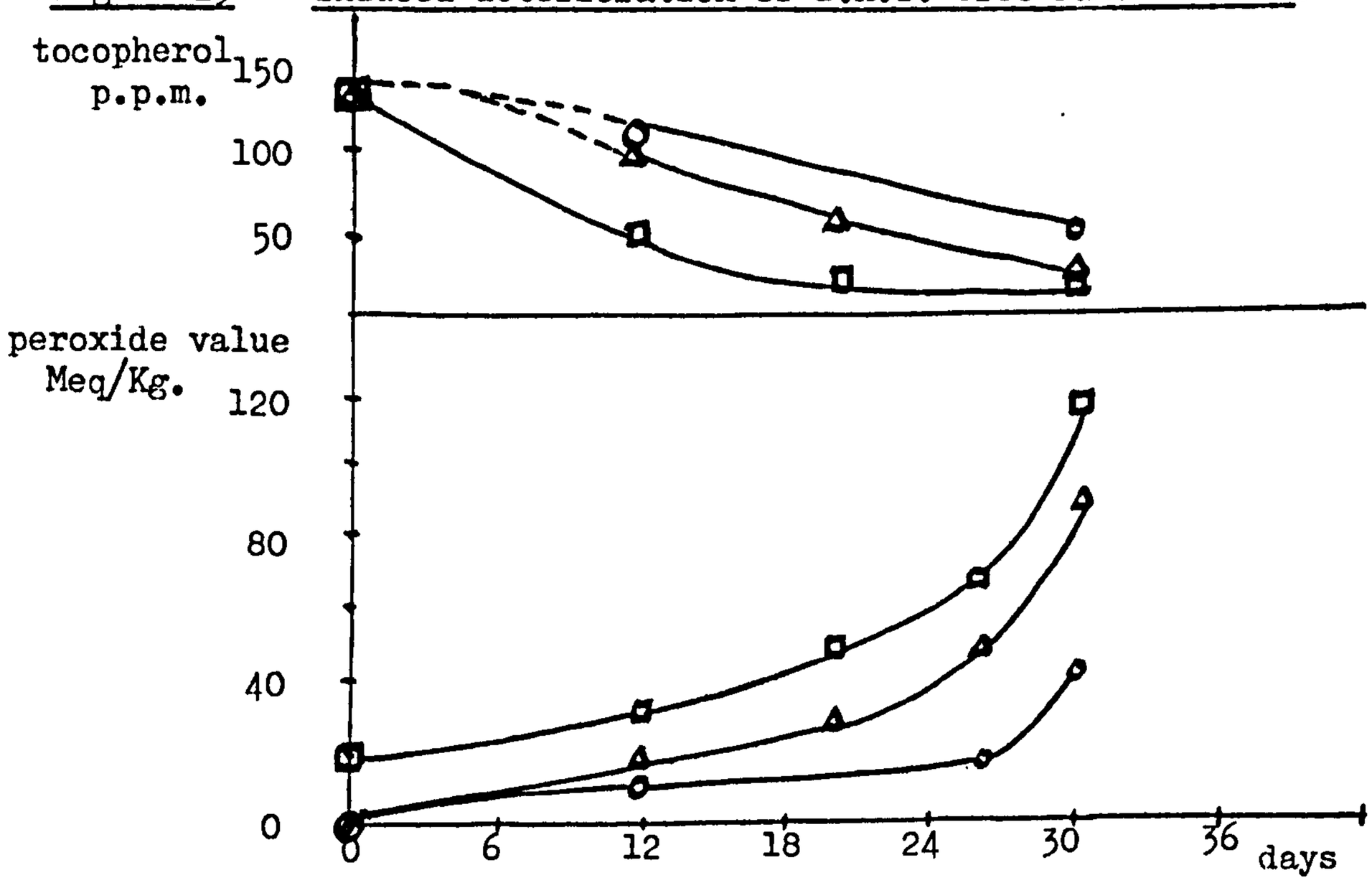


Figure 29 Induced deterioration of B.H.T.-free Pale Extract.



o - control sample

Δ - sample containing added iron

□ - sample containing added peroxides

those containing no added peroxides showed a more rapid rate of rising peroxide concentration initially but this slowed down on having attained a peroxide value of between 10 and 20 milli-equivalents per kilogram. This point corresponded to the end of an apparent induction period with regard to tocopherol losses in those samples whilst the sample containing added peroxides showed an immediate loss of tocopherol, the rate of which was constant (Figure 28). This induction period was more probably a period of slow but increasing rate of tocopherol loss. These results are thus consistant with the rate of inhibitor loss increasing with time when a chain initiator is absent, but constant when a sufficient initial concentration of hydroperoxides is present and equal to the rate of free radical formation from the hydroperoxides[188].

Of the B.H.T.-free samples those containing no added peroxides exhibited a decrease in the rate of rising peroxide concentration at a peroxide value of between 10 and 20 milliequivalent per kilogram similar to the corresponding samples containing B.H.T. An increasing rate of peroxide accummmulation was quickly reestablished however, unlike the B.H.T.-containing samples, and was correlated to the tocopherol concentration falling below about 60 p.p.m. The loss of tocopherol from these samples probably exhibited an initial induction period (very low rate of loss), a conclusion drawn from the extrapolation of the tocopherol decomposition curves (see Figure 29). The sample containing added peroxides exhibited an increasing rate of peroxide accummulation throughout the period of study and a rapid rate of tocopherol consumption.

Samples containing B.H.T. showed only a slight loss of B.H.T. during the period of study; the only significant loss being recorded in the sample containing added peroxides (see Table 33).

The rethrin content was monitored during the study by the ultra-violet spectrophotometric method[95] (see 1.4.4.) and at the start and end by the developed g.l.c. method using internal standard (see 2.1.2.). In the B.H.T.-containing samples the former method only recorded a significant decrease in rethrin concentration in the sample containing added peroxides. This was confirmed by the g.l.c. method (Table 33). The ultra-violet spectrophotometric method similarly indicated only slight losses of rethrin content in the B.H.T.-free samples but greater losses were recorded by the g.l.c. method in the samples containing added pro-oxidants (see Table 34). This may be attributed to the deficiency of the former method for analysing autoxidising systems since conjugated dienes, products of autoxidised unconjugated dienes such as linoleates, absorb in the region of rethrin absorption (~ 230 nm) (see 1.4.8.).

Tables 35 and 36 show the relative concentrations of the rethrins. The pyrethrin-cinerin and rethrins I-rethrins II ratios did not indicate any significantly greater losses of any particular group of rethrins.

The results of the induced-degradation study indicated that the presence of small quantities of iron and peroxides enhance the rate of overall autoxidation deterioration of refined pyrethrum extracts, thus shortening the induction period in respect of rethrin degradation, and of rethrin loss in unstabilised extracts. This is characterised by an increase

Table 35 Effect of pro-oxidants on rethrin stability in a commercial Pale Extract concentrate containing B.H.T. - after 6 weeks of storage

Additive	% involatiles	% PI	%CJI	% RJ	%PII	%CJII	%RII	% R	P/CJ	RI/RII	% loss
Original sample	63.5	10.1	5.9	24.0	0.5	4.35	12.85	36.05	2.60	1.865	-
No additives		10.7	5.4	24.1	8.35	4.35	12.7	36.8	2.77	1.89	(0.1)
Ferric stearate		10.6	5.1	23.7	9.05	4.35	13.4	37.1	2.93	1.77	(-0.4)
Peroxidised linseed oil		17.1	5.1	22.2	0.05	4.3	12.35	34.55	2.67	1.79	6

P = pyrethrin, CJ = cinerin + jasmolin, R = rethrins

Table 36 Effect of pro-oxidants on rethrin stability in a Pale Extract containing no B.H.T. - after 4 weeks of storage

Additive	% involatiles	% PI	%CJI	% RI	%PII	%CJII	% RII	% R	P/CJ	RI/RII	% loss
Original sample	55.0	11.35	3.35	14.7	7.6	2.05	10.45	25.1	3.06	1.405	-
No additive		10.9	2.9	13.8	6.4	2.75	9.15	22.95	3.06	1.51	8
Ferric stearate		10.5	2.6	13.1	6.0	2.5	8.5	21.6	3.23	1.54	14
Peroxidised linseed oil		8.95	2.35	11.3	5.6	2.3	7.9	19.2	3.13	1.425	23

p = pyrethrin, CJ = cinerin + jasmolin, R = rethrins

in the peroxide content but the accumulation of large quantities is inhibited by the presence of antioxidants. The tocopherols, present as naturally occurring antioxidants, temporarily inhibits their accumulation but is rapidly degraded. In the absence of B.H.T. the peroxide concentration increases as the destruction of the tocopherols is almost completed. Similar observations have been reported by Golumbic[228] for the antioxidant behaviour of α -tocopherol and by other workers using different phenolic antioxidants[278,279].

The tocopherols appeared to be less effective than B.H.T. in inhibiting rethrin degradation in extracts but samples containing B.H.T. exhibited no significant losses over six weeks of induced extract deterioration conditions. However both antioxidants probably supported each other to some degree and the presence of the tocopherols may decrease the rate of B.H.T. loss. Although presumably shortening the induction period of rethrin degradation, the presence of pro-oxidants did not induce rapid rethrin loss when B.H.T. was initially present.

2.3.5. Colour changes

2.3.5.1. Visible spectra

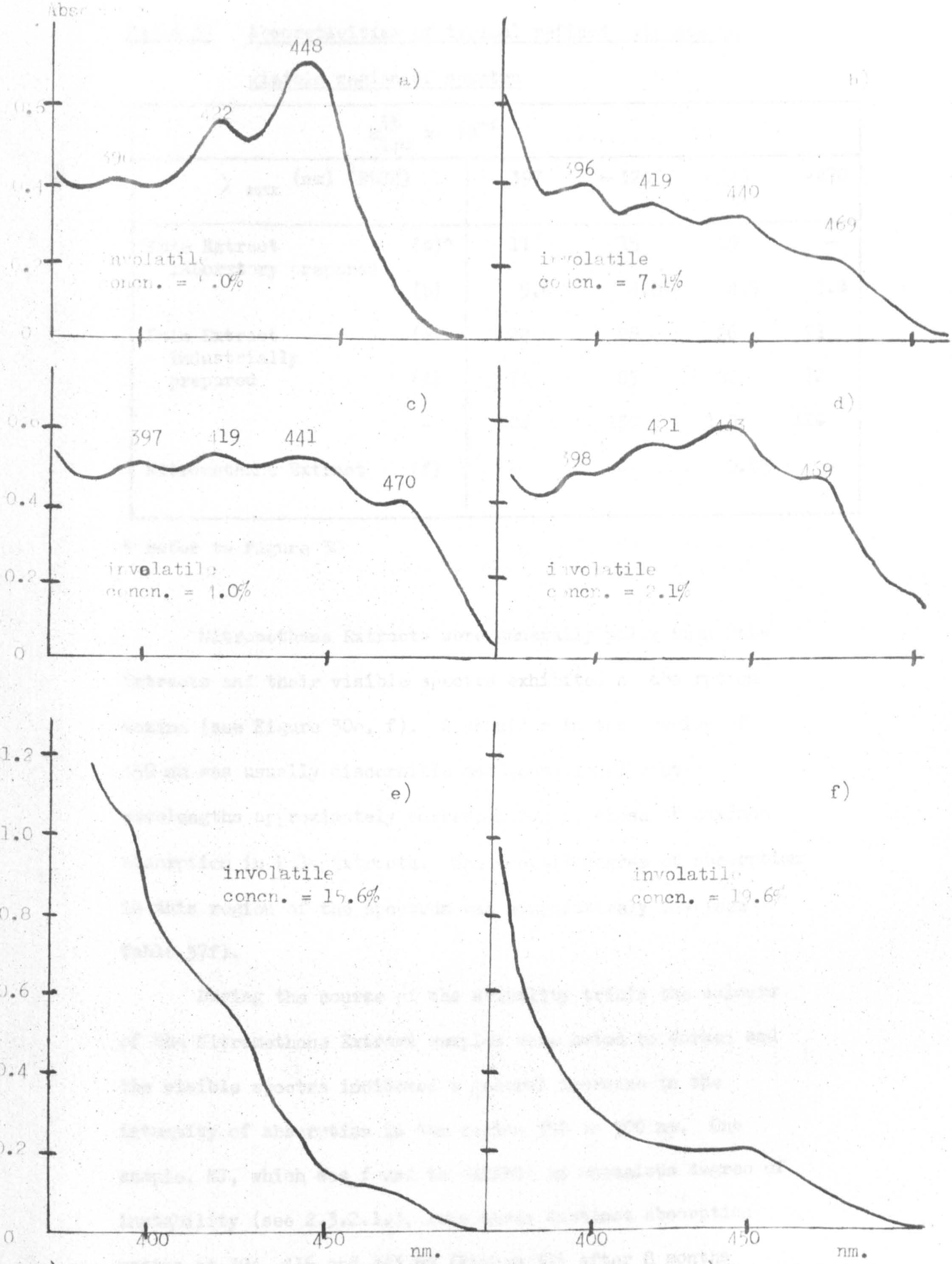
Although described as 'pale' or 'decolorised', refined pyrethrum extracts are not colourless. Repeated mixing with charcoal and filtering or passing through a charcoal column improves decolourisation but is reported to lead to rethrin losses by adsorption on the charcoal[14]. The extent of decolorisation may therefore vary between different extracts and, more particularly, between laboratory and industrially prepared extracts. This was illustrated by comparison of Pale Extract samples supplied by the Pyrethrum Marketing

Board of Kenya and some extracts prepared during this work when the former were noted to be more intensely orange-coloured. Examination of their visible spectra indicated significant differences in absorption characteristics (see Figure 30).

Four discrete absorption maxima were observed, the precise position of which appeared to vary slightly dependent on the concentration. The approximate wavelengths of maximum absorption, determined in absolute ethanol, were 397, 420, 445 and 470 nm. The absorption at 445 nm in particular appeared susceptible to changes in concentration. Head[280] has attributed absorption in this region to carotenoid constituents of pyrethrum oleoresin.

The absorptivities at the wavelength of maximum absorption for typical extracts are shown in Table 37, calculated on the concentration of involatile material (solvent removed at room temperature at reduced pressure) in the extracts. Further decolorisation of a laboratory prepared Pale Extract caused a reduction in intensity of absorption (see Figure 30a, b; Table 37a, b). Industrially prepared Pale Extracts invariably exhibited greater absorption (see Figure 30c, d; Table 37c, d) and varied widely between different samples. Since the carotenoids are potential labile substrates for autoxidation and it is known that non-decolorised pyrethrum extracts have lower stability (see 1.5.) some degree of standardisation of the decolorising procedure would seem appropriate.

Figure 30 Visible spectra of refined extracts (in ethanol).



a) Laboratory prepared Pale Extract partially decolorised ; b) Extract a further decolorised
c),d) Pale Extracts industrially prepared ; e),f) Nitromethane Extracts

Table 37 Absorptivities of typical refined extracts in visible region of spectra

$E^{1\%}_{1cm} \times 10^{-2}$					
λ max (nm) (EtOH)		~397	~420	~445	~470
Pale Extract laboratory prepared	(a)*	11	15	19	-
	(b)	5.6	4.8	4.5	3.0
Pale Extract industrially prepared	(c)	28	28	26	21
	(d)	74	85	92	72
	-	104	130	148	120
Nitromethane Extract	(f)	-	-	0.85	-

* refer to Figure 30

Nitromethane Extracts were generally paler than Pale Extracts and their visible spectra exhibited no absorption maxima (see Figure 30e, f). A shoulder in the region of 450 nm was usually discernible and occassionally at wavelengths approximately corresponding to those of maximum absorption in Pale Extracts. The overall degree of absorption in this region of the spectrum was comparatively low (see Table 37f).

During the course of the stability trials the colours of the Nitromethane Extract samples were noted to darken and the visible spectra iddicated a general increase in the intensity of absorption in the region 350 to 500 nm. One sample, NJ, which was found to exhibit an anomalous degree of instability (see 2.3.2.1.), gave three distinct absorption maxima at 394, 416 and 443 nm (Figure 31) after 8 months

Figure 32 Changes in the visible spectra of Pale extracts.

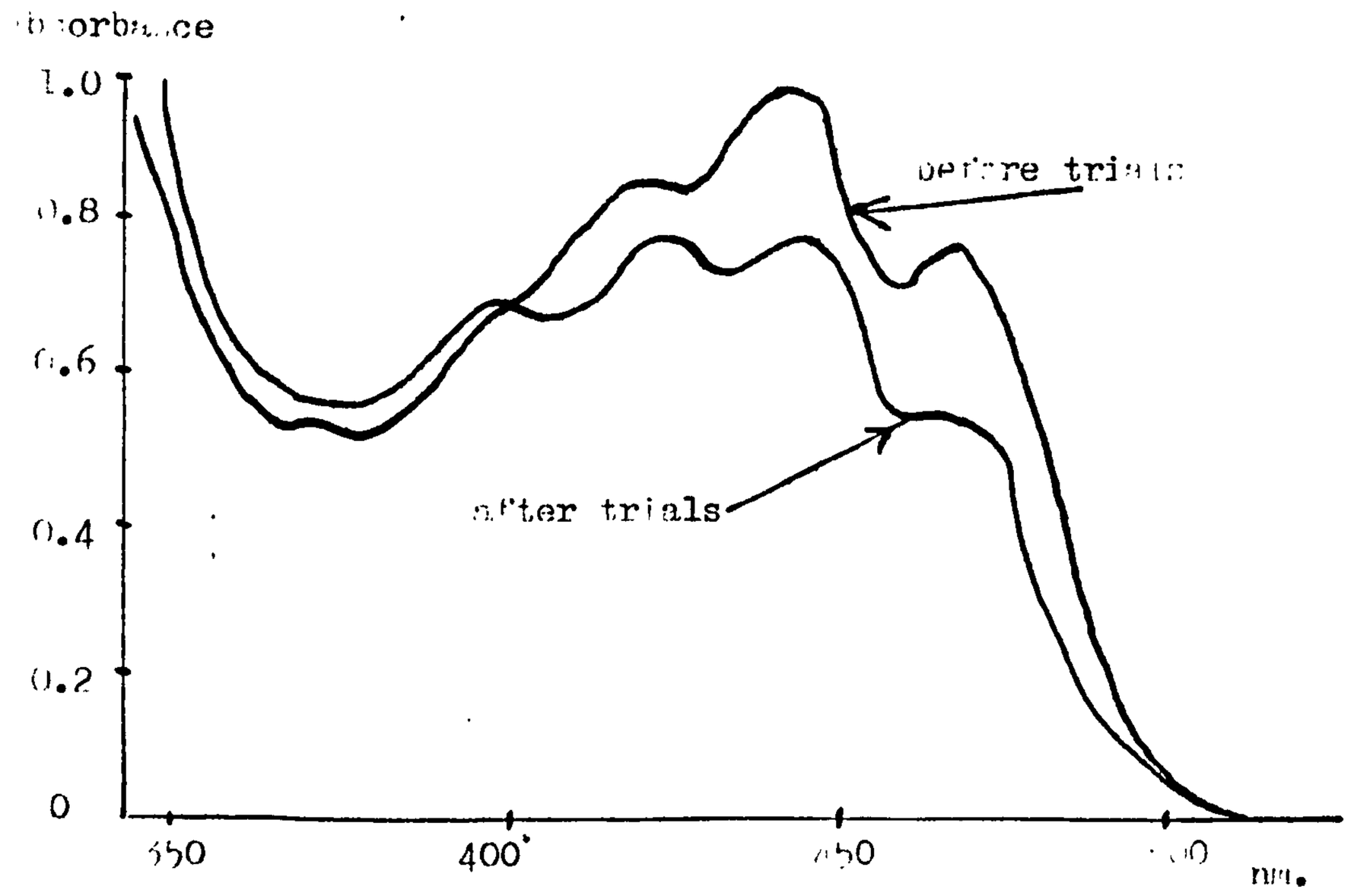
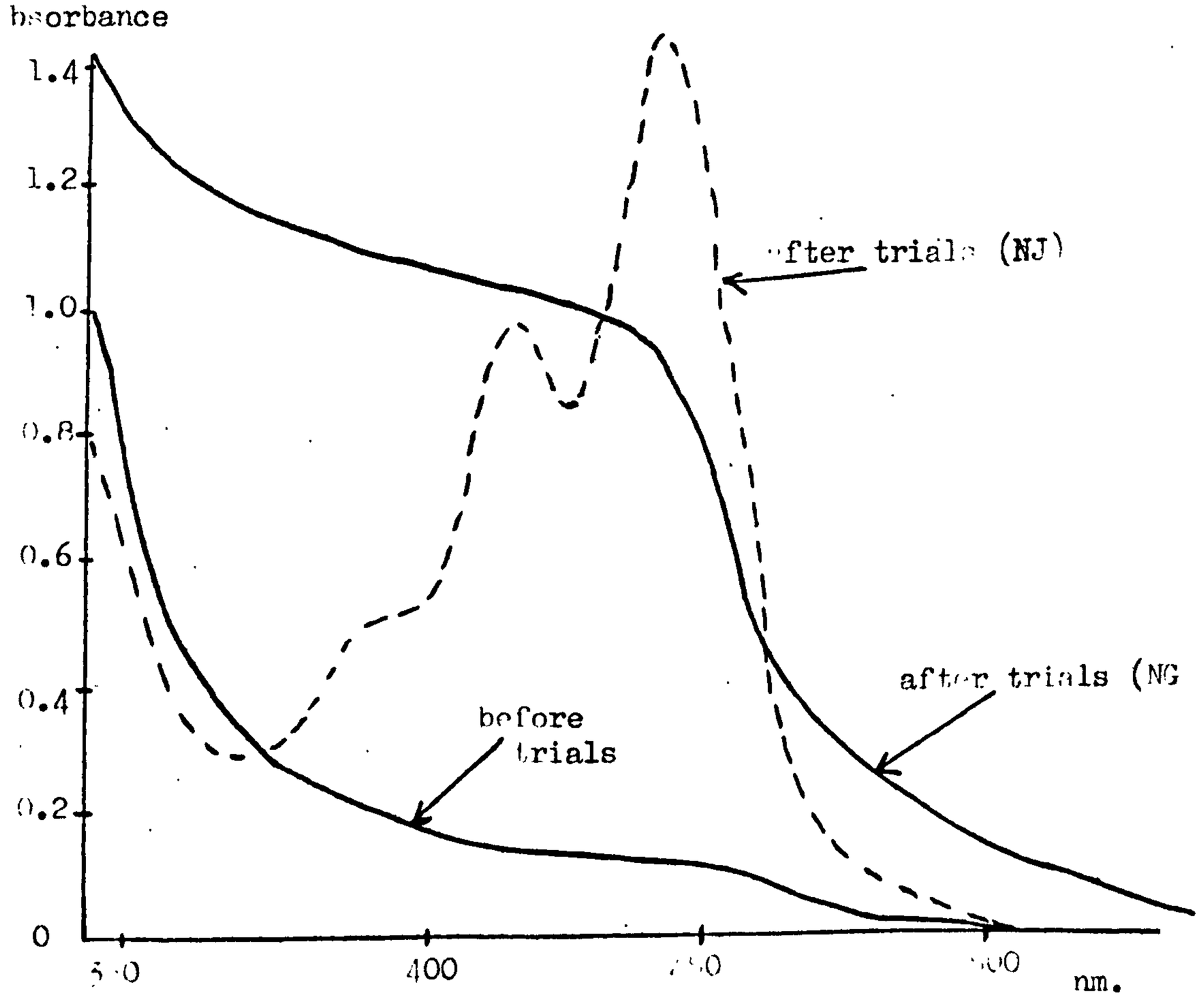


Figure 31 Changes in the visible spectra of Nitrometh.ne Extracts.



storage but this was not found in any other sample.

Pale Extract samples conversely exhibited a slight decrease in absorption intensity at most wavelengths of maximum absorption during stability trials (see Figure 32) but more particularly at 445 and 470 nm. This would be in accordance with degradation of carotenoids during the course of extract deterioration. No appreciable change in colour was noted.

2.3.5.2. Turbidity

In the marketing of Pale Extracts turbidity of the solution upon dilution of the Extract to about 2 per cent w/w with hexane (or petroleum spirits) is held as indicating the onset of rethrin degradation[14] and due to the formation of polimeric material.

Campbell and Mitchell[141] have reported the formation of resinous deposits, assumed to be polimeric material, in nitromethane concentrate samples exposed to daylight radiation (see 1.5.2.). This material was also insoluble in petroleum spirits and refined kerosene. In the present stability trials a solid resinous material separated out of the antioxidant-free Pale Extract sample AF/PF after about 15 months storage and constituted about one-fifth of the sample volume after 19 months storage. At this stage the rethrin content was 37 per cent of its original value (see 2.3.2.4.). No other sample however exhibited this phenomenon. These would appear to be extreme cases however.

With regard to turbidity upon dilution of the extract samples the majority of stored Pale Extract samples exhibited some degree of 'clouding' but this applied to samples which had undergone no rethrin loss as well as those that exhibited serious rethrin degradation upon longer storage. Conversely, Nitromethane Extract samples did not show this phenomenon, with the exception of the antioxidant-free sample AF/NA after 18 months storage, despite significant rethrin losses being recorded for some of the samples.

Turbidity of the solution upon dilution of pyrethrum extracts could not be held therefore to indicate extract deterioration in respect of rethrin degradation. The formation of polimeric material from rethrins may, in some cases, be the cause of the turbidity to some extent, but polar material formed by the degradation of the non-rethrin constituents are most probably the cause in Pale Extracts although 'clouding' of the Nitromethane sample (AF/NA) was more probably the result of rethrin degradation.

Small quantities of the autoxidation products of unsaturated fatty acid derivatives are insoluble on dilution with a non-polar solvent and autoxidised linseed oil (see 2.3.4.3.), for example, was also found to exhibit this phenomenon. The turbidity of diluted extracts which have been commercially produced and stored for a relatively short period of time (a few months) most probably indicates the onset of extract deterioration in respect of non-rethrin material and therefore indicate a relatively advanced stage of the induction period observed for rethrin degradation (see 2.3.2.).

2.3.6. Summary of stability studies

The overall stability of Pale Extract concentrates in respect of rethrin degradation was found to be much greater than had been inferred by the results of earlier workers (see 1.5.2. and 2.3.2.). Induction periods of more than six months were indicated for refined extracts. However the greater stability of Nitromethane Extracts in respect of the rate of rethrin degradation after the induction period indicated that improved refining to remove extraneous matter would further improve stability. Diluted forms of the extracts studied invariably showed greater stability than the concentrates over long periods (two years) but this is a general phenomenon of substrates labile to autoxidation.

In as much as the changes in concentration of the individual rethrins were significant the pyrethrins appeared to be less stable than the cinerins and the rethrins I content decreased faster than that of the rethrins II.

The stabilisation of pyrethrum extracts by B.H.T. was confirmed but its rate of loss from Pale Extracts was greater than from Nitromethane Extracts which inferred that the degradation of the added antioxidant, as well as the degradation of the rethrins, was enhanced by the extraneous material in Pale Extracts. The stability of pyrethrum extracts may be further due to the natural antioxidants, the tocopherols, which were shown to be present in crude extract and retained in the refining process for the production of Pale Extract.

The extraneous matter of refined pyrethrum extracts was shown to include a relatively large proportion of unsaturated fatty acid material which is known to be susceptible to autoxidation and would therefore contribute to the long-term instability of the rethrins in pyrethrum extracts.

Catalysis of the autoxidation processes is promoted by metal ion contamination and the possibility of such contamination due to the relatively high free acidity in both crude and refined pyrethrum extracts was noted. The iron content was concluded to be generally of the same order as that found naturally and therefore contamination did not appear to be a problem. However since iron contents greater than about 0.1 p.p.m. are known to catalyse autoxidation reactions it is possible that a lower iron concentration than that found naturally would lead to longer induction periods of rethrin degradation. The inclusion of small quantities of iron or addition of peroxides was shown to significantly reduce the stability of extracts in terms of peroxide accumulation and antioxidant consumption and such changes in extract composition would undoubtedly lead to greater instability in respect of the rethrins. This was supported by the results on the B.H.T.-free samples where a small but significant greater loss of rethrins was noted in samples containing added pro-oxidants.

2.4. Investigation of a novel procedure for the reconstitution of the rethrins

2.4.1. Review of present methods

The acids and alcohols which constitute the rethrin esters (see Figures 3 and 4) have been synthesised by various workers whose work has been reviewed by Elliott[64]. Pyrethrum extract is a more convenient, natural source of some of these compounds.

Pyrethrolone has been isolated from extracts by treatment to form the semicarbazone (to prevent formation of the dimeric cyclopentadienone that subsequently occurs when the carbonyl group is unprotected) followed by hydrolysis[281]. Regeneration of the alcohol yields the crystalline monohydrate which is easily separated by recrystallisation from the other, more soluble, alcohol hydrates. The pure alcohol may be obtained by dehydration, under reduced pressure, over phosphorus pentoxide[282]. A modified laboratory scheme has more recently been published by Maciver[283]. Cinerolone may be obtained pure from the mother liquors of pyrethrolone hydrate recrystallisation by acetylation and fractional distillation of the acetate mixture, when pyrethrolone acetate impurities have been found to be polymerised[282]. Jasmolone, however, is more readily obtained synthetically, by the procedure of Crombie et al[284].

Chrysanthemic acid may be obtained by the alcoholic hydrolysis of pyrethrum extract but is prepared commercially by a procedure based on the synthesis reported by Campbell and Harper[285]. Pyrethric acid is more conveniently obtained by the isolation procedure of La Forge et al[286]. The natural

isomers of the compounds are readily obtained when natural pyrethrum is the source and in the case of the synthesised compounds, purification procedures have been developed[287,288].

The reported reconstitution procedures, to form the rethrins, have to date involved the acid chloride as an intermediate[32,289-291]. The chrysanthemum acid is treated with thionyl chloride in refluxing benzene to form the acid chloride, and excess reagent, solvent and volatile side products are removed by distillation under reduced pressure. The acid chloride is esterified with the alcohol in benzene solvent containing pyridine and the product separated from the remaining reactants by chromatography on a silica column[32].

2.4.2. Reconstitution of the pyrethrins and allethrins

In the present work, it was initially thought to be advantageous to have pure samples of the principle active components of pyrethrum extract, namely pyrethrin I and pyrethrin II, in order to compare their stability in the pure state, with that in the pyrethrum extracts and thus determine whether the problem of extract deterioration was due to rethrin instability or their instability in the presence of extraneous material found in pyrethrum extracts. (This was later achieved by comparison with highly refined Nitromethane Extracts - see 2.3.2.). Further, pure compounds are generally required as standards for the development of an accurate method of analysis and it later became necessary to have reconstituted samples of the allethrins, in particular allethrin II, for the quantification of the developed g.l.c. method of rethrin analysis (see 2.1.2.5.). This prompted investigations into the reconstitution procedures.

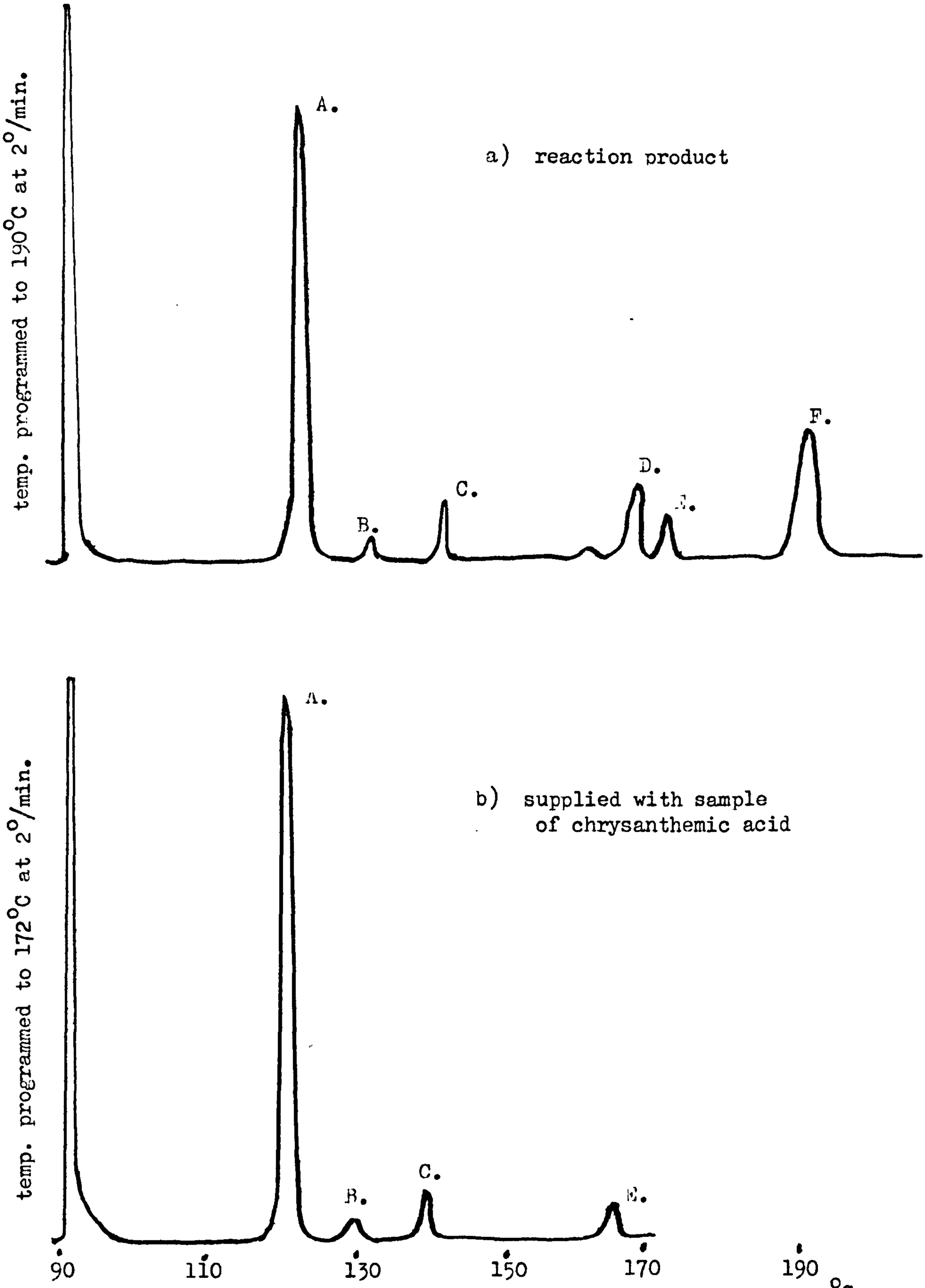
2.4.2.1. Purity of samples of the constituent acids and alcohols

Samples of (+)-trans chrysanthemic acid, (+)-trans pyrethric acid (Figure 3), (+)-cis pyrethrolone monohydrate (Figure 4) and, later, (+)-allethrolone (Figure 5; VIII) were available and their chemical purity was established by t.l.c. and g.l.c. (The isomeric purity of the samples was not important from the viewpoint of this work).

With the exception of the sample of chrysanthemic acid, all the samples were of greater than 95 per cent purity. In order to demonstrate that the major component of the impure sample was chrysanthemic acid, portions of the sample were methylated by refluxing with methanol containing one per cent mineral acid and gas chromatographed for comparison with chromatograms of methyl chrysanthemate, provided with the sample (see Figure 33). This demonstrated that methyl chrysanthemate was the major product. Small amounts of chrysanthemic acid, pyrethric acid and methyl pyrethrate were identified in the reaction mixture as well as a compound identified in the chromatograms supplied with the acid as the methyl methoxy ester of chrysanthemic acid (XXIII), a by-product of the reaction[122,292].

The nuclear magnetic resonance (n.m.r.) spectrum (Figure 34), of the reaction product was consistant with it being predominantly methyl chrysanthemate and the proton assignments shown in Table 38, are in good agreement with those which have since been published by Crombie et al[293] for methyl chrysanthemate.

Figure 33 Gas-liquid chromatograms of methylated chrysanthemic acid.

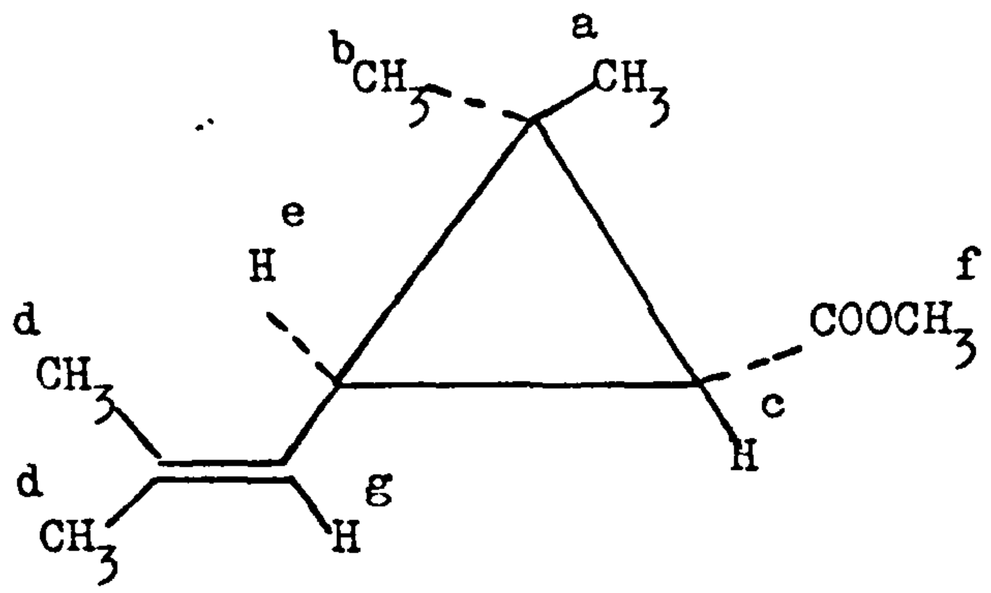


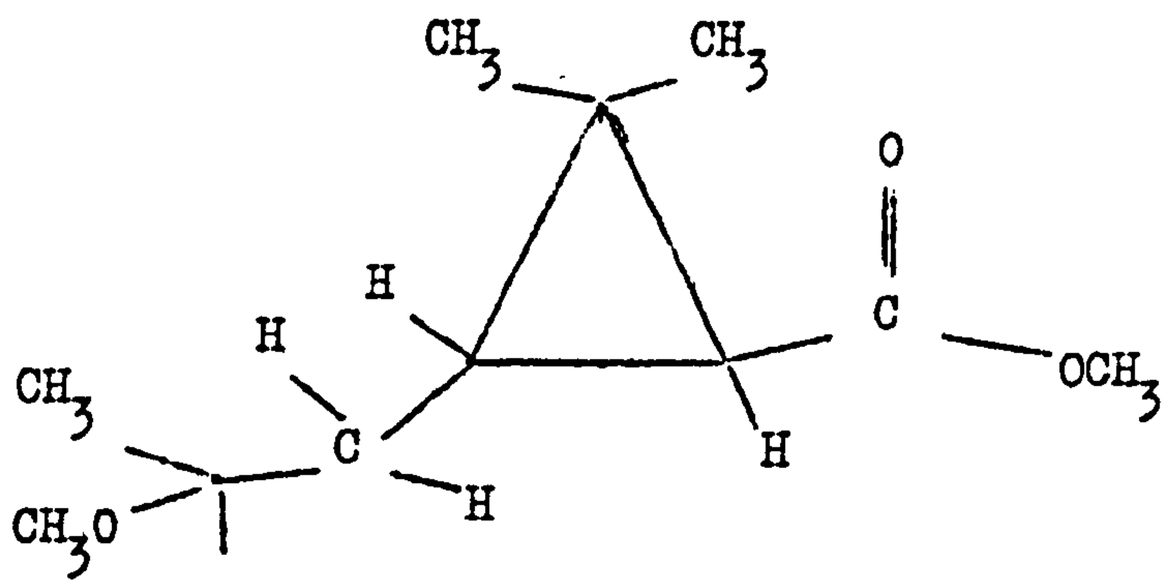
a) reaction product

b) supplied with sample of chrysanthemic acid

A = methyl chrysanthemate ; C,F = unknown found in acid ;
B = methyl methoxy ester of chrysanthemic acid ;
D = chrysanthemic acid ; E = methyl pyrethrate

Figure 34 N.m.r. spectrum of the product of chrysanthemic acid methylation.





methyl methoxy ester

of chrysanthemic acid

XXIII

Table 38 Proton assignments in n.m.r. spectrum of methylated chrysanthemic acid

Figure 34		Assignment	Crombie et al[293]	
Chemical shift	Coupling constant		Chemical shift	Coupling constant
τ	J c.p.s.		τ	J c.p.s.
8.9		$^{a}CH_3$	8.89	
8.8		$^{b}CH_3$	8.75	
8.65	d (J~4)	^{c}H	8.63	d (J~5.5)
8.3	d (J~1.5)	$^{d}CH_3$	8.31	d (J~1.5)
7.95	dd (J=4,7)	^{e}H	7.9	dd (J=5.5,8)
6.4		$^{f}CH_3$	6.33	
5.15	d (J~7)	^{g}H	5.09	d (J~8)

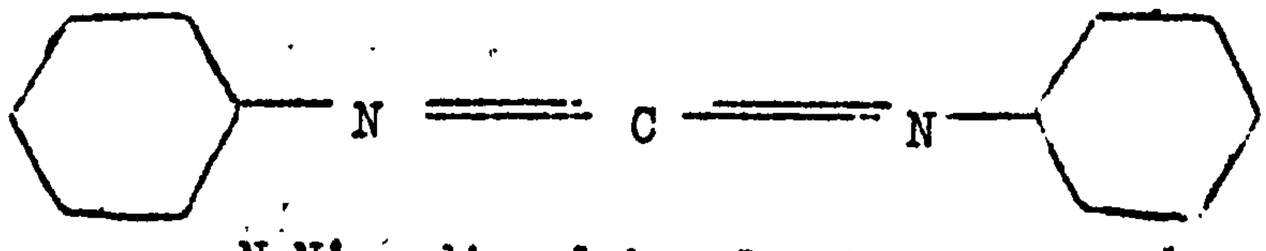
d = doublet

This sample of chrysanthemic acid was used in preliminary experiments on reconstitution but a purer batch was obtained for later studies.

2.4.2.2. Disadvantages of the Acid Chloride method and the potential of the N,N'-dicyclohexylcarbodiimide (D.C.C.I.) reagent

Samples of pyrethrin I, pyrethrin II, allethrin I and allethrin II were prepared by the Acid Chloride method following the procedure of Elliott and Janes[291] (see 3.11.2.). There were, however, several problems encountered in preparing small quantities of these rethrins which prompted an investigation into an improved method of reconstitution. In the acid chloride procedure, the component acid and alcohol are not reacted directly together since the former is first converted to its acid chloride and, in the case of pyrethrolone, the latter requires dehydration. The reaction is therefore a two or three-stage process which is also time consuming. Furthermore, in dealing with milligram quantities, the compounds are spread as thin films for a period of time, which makes them prone to autoxidative or photoxidative degradation (see 1.5.2.).

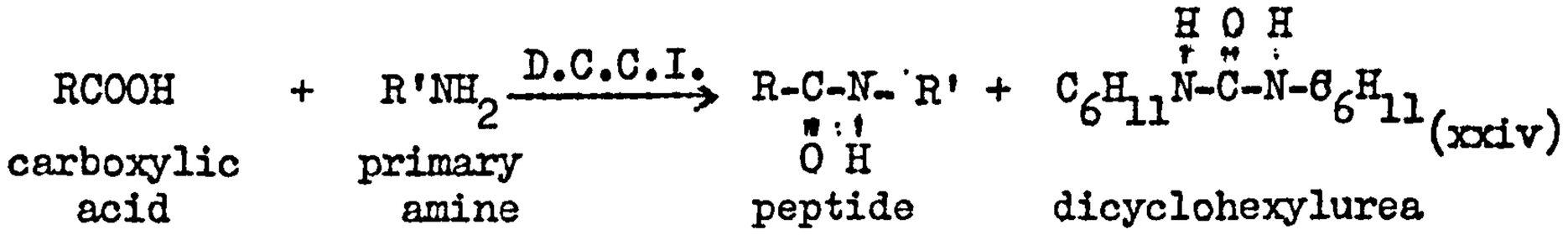
In considering the possibility of direct esterification between the acid and alcohol, the use of N,N'-dicyclohexylcarbodiimide (D.C.C.I.) (XXIV) as a condensing agent was investigated to obviate the need for pyrethrolone dehydration in pyrethrin reconstitutions and to reduce the procedure to a one-step process.



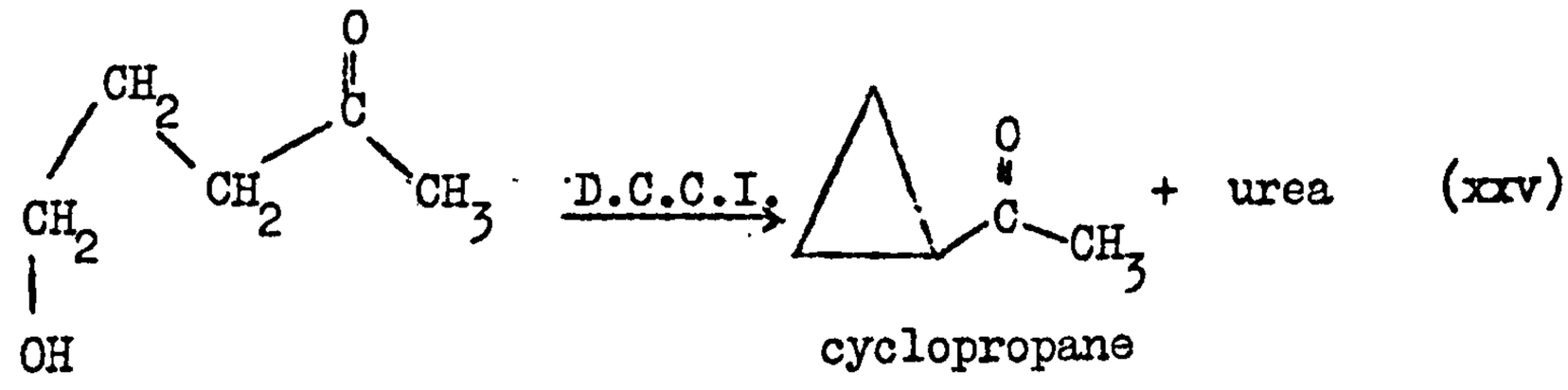
N,N' - dicyclohexylcarbodiimide (D.C.C.I.)

XXIV

This reagent has been primarily used in peptide link formation[294] (xxiv) giving improved yields over other methods, although recently it has been reported as an intramolecular dehydrating agent for ketols[295] (xxv). It was therefore considered as a dehydrating agent for alcohols and acids in the formation of esters in particular for the reconstitution of the rethrins (xxvi).

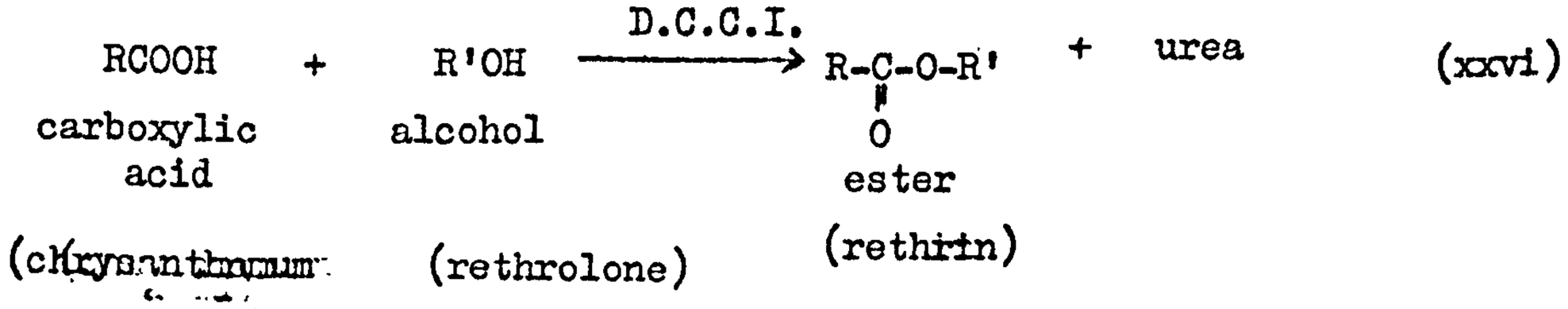


XXV



γ - hydroxy ketone

cyclopropane derivative



2.4.2.3. Attempted reconstitution of the rethrins using D.C.C.I.

Although the chrysanthemum acids were found to be soluble in a wide range of solvents, the sample of pyrethrolone monohydrate was found not to dissolve appreciably in petroleum spirits (b.p. 40 - 60°) or acetone, but was more soluble in methanol, chloroform and 1,4-dioxan. The best solvent found was chloroform : 1,4-dioxan (1:4 v/v) which was therefore used in subsequent reactions involving pyrethrolone monohydrate although chloroform was satisfactorily used for allethrolone.

Mixing of pyrethric acid, pyrethrolone monohydrate and D.C.C.I. (1:1:2 molar equivalents) in a minimum amount of solvent resulted in the formation of a white precipitate after approximately two minutes. The infra-red spectrum and melting point determination of this precipitate indicated it to be the urea by product (XXV) of the reaction. Comparison with authenticated dicyclohexyl urea by infra-red spectroscopy, t.l.c. and g.l.c. confirmed this assignment.

T.l.c. examination of the filtrate indicated a component of Rf value equivalent to that of the rethrins II although unreacted reagents appeared to still be present. The ultra-violet spectrum of this component, extracted from several chromatographic plates, showed maximum absorption at 233 nm (c.f. rethrins II) but the infra-red spectrum showed only slight similarity to that published for pyrethrin II by Elliott[296].

G.l.c. examination of the filtrate on polar (N.P.G.S.) and non-polar (Apiezon L) stationary phases confirmed the presence of the reagents, a small quantity of the urea by product

and two other components. One of these components was shown to elute with a similar retention time to that of pyrethrin II by comparison with a refined pyrethrum extract. The other component (E.C.L. = 16.0 on Apiezon L) however was clearly the major product of the reaction. As a proportion of the major product, the pyrethrin II content was estimated to be approximately 10 per cent, assuming an equal weight-response.

It was concluded therefore that D.C.C.I. was potentially a suitable reagent for the reconstitution of the rethrins from the alcohol and acid components but unless the reaction could be controlled to prevent the formation of the major product it would be an inefficient process.

Further work on this reaction was carried out using allethrolone and chrysanthemic acid since the desired product, allethrin I, has shorter retention time on g.c. columns than pyrethrin II and therefore elutes closer to the other components in the reaction system.

A gas chromatographic investigation (Figure 35) of the reaction involving allethrolone, chrysanthemic acid and D.C.C.I. in equimolar quantities confirmed the earlier work in demonstrating the formation of the corresponding rethrin as a minor product as well as another, major product (E.C.L. = 15.7 on Apiezon L). The allethrin I appeared to be present in a smaller quantity however, relative to the major product. Using methyl myristate as internal standard, present throughout the reaction, the allethrin I content was calculated to be formed in about 2 per cent yield (assuming equal weight response). On leaving to stand for a period up to 6 weeks a slight increase in the

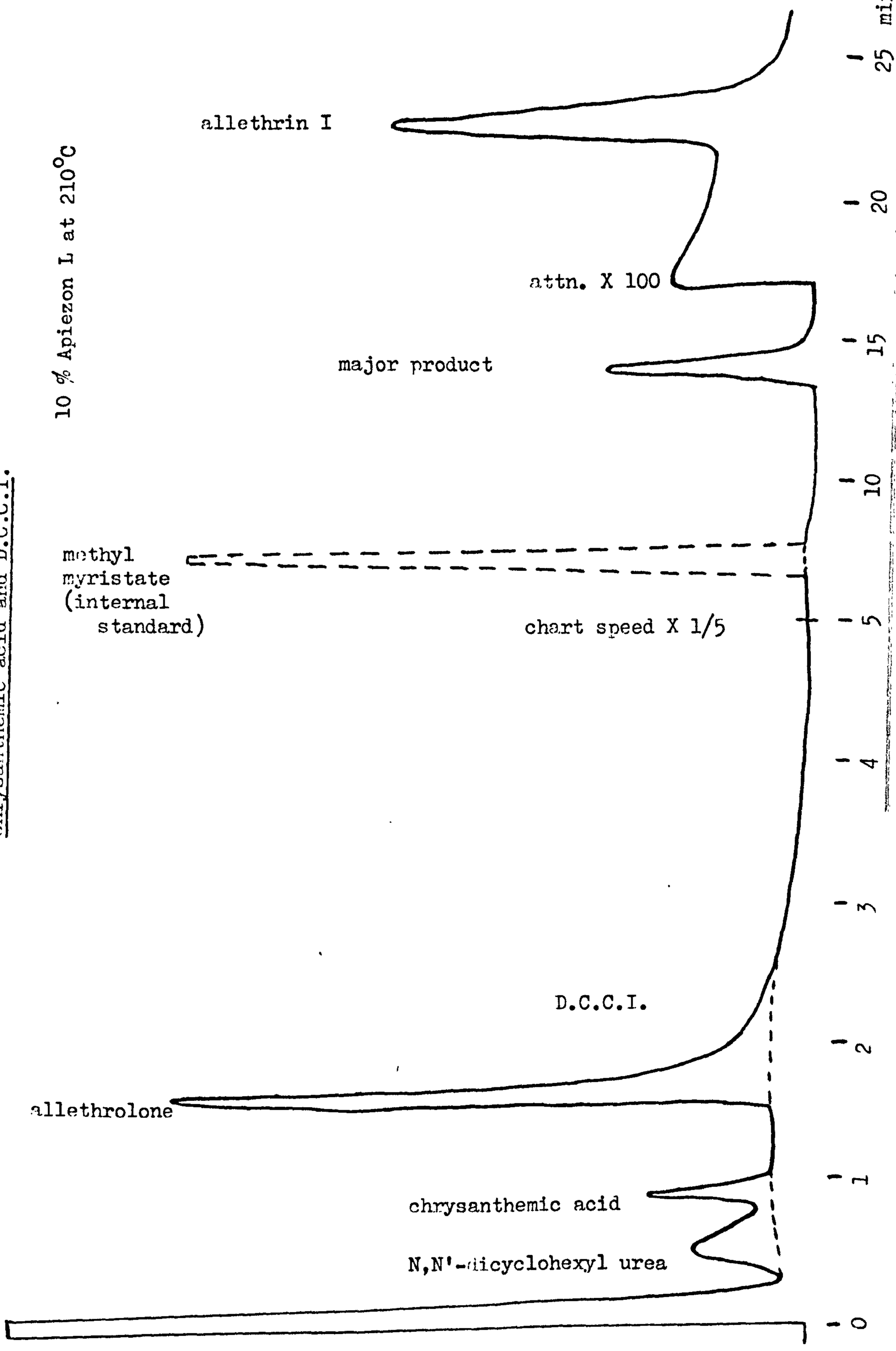


Figure 35 Gas-liquid chromatogram of the reaction mixture of allethrolone, chrysanthemic acid and D.C.C.I.

allethrin I content was noted. Maintaining a sample at 40°C for 3 weeks showed an increase from 0.5 per cent to 4 per cent yield after 9 days but this then fell to 2 per cent. This was a small but significant change in respect of the chromatogram characteristics. The concentration of the major product appeared to remain constant.

2.4.3. Reaction of chrysanthemic acid with D.C.C.I.

In order to identify the major product encountered in the reconstitution reactions involving D.C.C.I. (2.4.2.3.) the interaction of each of two of the reagents was noted. Chrysanthemic acid and D.C.C.I. in chloroform solution produced the characteristic formation of dicyclohexyl urea after 2 minutes shaking and the gas chromatogram of the reaction solution was similar to that shown in Figure 35 with the omission of the allethrin I and allethrolone components.

T.l.c. examination of the reaction mixture showed the presence of three spots with Rf values 0.0, 0.52 and 0.63 (Kieselgel HF; ethyl acetate - hexane (1:3)). The first two fractions corresponded to the urea by product and unused acid respectively. The Rf value of the product (0.63) was noted to be close to the position at which allethrin I was shown to occur (Rf 0.61) but as allethrolone was not present in the reaction allethrin I could not be the product. The similarity of the t.l.c. characteristics of the product with rethrins I in the present reaction and with rethrins II in the pyrethrin II reconstitution reaction (see 2.4.2.3.) indicated the unknown compound to be a chrysanthemumate-type compound. This undoubtedly explains the anomaly between the results of the t.l.c.

and g.l.c. analyses of pyrethrin II reconstitution reaction mixture when the former inferred relatively large yields of the required product which were not confirmed by the latter.

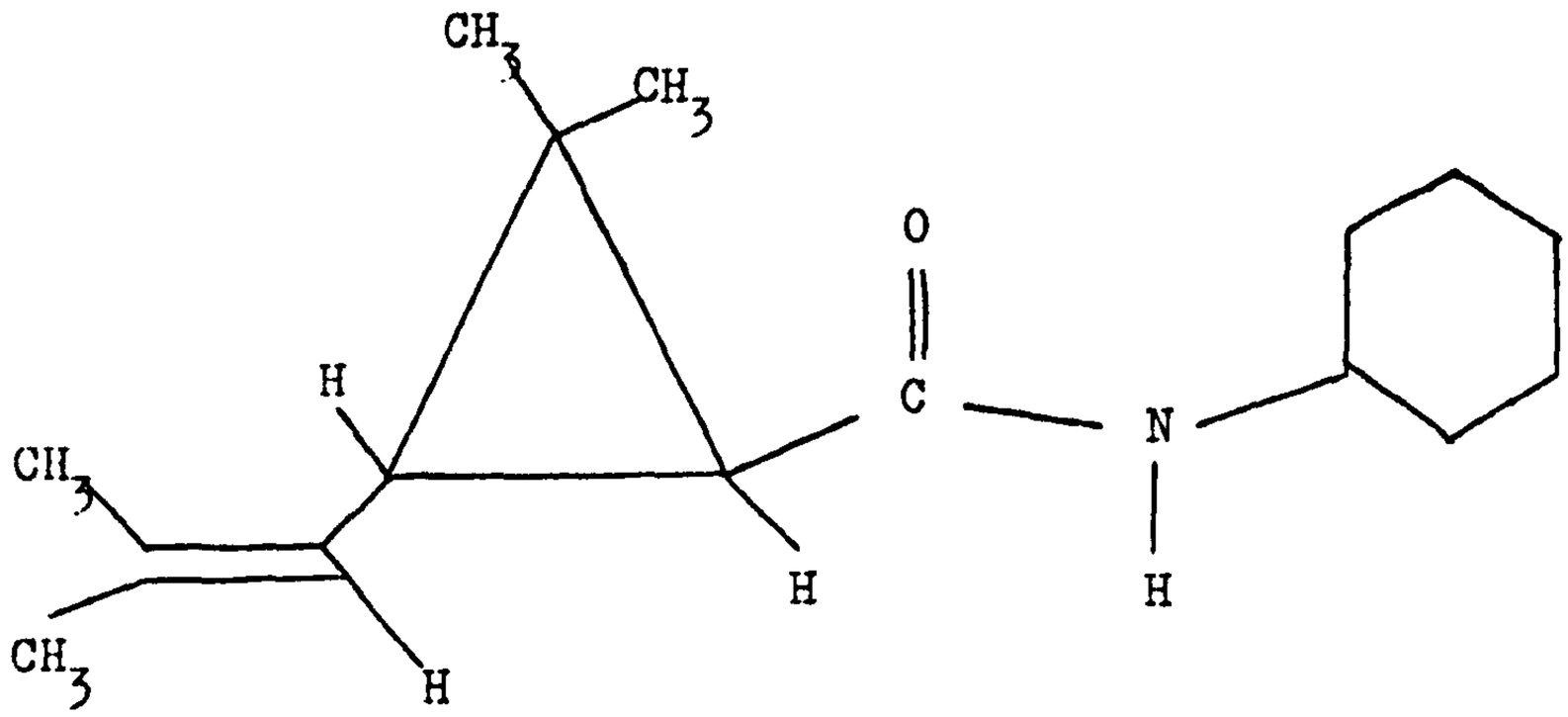
The three bands separated by t.l.c. were individually examined by g.l.c. This confirmed the assignment of urea and acid to the first two components and the least polar component (Rf 0.63) had an E.C.L. value of 15.7 (Apiezon L) but was found to be contaminated with a minor component of retention time close, but not the same as, that of chrysanthemic acid. Its presence in chromatograms of the reaction mixture was obscured by the broad elution band of the D.C.C.I. T.l.c. of the fraction confirmed only one band at Rf 0.63.

2.4.3.1. Identification of the reaction product

Since t.l.c. failed to isolate the reaction product, g.l.c. - mass spectrometry was used in an attempt to identify the compound. The mass spectrum (Figure 36) showed the parent ion at mass 249 and Figure 37 shows that the assignment of N-cyclohexyl chrysanthemamide (XXVI) is consistant with the fragmentation pattern of the mass spectrum.

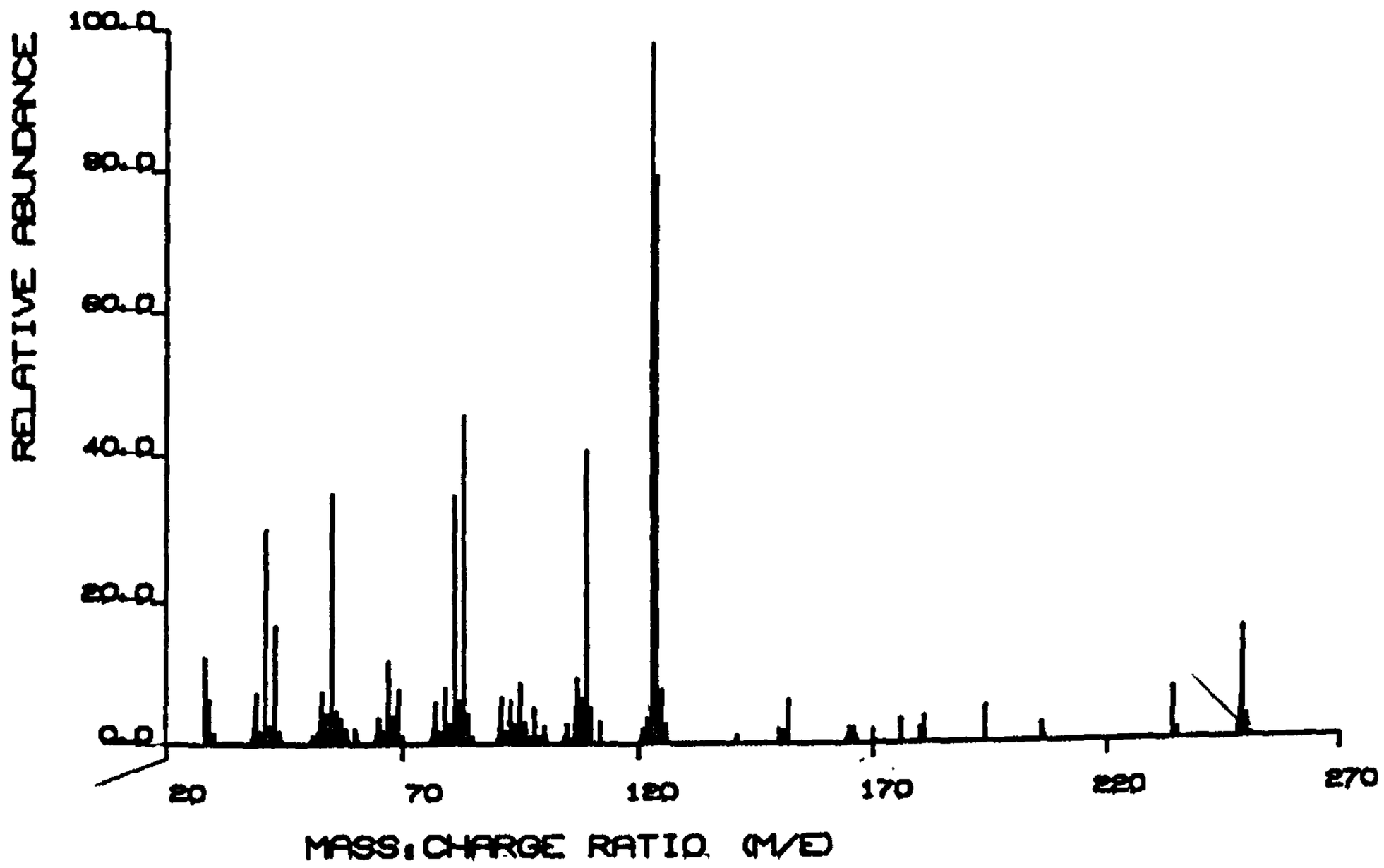
For confirmation of this assignment, chrysanthemoyl chloride was reacted with cyclohexylamine to form the amide and the product examined by g.l.c., on polar and non-polar stationary phases, by t.l.c. and by infra-red spectrometry. The gas chromatograms showed that the product had a retention time similar to that of the D.C.C.I. - chrysanthemic acid reaction product (E.C.L. = 15.7 on Apiezon L) and the thin layer chromatogram showed a single component at Rf = 0.63. The infra-red spectrum (Figure 38) showed absorption maxima attributable to a secondary

Figure 56 Mass spectrum of the major chrysanthemic - D.C.C.I. reaction product.



N - cyclohexyl chrysantemamide

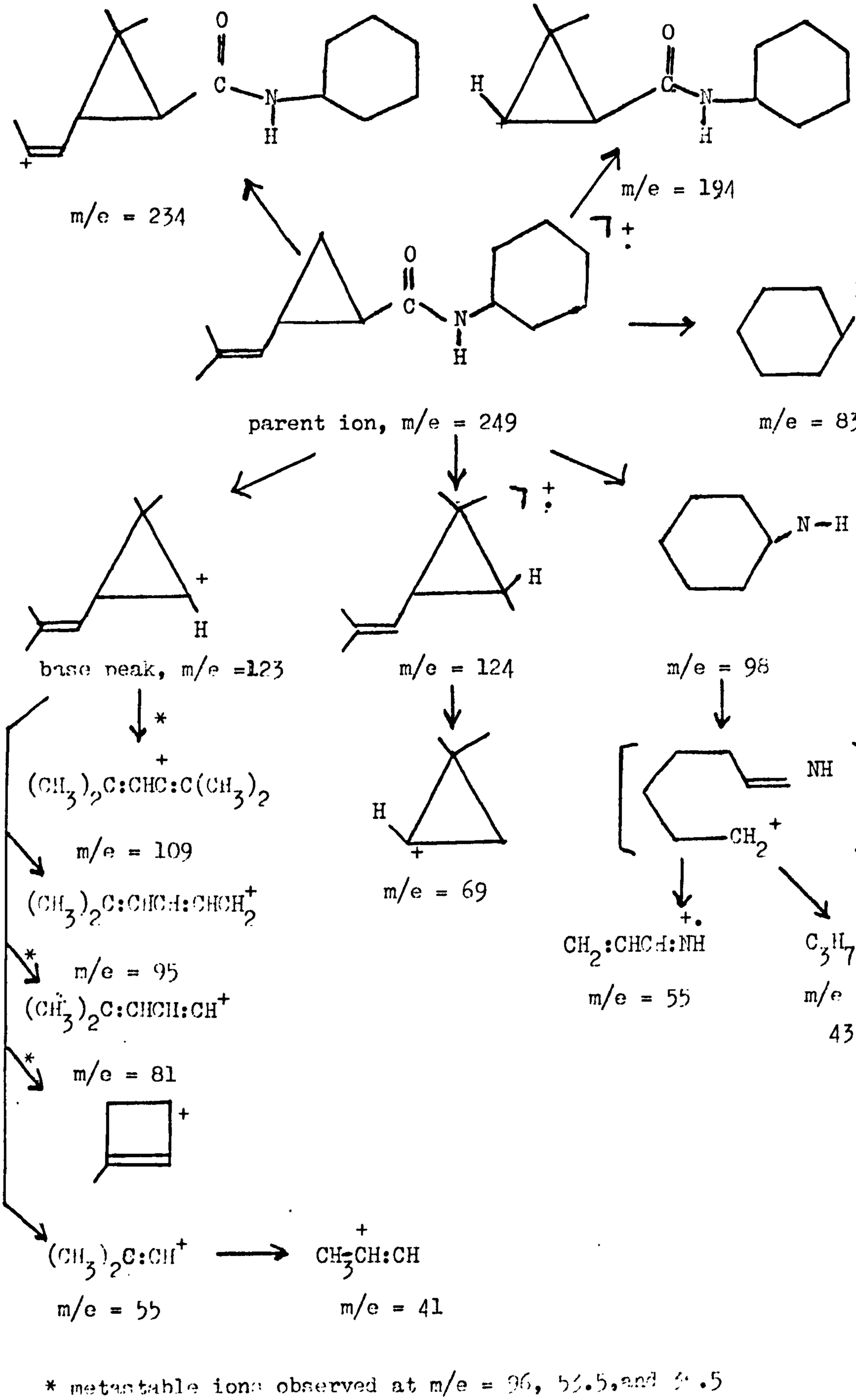
XXVI



Parent peak, m/e = 249 ; Base peak, m/e = 123 ;

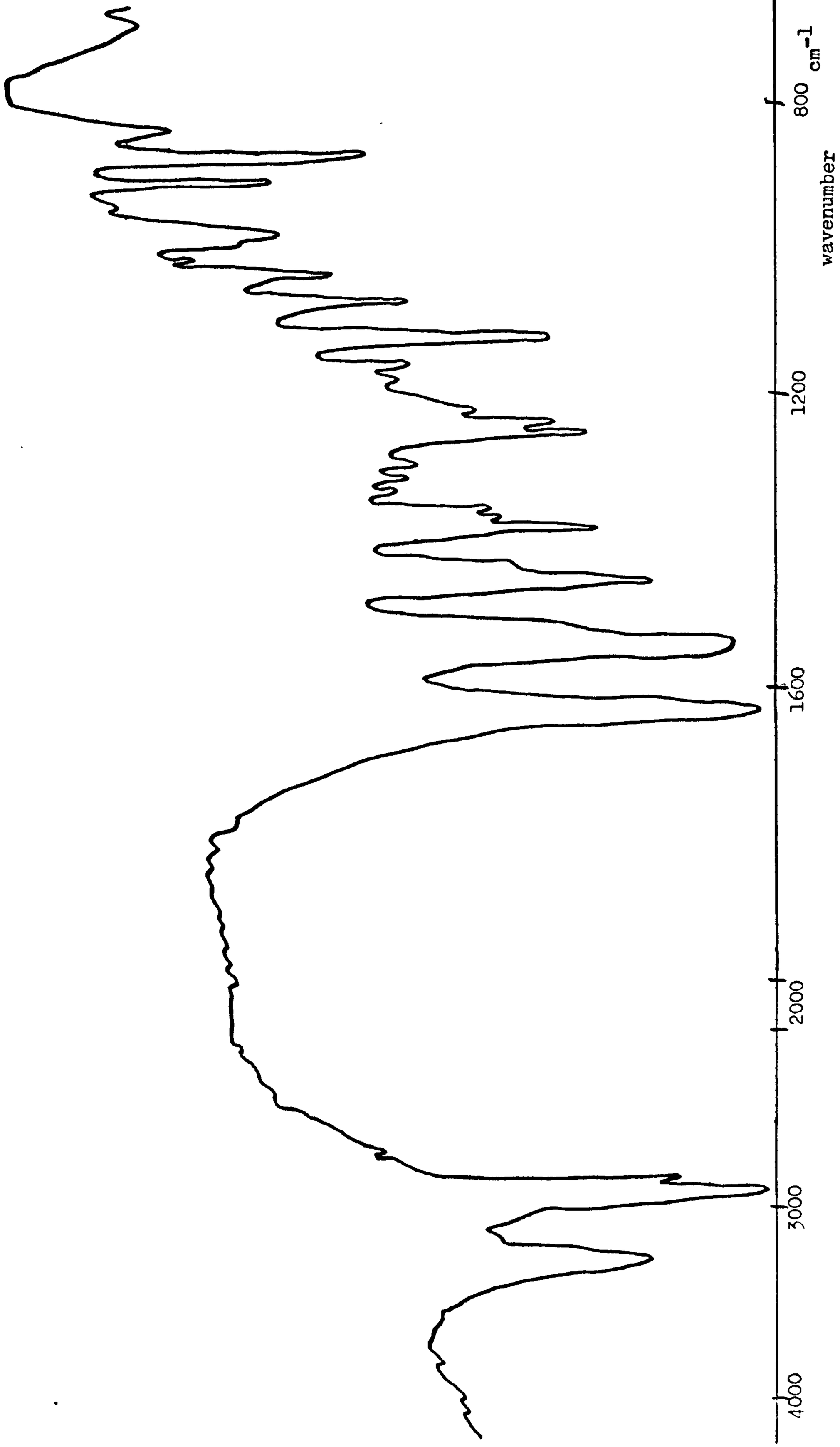
	Intensity relative to parent peak
parent peak + 1	18 - 19 %
parent peak + 2	2 %

Figure 37 Probable fragmentation pattern of N-cyclohexyl chrysanthemamide.



* metastable ions observed at m/e = 96, 55.5, and 41.5

c.f. references [297-299]



Infra-red spectrum of chrysanthemoyl chloride - cyclohexylamine reaction product.

Figure 38

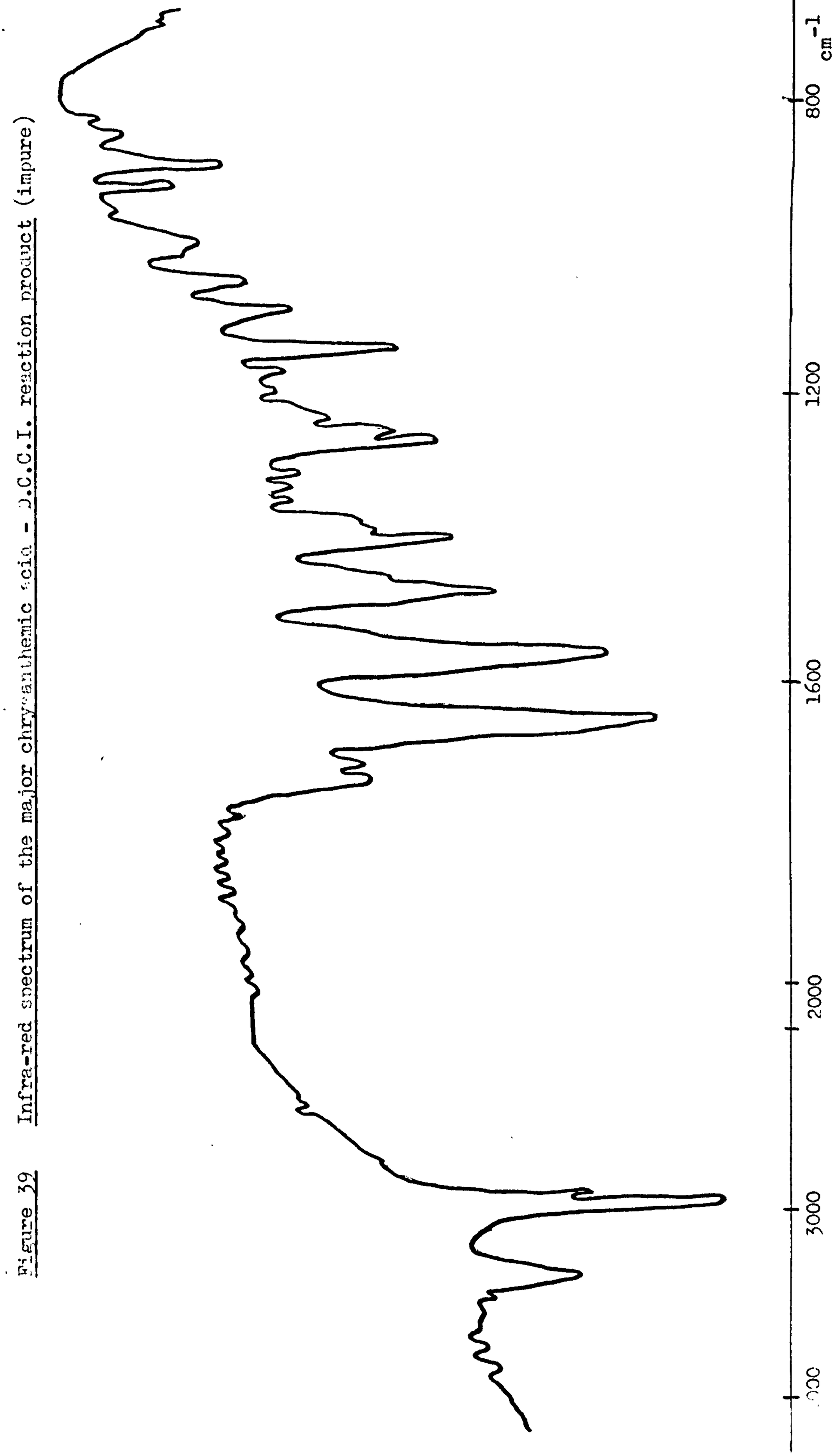


Figure 39 Infra-red spectrum of the major chrysanthemic acid - D.C.C.I. reaction product (impure)

Figure 40 Possible reaction scheme for the D.C.C.I. reactions

amide and was almost identical with the infra-red spectrum (Figure 39) of the component separated from the D.C.C.I. - chrysanthemic acid reaction by t.l.c. (Rf = 0.63) (impure - see 2.4.3.).

The major product of the reaction of chrysanthemic acid and D.C.C.I. was therefore concluded to be N-cyclohexyl chrysanthemamide and that this is the main product of the reaction in the attempted reconstitution of allethrin.

A possible reaction scheme for its formation from chrysanthemic acid and D.C.C.I. is shown in Figure 40. The intermediate (XXVII) initially formed by the reaction of D.C.C.I. and the chrysanthemum acid (equation xxvii) may undergo several reactions. By reacting with an alcohol such as the rethrolones (equation xxviii) the ester (XXVIII) and the dicyclohexyl urea (XXV) are formed. Competing reactions however include reaction with a second acid molecule (equation xxviii; R"OH = chrysanthemum acid) whereby the product is the anhydride (XXX). An intra-molecular reaction (equation xxix) may yield the amide (XXVI) and an isocyanate (XXIX) and an inter-molecular reaction (equation xxx) involving two molecules of the intermediate (XXVII) also yields the amide and products which subsequently yield dicyclohexyl urea (XXV), the acid anhydride (XXX) and cyclohexyl isocyanate (XXIX) (equation xxxi). The formation of chrysanthemum acid anhydride or cyclohexyl isocyanate was not however investigated.

3. EXPERIMENTAL.

3.1. General

3.1.1. Solvents

All solvents were of reagent grade unless otherwise stated.

Diethyl ether was dried with molecular seive (size 4A) for 24 hours and then allowed to stand in contact with sodium wire. Benzene was dried with sodium wire. Hexane was "low in aromatic content" grade. Chloroform and acetic acid were Analar reagents. Analar 1,4-dioxan was passed down a silica column before use. Absolute alcohol was shown to be spectroscopically pure (no absorption in ultra-violet spectrum). Shellsol T was a commercial, iso-paraffinic solvent marketed by Shell (b.p. 181 - 187°C (> 95%); free of alkenes by infra-red absorption (no bands at > 1600 cm^{-1} (C=C stretch) or > 3000 cm^{-1} (alkene C-H stretch).

3.1.2. Instrumentation

The majority of gas-liquid chromatograms were obtained on Philips PV 4000 series chromatograph fitted with a flame ionisation detector (F.I.D.) for which the recommended hydrogen and air flow rates were 20 and 240 ml. per minute respectively. The carrier gas used was nitrogen unless otherwise stated.

Infra-red absorption spectra were obtained on either a Unicam SP 200 or a Perkin-Elmer 457 spectrophotometer as liquid film samples or Nujol mulls on rock salt plates or as potassium bromide discs. Calibration was achieved using a polystyrene film.

Ultra-violet and visible absorption spectra were obtained on a Unicam SP 800 or SP 8000 recording spectrophotometer or, for fixed wavelength absorption measurements, on a Unicam SP 500

spectrophotometer. Solutions were made up in absolute alcohol unless otherwise stated and solvent was used as reference. Calibration was achieved using a Holmium filter.

Nuclear magnetic spectra were obtained on a Perkin-Elmer R10 N.M.R. spectrometer operated at 60 MHz using approximately 20 per cent solutions in carbon tetrachloride with tetramethyl silane as internal standard.

The mass spectrum was obtained on a g.l.c. (Perkin-Elmer F11) - mass spectrometer (Perkin-Elmer Hitachi) linked instrumentation. Blank corrections were made and the spectra plotted by the use of the computer program, MSPEC (by Dr. J. Wilson).

The computer used throughout this work was an Elliott 4120 model with 16 K store. Input was by paper tape and line printer, digital plotter and magnetic tape peripheral devices were available.

3.1.3. Thin layer adsorption chromatography

3.1.3.1. Preparation of thin-layer chromatographic plates

Distilled water (~ 60 ml) was added to silicic acid (Kieselgel H (Merck)) (25 g.) to form a slurry. Layers of 0.025 cm. were produced on grease-free glass plates (5/10/20 cm. x 20 cm. x 0.3 cm.) by spreading the slurry onto the plates using a commercial applicator. These were heated in an oven (with vent holes open) at 110°C for 2 hours and then allowed to cool.

Chromatographic plates of stationary phase containing a fluorescent indicator or silver nitrate were similarly prepared using Kieselgel $HF_{254+366}$ (Merck) or Kieselgel H containing 2% silver nitrate (added during the slurry formation), 300 respectively.

The latter type of plate was stored in light-proof paper or aluminium foil.

3.1.3.2. Development of chromatographic plates and viewing methods

A solution of the sample was spotted onto a baseline 1.5 cm from one end of the plate and the solvent allowed to evaporate. The plate was then placed in a glass tank lined with filter paper containing the eluant and the sample eluted on the plate in an ascending manner over 16 cm. The components of the sample were then viewed by one or more of several methods after allowing the eluant to evaporate. The following methods were used.

(i) Viewed directly under ultra-violet light of wavelength 254 nm. (only when stationary phase contained fluorescent indicator). Ultra-violet absorbing compounds appeared as dark spots on a green fluorescent background.

(ii) Sprayed with 0.1% dichlorofluorescein (sodium salt) in ethanol and viewed under ultra-violet light of wavelength 254 nm. Ultra-violet absorbing compounds appeared as yellow-green spots.

(iii) Sprayed with 12% phosphomolybdic acid in ethanol and heated. Unsaturated components (and saturated ones on strong heating) appeared as green spots on a yellow background.

(iv) Sprayed with 50 per cent sulphuric acid and heated at 150°C for 10 minutes. All compounds are carbonised and appear as brown-black spots.

3.1.4. Column adsorption chromatography

Glass columns, with a sintered glass disc near the botton end, were packed by slowly adding a slurry of the adsorbent to the top of the column full of hexane and running out excess solvent at the botton of the column.

For alumina packed columns the activity of the adsorbent is often critical. The activity of the alumina was prepared as Grade III alumina by sprinkling distilled water (20 ml) on Woelm Grade I alumina (500 g.) and the mixture rotated on a rotary evaporator for at least one hour. The activity of this alumina was determined by the elution of Sudan Red, Sudan Yellow and methoxyazobenzene. Each of these components (3 x 40 mg.) were dissolved in benzene - petroleum spirit (b.p. 40 - 60°C) (1:4) (100 ml.). A portion of this dye solution (10 ml.) was eluted down a column of the alumina (5 cm. x 15 mm) with benzene - petroleum spirit (b.p. 40 - 60°C) (1:5) (20 ml.). The Sudan Red was retained at the top of the column, Sudan Yellow eluted partly down the column and methoxyazobenzene was completely eluted from the column. This indicated the alumina to be Grade III on the Woelm scale and was stored in dessicators until used.

3.1.5. Gas-liquid chromatography

3.1.5.1. Chromatographic columns

Gas chromatographic analyses were carried out using columns containing the following types of packing:

(i) 10 per cent Apiezon L grease on 80 to 100 mesh Diatomite C or 100 to 120 mesh Chromosorb W cured at 270°C.

(ii) 12.5 per cent polyethylene glycol adipate (PEGA) on 85 to 100 mesh Celite, cured at 190°C.

(iii) 1 per cent neopentyl glycol succinate (N.P.G.S.) on 80 to 100 mesh Chromosorb W, cured at 200°C.

(iv) 3 per cent methyl silicone gum (5 SE-30) on 80 to 100 mesh Chromosorb W, cured at 250°C.

The column packings were prepared 'by adding the required percentage weight of stationary phase to a slurry of the stationary phase support in chloroform. With gentle stirring of the mixture the chloroform was evaporated off by gentle heating and the last traces of chloroform allowed to evaporate overnight. The coated support was seived to obtain the correct particle size with gently brushing of the material to avoid breaking-up the particles.

All the columns were stainless steel and were 6 ft by 0.25 in. i.d. except for those containing N.P.G.S. which were 3 ft by 0.25 in. i.d. unless otherwise stated. The columns were packed by applying a slight vacuum to oneend and adding the packing material at the other whilst gently vibrating the column. Both ends of the column were stoppered with fine glass wool. The columns were then cured of column bleed at the temperature limits shown for each type of packing.

3.1.5.2. Preparation of g.l.c. standard methyl esters

The methyl esters of lauric, myristic, palmitic and stearic acids were prepared by the following procedure.

The fatty acid (100 mg) was heated under reflux with 10% boron trifluoride in methanol '15 ml) for 2 minutes. The mixture was cooled, distilled water (20 ml) added and washed twice with petroleum spirit (b.p. 40 - 60°C) (2 x 15 ml.). The combined washings were washed free of acid with distilled water and then dried over anhydrous magnesium sulphate for more than 2 hours. After filtration, the solvent was removed under reduced pressure at room temperature on a rotary

evaporator. The product was shown to be free of the acid component by infra-red spectroscopy (absence of absorption bands at 1700 cm^{-1} (acidic C=O stretch) and 2700 to 3500 cm^{-1} (acidic O-H stretch)) and to be characteristic of the ester (strong absorption at 1740 cm^{-1} (ester C=O stretch)). Chromatographic examination by g.l.c. and t.l.c. (viewed by spraying with 50% sulphuric acid and heating) indicated only one component.

3.1.5.3. Equivalent chain length (E.C.L.) determinations

The E.C.L. of the components of gas chromatographed samples were determined by reference to a series of n-alkanes or methyl esters of fatty acids.

The reference series of compounds were chromatographed under the same conditions as the sample preferably immediately prior to and/or after the sample. A plot of the logarithm of their relative retention times against their carbon number (no. of carbon atoms per molecule of alkane or acid) was used to provide a calibration graph. The E.C.L. of a component was then determined as the carbon number corresponding to its relative retention time.

3.2. Pyrethrum Extracts

3.2.1. Extracts supplied by the Pyrethrum Marketing Board (P.M.B.)

Table 39 lists the samples of pyrethrum extracts which were supplied by P.M.B. and their reported rethrin content as determined by the A.O.A.C. 8th Edition method of analysis[77]. Rethrin analyses, determined by the developed g.l.c. method, and the involatile content are also shown where these were determined. All the samples were dispatched by air from Kenya and stored at -5°C on receipt.

Table 39 Pyrethrum Extracts supplied by P.M.B.

Extract sample	% Rethrin content		% Involatile content (w/w)	Container type
	A.O.A.C. RI ; RII	g.l.c. RI ; RII		
Commercial oleoresin	~ 22	-	-	metal
B.H.T.-free oleoresin	16.6; 14.5	-	-	glass
Commercial Pale Extract concentrate	20.24; 20.86	24.0; 12.85	63.5	metal
B.H.T.-free Pale Extract (1)	12.6; 14.0	15.3; 9.2	52.7	glass
B.H.T.-free Pale Extract (2)	-	14.7;10.45	55.0	glass
World Standard Extract *	11.4; 9.9	10.6; 6.1	~30	glass

* a blend of Pale Extracts[175]

3.2.2. Laboratory preparation of Nitromethane Extracts

A procedure based on the method reported by Barthel, Haller and La Forge[16] was used. Commercial oleoresin (100 g.) (ex. P.M.B.) was shaken with hexane or petroleum spirit (b.p. 40 - 60°C) (50 ml.) and stirred for 10 minutes. This solution was extracted three times with nitromethane (3 x 125 ml.) by stirring for 30 minutes. The mixture was allowed to settle (~ 10 minutes) and the botton nitromethane layers separated from the hydrocarbon layer.

The combined nitromethane solutions, which were dark green in colour, were stirred for 30 minutes with charcoal (7 g.) (ex P.M.B.) and filtered through a bed of cellulose to yield a pale yellow-green filtrate. Retreatment with charcoal produced a small improvement upon the degree of decolourisation. The use of other, commercially available samples of charcoal was less efficient and generally resulted in some of the charcoal passing through into the filtrate.

The solution was concentrated under reduced pressure at room temperature on a rotary evaporator and the resulting solution made up to volume in a graduated flask (50 ml.). The concentration of involatile material was determined on an aliquot of the solution and found to be approximately 30 per cent of the oleoresin sample.

3.2.2.1. Demonstration of the efficiency of extraction

The combined nitromethane extraction solutions and the remaining hydrocarbon solution were examined by t.l.c. on silicic acid containing fluorescent indicator (Kieselgel $HF_{254+366}$) using ethyl acetate - hexane (1:3) as eluant[12,118,301,302] (see Table 40). The rethrins appeared as dark spots on a green fluorescent background when viewed under ultra-violet light (wavelength = 254 nm.). Spraying with 12 per cent phosphomolybdic acid and heating indicated unsaturated compounds and heating at 150°C after spraying with 50 per cent sulphuric acid indicated all the compounds present by their carbonisation.

Table 40 T.l.c. examination of rethrin extraction solutions

Viewing system	1		2		3	
Solution	CH_3NO_2	alkane	CH_3NO_2	alkane	CH_3NO_2	alkane
Rf					0.82 vf	
		0.78 v		0.77		0.78 s
			0.47		0.68 vf	
	0.53 s		0.51		0.53	
			0.41	0.41	0.42 f	0.42 s
	0.36 s		0.34		0.36	
			0.28	0.28	0.29	0.29
						0.18
			0.13		0.14	0.14

1. = directly under u.v.

2. = 12% phosphomolybdic acid spray and heat

3. = 50% sulphuric acid spray and heat at 150°C

v = visible, s = strong, f = faint, v.f. = very faint

The components eluting at Rf 0.36 and 0.53 were removed from the plate (see 3.1.3.) and examined by g.l.c. (see 3.3.3.1.). The less polar spot (Rf = 0.53) was found to be rethrins I and the more polar one to be rethrins II.

In an attempt to achieve further resolution of the individual rethrins by t.l.c. the nitromethane fraction was examined on a 2% silver nitrate/silicic acid chromatographic plate^{300} (see 3.1.3.). Plates were developed with a range of eluants (Table 41) and viewed under long wavelength (366 nm.)

Table 41 Attempts to resolve the individual rethrins by t.l.c.

Eluant	Rf values of components observed under u.v.	
1:19 ethyl ether - petroleum spirit (40 - 60°C)	0.08	0.03
1:3 ethyl acetate - hexane	0.29	0.16
1:1 ethyl acetate - hexane	0.51	0.42
7:3 ethyl acetate - hexane	0.73	0.60

2% silver nitrate - silicic acid chromatographic plates

ultra-violet light after spraying with 0.1% dichlorofluorescein. No further resolution of the rethrins was achieved.

The results of this investigation indicated that most of the rethrin content was extracted from the alkane solution of commercial oleoresin by three portions of nitromethane.

3.2.3. Laboratory preparation of Pale Extracts

A laboratory procedure based on the outline[14] of the commercial process for the refining of pyrethrum extracts was developed for the preparation of Pale Extracts.

Commercial oleoresin (100 g) (ex P.M.B.) was extracted twice with methanol (2 x 600 ml.). by stirring for 30 minutes. Solid carbon dioxide was periodically added to saturate the system with carbon dioxide and maintain a temperature of - 10 to -20°C. The mixture was allowed to settle (- 10 minutes)

and the liquid containing a suspension of bright yellow flakes was decanted from the dark brown residue. The combined solutions were filtered through a sintered glass funnel at -20°C. The solvent was removed from the filtrate under reduced pressure at room temperature on a rotary evaporator to yield a yellow-brown viscous oil.

Hexane (low in aromatic content, G.P.R. Hopkins and Williams) (150 ml.) and charcoal (ex P.M.B.) (10 g) were added to the methanol concentrate and the mixture stirred for 30 minutes. This was then filtered through a bed of cellulose to yield a pale yellow-green filtrate which was retreated with charcoal to yield a paler solution.

The solution was concentrated under reduced pressure at room temperature on a rotary evaporator and the resulting solution made up to volume in a graduated flask (50 ml.). The concentration of involatile material was determined on an aliquot of the solution and found to be approximately 34 per cent of the oleoresin sample.

In commercial samples the decolourised concentrate is diluted with colourless kerosene or Shellsol T (a commercial iso-paraffinic solvent).

3.2.4. Extracts for stability studies

3.2.4.1. Long term stability trials (see 2.3.)

A Nitromethane Extract and a Pale Extract were prepared from commercial oleoresin as described in 3.2.2. and 3.2.3. respectively. The concentration of involatile material in each extract was determined by removing the solvent from an aliquot under reduced pressure at room temperature. Portions of the extracts

were then suitably diluted with the appropriate solvent (nitromethane or hexane) to yield samples NK, NJ, NG, NF and NA and PC and PB respectively (see Table 42). A further Nitromethane Extract yielded sample NE and a Nitromethane Extract prepared from B.H.T.-free oleoresin was used to prepare samples AF/NA and AF/NB. The B.H.T.-free Pale Extract sample was supplied by P.M.B. (see 3.2.1.) and was used without dilution.

The concentration of non-volatile material per unit volume in each sample was calculated by determination of the density of the sample (see Table 42). This was achieved by weighing a portion in a weighed 50 µl. pipette.

The samples were stored in half-full, screw-top glass containers (12 ml. capacity) on a bench in the laboratory. (Sample NG was stored in a full container). Any remaining extract solution was stored in the dilute form in the refridgerator.

3.2.4.2. Enhanced deterioration studies (see 2.3.4.5.)

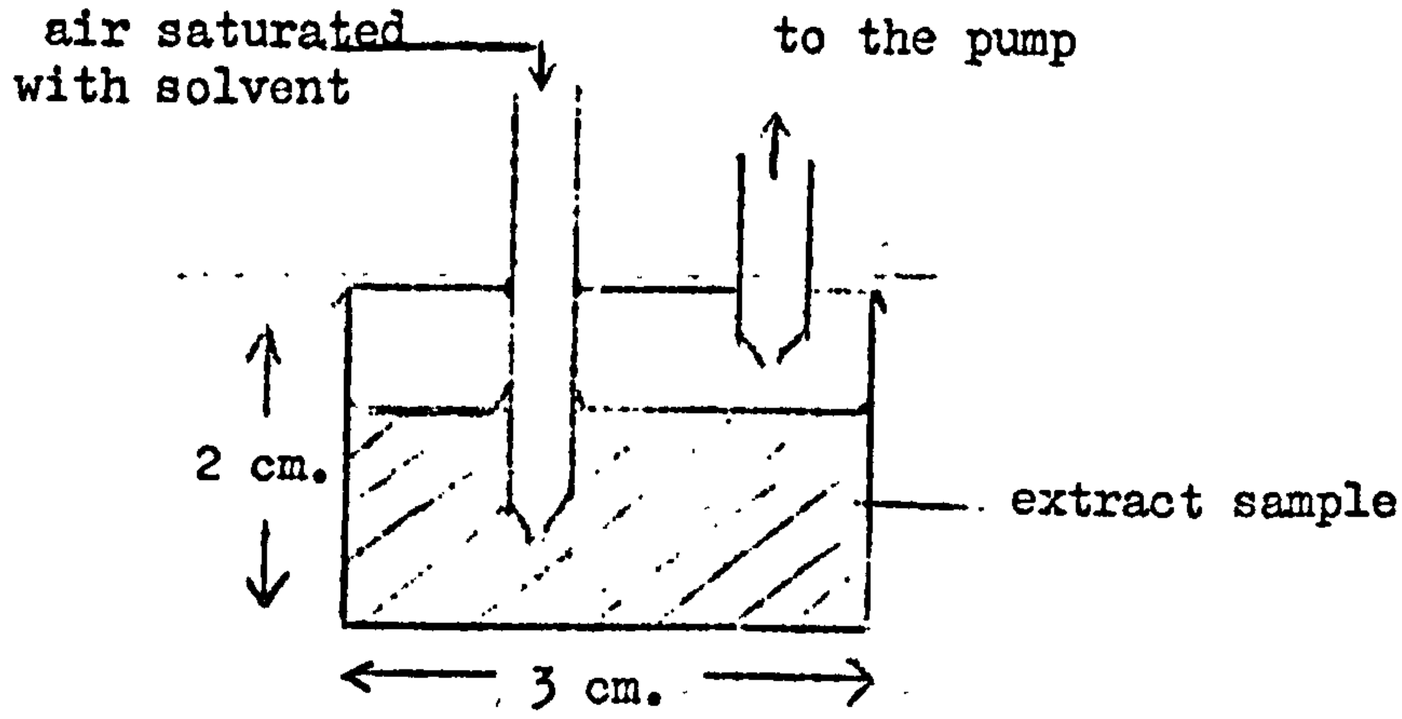
Portions of the Pale Extract concentrate and of the B.H.T.-free Pale Extract (2) (see 3.2.1.) were each mixed with a known weight of ferric stearate ($(C_{17}H_{35}COO)_3Fe$) solution (0.1% in Shellsol T) so that the iron content of each sample was approximately 2 p.p.m. Peroxidised linseed oil (peroxide value = 600 milliequivalents per kilogram) was added to one further portion of each extract so that the peroxide value of each sample was approximately 20 milliequivalents per kilogram. A further portion of each extract were used as control samples.

Table 42 Refined extract samples for stability studies

Sample	Non-volatile content % w/w	Density g./ml.	Non-volatile content % w/v
Nitromethane Extract (1)			
NK	63.8	1.07	68.3
NJ	63.8	1.07	68.3
NG*	63.8	1.07	68.3
NF	42.3	1.08	45.5
NA	19.65	1.09	21.4
Pale Extract			
PC	76.3	0.90	68.8
PB	44.9	0.76	33.8
Nitromethane Extract (2)			
NE	62.8	1.07	67.5
B.H.T.-free Nitromethane Extract			
AF/NA	79.0	1.07	84.5
AF/NB	32.0	1.07	34.2
B.H.T.-free Pale Extract			
AF/PF	52.7	0.89	47.2

* stored in full container

Figure 41 Container for induced degradation studies



Each sample was contained in a shallow glass container as shown in Figure 41 and air, saturated with solvent (Shellsol T) was drawn through the sample (~ 10 ml. per min.). The weight of each container-plus-sample was checked periodically (~ daily) and maintained at its original weight by the addition of solvent if necessary. (Allowance was made for the removal of portions for analyses).

3.3. The developed g.l.c. method of rethrin analysis (see 2.1.)

3.3.1. Purity of allethrins (see 2.1.2.9.)

Two samples of commercial allethrin (allethrin I) were supplied by the Cooper Technical Bureau, Berkhamsted and the Pyrethrum Marketing Board, kenya respectively.

The purity of these samples, used as internal standards for rethrin analysis, was determined by a procedure based on two variants of the D.N.P. method described by Head[100,101] and further demonstrated by chromatographic investigations.

3.3.1.1. The D.N.P. methods

Procedure 1

A weighed portion of the sample (containing ~30 mg of allethrin I) was dissolved in chloroform (Analar, 3 ml.). Dinitrophenylhydrazine (D.N.P.) reagent (1.98 g Analar in 210 ml. methanol and 3 ml. 10 N hydrochloric acid) (7.5 ml.) was added and the mixture periodically shaken for 3.5 hours. This was then extracted with hexane (1 x 100 ml, 5 x 20 ml) and the combined hexane solutions were washed with distilled water (2 x 30 ml.). After filtration through dry cotton wool the solution was evaporated to dryness under reduced pressure at room temperature on a rotary evaporator.

The residue was dissolved in ethyl acetate - hexane (1:19) (30 ml.). This was slowly added to a column of alumina (prepared as in 3.1.4.) in hexane (30 cm. x 1 cm.) and eluted with ethyl acetate - hexane solvent (1:19) until the third yellow band of the forerun had eluted from the column. The allethrin I d.n.p.h. was eluted with ethyl acetate - hexane solvent (1:9). This fraction was made up to volume in a graduated flask (100 ml.). An aliquot (5 ml.) was evaporated to dryness at room temperature under reduced pressure and the residue dissolved in spectroscopically pure absolute ethanol and made up to volume (100 ml.). The absorbance of this solution was determined on a Unicam SP 500 spectrophotometer at 377 nm. (confirmed as λ_{max} on a recording spectrophotometer) in a 1 cm. cell.

Procedure 2 ('rapid' variant[101])

A weighed portion of the sample (containing ~ 30 mg. allethrin I) was dissolved in chloroform (Analar, 1 ml.) and D.N.P. reagent (see Procedure 1) (5 ml.) added. The mixture was refluxed (15 minutes) and hexane (10 ml.) added. On cooling to room temperature the mixture was made up to volume in a graduated flask (100 ml.) and allowed to settle (~ 15 minutes). Excess D.N.P. separated out at the bottom of the flask.

A portion (5 ml.) of the supernatant liquid was evaporated to dryness at room temperature under reduced pressure and the residue dissolved in spectroscopically pure absolute alcohol and made up to volume (100 ml.). The absorbance of this solution and of a blank was determined on a Unicam SP 500 spectrophotometer at 377 nm. (λ_{max} confirmed as λ_{max} on a recording spectrophotometer) in a 1 cm. cell.

Using either Procedure 1 or 2 the percentage purity of the sample was calculated by the expression (xxxii) derived as follows:

$$\text{Concn. of allethrin I in cell} = \frac{W}{M} \times \frac{x}{100} \times \frac{f}{n} \times 100 \quad \text{moles/litre}$$

$$= \frac{A}{E \times l} \quad \text{(Beer-Lambert law)}$$

$$\therefore \quad x = \frac{M}{W} \times 100 \times \frac{n}{f} \times \frac{A}{E \times l} \quad \%$$

$$= 2.16 \times \frac{A}{W} \quad \% \qquad \text{(xxxii)}$$

where x = percentage purity of sample

W = weight of sample (g)

f = fraction of sample solution used (5/100)

n = final volume of solution (100 ml.)

M = molecular weight of allethrin I (302)

E = molar absorptivity (for rethrin = 28000)

l = cell path length (1 cm.)

A = measured absorbance

Results of analyses on the samples of allethrin I are shown in Table 9.

3.3.1.2. Chromatographic investigations

The allethrin I samples were examined by t.l.c. on silicic acid containing fluorescent indicator (Kieselgel $HF_{254+366}$) using ethyl acetate - petroleum (b.p. 40 - 60°C) (1:3) as eluant (see Table 43). Components were viewed under ultra-violet light (wavelength = 254 nm) and heating at 150°C after spraying with 50 per cent sulphuric acid. The results were compared with samples of chrysanthemic acid and allethrolone which were chromatographed on the same plate. Portions of the d.n.p.h. fractions which eluted from the chromatographic column in Procedure 1, 3.3.1.1. were also compared (Table 43).

The g.l.c. examination of the allethrin I samples was carried out on 10 per cent Apiezon L stationary phase. The column was temperature programmed from 170° to 240°C at 2° per minute and then isothermally. The carrier-gas flow rate was 20 ml per minute.

Table 43 T.l.c. examination of allethrin I samples

allethrin I Sample A	allethrin I Sample B	allethrolone	chrysanthemic acid	D.N.P. reagent	D.N.P. product
	0.81				
	0.72 uv/f				0.73 v/s
0.66 uv/s	0.66 uv/s				
			0.59 f		
				0.43 v/s	
	0.30 uv/f		0.30 uv/s		
	0.18 uv/f				
	0.12 uv/f				
		0.08 uv/s		0.08 v	

v = seen under visible light (coloured)

uv = seen under ultra-violet light

f = faint; s = strong

3.3.2. Relative response factor determination of rethrins II to rethrins I (see 2.1.2.5.)

Samples of allethrin I and allethrin II were reconstituted from their component acids and alcohol by the Acid Chloride method (see 3.11.2.) and stored in hexane at 0°C. The concentration of each solution (~ 2%) was determined by evaporating an aliquot to dryness at room temperature under reduced pressure. Their purity (see Table 5) was determined by the D.N.P. method of analysis (3.3.1.1., Procedure 2) on portions of the allethrin solutions. (For allethrin II analysis, molecular weight = 346 therefore 2.16 in equation (xxvii) is replaced by 2.48).

Chromatographic demonstration of their parity was achieved by comparison with samples of allethrolone, the chrysanthemum acid and the chrysanthemum acid chloride using g.l.c. (10% Apiezon L stationary phase, temperature programmed from 170° to 240°C) and by t.l.c. (Kieselgel $HF_{254+366}$, eluant = ethyl acetate - hexane (1:3)) when no component other than the respective allethrin was found to be present.

For the determination of the relative response factor between these two compounds, two samples were prepared containing known proportions of the allethrin I and allethrin II solutions (allethrin I : allethrin II = 1:3). These mixtures were concentrated by blowing a stream of nitrogen over the solution surface and then gas chromatographed (four times each sample) on 1 per cent N.P.G.S. stationary phase at 160°C. The response of each allethrin component was evaluated by the developed computerised method (see 2.2.) and the results are shown in Table 6.

3.3.3. Rethrin analyses

3.3.3.1. Gas chromatographic conditions (see 2.1.2.3.)

Gas chromatographic analyses of pyrethrum extracts were determined on a stainless steel column (3 ft by 0.25 inch. i.d.) packed with 1 per cent neopentyl-glycol succinate (N.P.G.S.) stationary phase on 60 - 80 mesh, acid washed Chromosorb W support (see 3.1.5.). The column temperature was 165°C and 193°C for rethrins I and rethrins II analyses respectively and the carrier-gas (nitrogen) flow rate (14 to 20 ml. per minute) was adjusted to yield optimum resolution (see Figures 6 and 7).

During rethrins II analyses the sensitivity was increased by a factor of ten between the elution of the pyrethrin I and cinerin II components. The accuracy of peak area determination for the rethrins was thereby improved. A negligible amount of error was introduced by the increased amplification of the response, shown by the observed linear range of the sensitivity settings used.

The column was 'cleaned' at 200°C and a carrier-gas flow rate of 30 to 40 ml. per minute for 30 to 45 minutes between each rethrins I analysis and each set of rethrins II analyses.

A pre-column (see 2.1.2.8.) (3 inch by 0.25 inch i.d.), packed with the same material as the main column and fitted between this and the injection port, was changed periodically after 5 to 10 analyses.

3.3.3.2. Sample preparation

Samples of pyrethrum extracts which had been stored at sub-zero temperatures were allowed to attain room temperature. All samples were well shaken prior to preparation of the sample for analysis. Weighed portions of the extracts (~50 mg. of extract nominally containing 50 per cent rethrins), were mixed with a weighed portion of an allethrin I solution of known concentration (~300 mg. of a 1% solution) prepared in a similar solvent to that of the extract (hexane or nitromethane). The analysis sample then contained approximately 10 per cent involatile material and a natural rethrin I to allethrin I content ratio of approximately four. Such samples were found to yield the optimum chromatograms for rethrins I analyses when an injection volume of about 0.4 µl was used (~ 10^{-6} g. of allethrin I).

In general the same sample was not used for rethrins II analyses because the presence of allethrin I generally made construction of a baseline to the rethrins I (namely pyrethrin I) more difficult. Therefore a sample was prepared for analysis by direct dilution of the extract with solvent

3.3.3.3. Evaluation of rethrin chromatograms

The equivalent chain lengths (E.C.L.) of the rethrins (Table 4) were obtained by reference to the even-numbered n-alkanes C_{24} to C_{32} (see 3.1.5.3). Their retention times relative to cinerin I (Table 4) were determined as the ratio of the retention time of the rethrin to that of cinerin I.

The number of theoretical plates and height equivalent to one theoretical plate (H.E.T.P.) relative to the pyrethrin II component (see 2.1.2.3. and 2.1.2.8.) were determined from the expressions:

$$\text{no. of theoretical plates, } n = 16 \times \left\{\frac{t}{(w)}\right\}^2 \qquad \text{(xxxiii)}$$

where t = retention time (from sample injection)

w = peak width at base determined by tangents drawn at the inflection points of the peak (in terms of time)

$$H.E.T.P. = \frac{L}{n} \qquad \text{(xxxiv)}$$

where L = colum length

The concentration of the rethrins in extract samples was determined from equations (xxxv) and (xxxvi) derived from equation (iii) (see 2.1.2.1.).

$$\% \text{ concn. of component} = \frac{\text{wt. of component}}{\text{wt. of sample}} \times 100$$

$$\text{Wt. of rethrins I} = k_{R/A} \times \frac{\text{rethrin I response}}{\text{allethrin I response}} \times \text{wt. of allethrin I}$$

$$\% \text{ concn. of rethrins I} = k_{R/A} \times \frac{R_R}{R_A} \times W_A \times \frac{C_A}{100} \times \frac{P_A}{100} \times \frac{100}{W_S}$$

$$= k_{R/A} \times \frac{R_R}{R_A} \times \frac{W_A}{W_S} \times \frac{C_A \times P_A}{100} \qquad \text{(xxxv)}$$

where R_R and R_A = responses of natural rethrins I and allethrin I respectively

W_A = weight of allethrin I solution

C_A = % concentration of allethrin I solution

P_A = % purity of allethrin I

W_S = weight of extract sample

$k_{R/A}$ = relative response factor (= 1, see 2.1.2.1.)

$$\text{Wt. of rethrins II} = k_{II/I} \times \frac{R_{II}}{P_I} \times \text{wt. of pyrethrin I}$$

$$\text{Wt. of pyrethrin I} = \text{wt. of rethrins I} \times \frac{RC_{PI}}{100}$$

$$\% \text{ concn. of rethrins II} = k_{II/I} \times \frac{R_{II}}{P_I} \times \frac{\text{wt. of rethrins I}}{W_S \times 100} \times 100 \times RC_{PI}$$

$$\times RC_{PI}$$

$$= k_{II/I} \times \frac{R_{II}}{P_I} \times C_I \times RC_{PI} \qquad \text{(xxxvi)}$$

where R_{II} and P_I = responses of rethrins II and pyrethrin I respectively

C_I = % concentration of natural rethrins I

RC_{PI} = pyrethrin I content as % of rethrins I

$k_{II/I}$ = relative response factor (= 1, see 2.1.2.5.)

The required rethrin responses were the average determined from three or more chromatographic analyses on the same sample. The areas of the component peaks were evaluated either by planimetry (early work) or by the developed computerised method (see 2.2. and 3.4.)

3.3.4. Demonstration of the precision and accuracy of the developed method (see 2.1.2.11.)

A sample of the World Standard Extract was used to evaluate the developed method and the composition of the samples prepared for analysis are shown in Table 44. Each of the six samples were chromatographed three times for each of the rethrins I and rethrins II analyses.

The mean of these analyses are shown in Table 11. Statistical treatment of the results was performed using the following equations:-

$$\text{standard deviation, } \sigma = \sqrt{\frac{\sum_1^n d^2}{n-1}} \qquad \text{(xxxvii)}$$

$$\text{coefficient of variation} = \frac{\sigma}{\text{mean}} \qquad \text{(xxxviii)}$$

$$95\% \text{ confidence limits} = t \times \frac{\sigma}{\sqrt{n}} \qquad \text{(xxxix)}$$

Table 44 World Standard Extract analysis samples

Allethrin I solutions (purity of allethrin I = 91.5%)

	Wt. of allethrin I (mg)	Wt. of hexane (mg)	Concn. of solution %
(i)	34.67	2177.7	1.592
(ii)	43.33	2455.9	1.764
(iii)	26.92	1018.7	2.642
(iv)	42.71	2362.4	1.808

Sample preparation

	Wt. of extract (mg)	Wt. of allethrin I soln. (mg)	Identity of allethrin I soln.
A	210.15	278.74	(i)
B	62.01	206.73	(ii)
C	131.79	186.42	(ii)
D	96.32	121.98	(iii)
E	88.90	138.87	(iv)
F	126.88	314.30	(iv)

where d = difference between value and mean

n = no. of data

t = Student's factor

= 4.30 for 3 data)
)
2.57 for 6 data) for 95% limits
)
2.20 for 12 data)

3.3.5. Thermal degradation studies (see 2.1.2.7.)

Weighed portions (~ 3 g.) of a commercial Pale Extract concentrate (see 3.2.1.) were heated under reflux for various time intervals in an oil bath maintained at a temperature of 195 ± 5°C (see Table 8). After cooling rapidly under the tap the sample was dissolved in spectroscopically pure absolute alcohol and made up to volume in a graduated flask (25 ml.). An aliquot (1 ml.) was diluted to 25 ml. with alcohol and an aliquot of this solution (1 ml.) was further diluted to 100 ml. Based on the original rethrin content of the extract (36.85% by g.l.c.) the original alcoholic solution contained approximately 10^{-5} g. of rethrins per µl., a suitable concentration for g.l.c. analysis (see 3.3.4.), and the most dilute solution contained approximately 10^{-3} g. per 100 ml., found to give 75% full scale absorbance in ultra-violet spectrophotometric analysis.

Each heated sample, suitably diluted as described above, was analysed by ultra-violet spectrophotometry using 5 mm. cells and solvent in the reference cell. The results are shown in Table 8 and Figure 8. A blank determination on Shellsol T, the probable solvent of the Pale Extract concentrate sample (see 1.1.2.), was found to give zero absorbance in the region 230 nm to 270 nm at a concentration of 10^{-3} g. per 100 ml. of ethanol. G.l.c. analyses of the suitably diluted samples were performed on a 1 per cent N.P.G.S. column (see 3.1.5.) at 193°C and a carrier-gas flow rate of 18 ml. per minute (see 3.3.3.1.). The results are shown in Figure 9.

3.4. Computerised evaluation of gas chromatograms (see 2.2.)

The computer program DIGIT was devised and written in Algol 60 programming language and is shown in the Appendix (see also Figure 11).

3.4.1. Operating instructions for use of the program DIGIT

The program DIGIT was suitable for compilation by an Elliott 4120 computer with a storage capacity in excess of 16000 words (16K). The program was compiled by the computer prior to a series of evaluations of punched tape output from gas chromatographic analyses.

Operator instructions for the evaluation of digital data are shown at the end of the program printout (see Appendix) with the detailed requirements for the supplementary data.

At the message LOAD BINARY DATA TAPE the digital data is input to the computer (see 2.2.2.2.(a)). At the message SECTION A a choice of key settings on the computer control panel is made and the supplementary data is input to the computer. When processing of the data (see 2.2.2.2.(b)) is chosen (by key setting 1 at message SECTION A) the message SECTION B subsequently appears when a further choice of key settings is made (for printout of the results).

3.4.2. Sampler-speed determination (see 2.2.2.1.)

The signal from a chromatographic detector was recorded three times on the paper tape output for known periods of time (3 x 15 minutes), as determined by a stop-watch, at each of the five sampling speeds available. The data recorded was totalled by the program DIGIT and the results shown in Table 45.

Table 45 Paper-tape punch sampling speeds

Sampling speed code	Sampling speed (av. of 3 determinations) (counts per sec.)
-1	0.290 ± 0.001
-2	0.490 ± 0.001
-3	0.794 ± 0.001
-4	1.349 ± 0.002
-5	2.213 ± 0.003

3.4.3. Test evaluation on a decane - undecane component system (see 2.2.3.)

The gas chromatographic analyses were carried out on an Apiezon L column (6 ft. x 0.25 in. i.d.) at 140°C with a carrier gas flow rate of 20 ml. per minute. Samples of n-decane and n-undecane were each chromatographed without dissolution in a solvent and shown to be pure by g.l.c.

Two mixtures were prepared containing these two compounds in a known proportion by weight and dissolved in hexane (shown by g.l.c. to contain no trace of decane or undecane). The samples, A and B, were chromatographed several times by injection of different volumes of sample (0.1 to 1.0 µl.) and sample B was also chromatographed at a variety of column temperatures (130°, 120° and 105°C). The detector output was recorded on punched tape (sampling rate = -4) as well as on the strip recorder. Evaluation of the chromatographic output was made by the developed computer method and by planimetry (average of 5 readings) of the component peaks in the strip chromatogram.

The results are shown in Table 14. Statistical treatment of the results using the expressions shown in 3.3.4. is shown in Table 15.

3.5. Rethrin content of samples in stability trials

3.5.1. By g.l.c.

Rethrin analyses were performed throughout the stability trials by the developed g.l.c. method described in 3.3.3. The initial rethrin content however was only determined on the most concentrated sample and the rethrin concentration in the more dilute samples was calculated accordingly.

3.5.2. By ultra-violet spectrophotometry

Rethrin analysis by ultra-violet spectrophotometry were determined during the induced degradation studies (see 2.3.4.5.). The procedure used the empirical value for the absorptivity of rethrin in pyrethrum extracts ($E^{1\%}_{227}$ (EtOH) = 1104) proposed by Ward and Newham[95] (see 1.4.4.).

The solvent was removed from a portion (~ 100 mg.) of the extract sample under reduced pressure at room temperature and the residue dissolved in spectroscopically pure absolute alcohol (~ 3 g.). A portion of the solution (~ 50 mg.) was further diluted with ethanol to yield a solution of an approximate rethrin concentration of 10^{-3} g. per 100 ml. (density of ethanol = 0.7893 g. per ml.). This solution was analysed on a recording spectrophotometer in the region 220 to 300 nm. with ethanol in the reference cell. The absorbance at 227 nm. was related to the concentration of the rethrins in the extract sample by equation (xl).

$$A = E \times c \times L \qquad \text{(Beer-Lambert law)}$$

$$\% \text{ concn. of rethrins in extract} = \frac{\% \text{ concn. of rethrins in cell solution } (c)}{\% \text{ concn. of extract sample in cell solution } (C)} \times 100$$

$$= \frac{A_{227} \times 100}{E \times L \times C} \qquad \text{(x1)}$$

where A_{227} = absorbance at 227 nm.

E = absorptivity = 1104

L = cell path length

C = concn. of extract in cell solution (g. per 100 ml.)

3.6. B.H.T. analyses (see 2.3.3.)

3.6.1. G.l.c. analysis conditions

Gas chromatographic analyses of the pyrethrum extracts to determine the concentration of B.H.T. were carried out on two types of stationary phase. The polar stationary phase was 12.5 per cent PEGA on 85 to 100 mesh Celite and the non-polar one was 10 per cent Apiezon L on 100 to 120 mesh Chromosorb W. The dimensions of both columns were 6 ft. by 0.25 in i.d. The former was operated at 150°C and the latter at 170°C. and the carrier-gas flow rates were approximately 25 ml. per minute. Under these conditions B.H.T. eluted with a retention time of approximately 6 minutes on both columns.

3.6.2. Qualitative investigation

Refined pyrethrum extracts, (Pale and Nitromethane) diluted to contain approximately 10 per cent involatile material (see 3.3.3.2.) were examined by g.l.c. (~ 0.4 µl. injection sample) using the conditions described in 3.6.1. (see Figures 17 and 18). The equivalent chain lengths (E.C.L.)

of the major components in the region of B.H.T. in the chromatograms were obtained (Table 21) by reference to the even numbered fatty acid methyl esters C_{10} to C_{16} (see 3.1.5.).

3.6.3. Quantitative analysis

Methyl myristate solution (~ 200 mg. of 2% solution) was added to a portion of the extract sample (~50 mg.). This sample was chromatographed as described in 3.6.1. The area responses of the B.H.T. and methyl myristate were evaluated by quadrature (see 2.2.1.) or by the developed computerised method (see 2.2. and 3.4.). The results of B.H.T. analyses of refined extracts are shown in Tables 22 - 24 and 33.

3.6.4. Relative response factor of B.H.T. - methyl myristate

B.H.T. (Analar) and methyl myristate (> 99% purity by g.l.c.) were each dissolved in nitromethane at a known concentration. Portions of these solutions were mixed in a known proportion by weight and gas chromatographed using the conditions described in 3.6.1. The samples were also chromatographed connected to a different flame ionisation detector (F.I.D.) and by using a variety of carrier gas flow rates. The results are shown in Table 46. The variation in the determined values indicated that within the limits of chromatographic detection, a single value of 0.84 could be used for the relative response factor of B.H.T. to methyl myristate but there appeared to be a small but significant difference between the detectors 1 and 2.

Table 46 Relative response factor of B.H.T. - methyl myristate

Stationary phase	10 % Apiezon L			12.5 % PEGA	
F.I.D. No.	1	1*	2	2*	1
No. of chromatograms	15	20	16	17	13
Mean	0.850	0.827	0.860	0.869	0.832
Standard deviation	0.028	0.018	0.034	0.054	0.036
95 % confidence limits	0.015	0.008	0.018	0.028	0.022

* - using a variety of carrier-gas flow rates

3.7. Tocopherol analyses (see 2.3.4.1.)

3.7.1. Attempted gas chromatographic and column adsorption chromatographic analysis

A hexane solution of α-tocopherol (> 98% purity, supplied by Beecham Research Laboratory, Surrey) was gas chromatographed on a 3 per cent SE30 column (see 3.1.5.) at 240°C (optimum in range 200 to 260°C) with a carrier-gas flow rate of 14 ml. per minute (optimum for peak symmetry). The tocopherol eluted after 23 minutes. A hexane solution of the World Standard Extract was analysed under the same conditions and yielded a chromatogram which was relatively complex in the region of α-tocopherol elution.

Partial purification of extracts in respect of tocopherol concentration was attempted by elution with ethyl acetate - hexane solvent (1:3) through a column of silica (12 in. by 0.5 in.) packed in hexane. The fraction eluting with a retention volume corresponding to that found with an α-tocopherol sample was

analysed by g.l.c. using the conditions described above. No significant simplification of the chromatogram had been achieved. T.l.c. examination of the collected fraction on Kieselgel $HF_{254+366}$ (eluant = ethyl acetate - hexane (1:3)) showed three spots (Rf = 0.70, 0.64 and 0.47) which were compared with the pure component Rf = 0.61.

The adsorption chromatography of extract samples was repeated using less polar eluants but this produced a greater amount of diffusion within the column and no reduction was observed in the complexity of the gas chromatogram of the fraction calculated to contain any α-tocopherol.

3.7.2. Sample preparation (saponification) prior to t.l.c. analysis

Pyrethrum extract (6 g.) was refluxed with alcoholic potassium hydroxide (20 ml., 12% solution) in the presence of pyrogallol[240] (0.3 g., equivalent to 5% w/w of the oil) for one hour under nitrogen. On cooling, portions (~ 1/3) of the saponified extract were added to distilled water (30 ml.) and washed four times with diethyl ether (4 x 10 ml.). Excess shaking was avoided on the first two washings to prevent formation of an emulsion. The combined ether fractions, from the treatment of all portions of the saponified extract, were washed free of alkali with water (60 ml portions). (Excessive shaking was again avoided).

The ether was removed under reduced pressure at room temperature and the residue dissolved in benzene and made up to volume in a graduated flask (10 ml.). The solution was a deep red-orange colour.

The concentration of involatile material was determined on an aliquot of the solution and the unsaponifiable content found, in the case of oleoresin, to be approximately 30 per cent of the original sample.

3.7.3. T.l.c.-spectrophotometric analysis of tocopherols

An aliquot (100 µl.) of the benzene solution prepared in 3.7.2. was spread as a thin band along one edge of a 20 cm. by 20 cm. t.l.c. plate (Kieselgel $HF_{254+366}$) by taking up 40 µl. portions in a syringe and expelling 25 µl. onto the plate. The plate was developed over 15 cms. in diethyl ether - benzene (1:19) with tocopherol as standard on the same plate. After allowing to dry in the air (~ 2 minutes) the plate was viewed under ultra-violet light (wavelength = 254 nm.). The two components with approximate Rf values 0.42 and 0.38 (the former corresponds to the Rf value of the standard tocopherol) were removed from the plate as the tocopherol fraction and the components eluted from the silica with absolute ethanol. The ethanol solution was concentrated under reduced pressure at room temperature on the rotary evaporator and then transferred to graduated flask (2 ml.) and made up to volume with solvent.

2,2'-dipyridyl solution (0.5 ml., 0.5% solution in ethanol) was added followed by ferric chloride solution (0.5 ml., 0.2% solution in ethanol freshly prepared)[234] and the mixture quickly placed in the dark chamber of a Unicam SP 500 spectrophotometer. Exactly two minutes after the addition of the ferric chloride solution the absorbance was determined at 520 nm. (wavelength of maximum absorption demonstrated on a recording spectrophotometer) against ethanol in the reference cell.

A blank area (near the solvent front) was similarly removed from the chromatographic plate and used as a blank for the spectrophotometric determination. The absorbance of the spectrophotometric reagents were also determined and fresh reagents prepared if the value exceeded 0.14[239].

The absorbance of the sample relative to that of the t.l.c. blank was related to the tocopherol concentration in the original sample by equation (xli):-

$$\text{Wt. of tocopherol in cell soln.} = \frac{A}{ExL} \times M \times V_c \times 10^{-3} \text{ g.} \quad \text{(by the Beer-Lambert law)}$$

$$\text{Wt. of sample on plate} = \frac{V_p \times 10^{-3}}{V_s} \times W \text{ g. of extract}$$

$$\therefore \text{ Concn. of tocopherol} = \frac{A \times M \times V_c \times V_s}{E \times L \times W \times V_p} \text{ g. per g.}$$

$$= 2450 \times \frac{A}{L} \times \frac{V_c \times V_s}{W \times V_p} \text{ p.p.m.} \quad \text{(xli)}$$

where A = absorbance at 520 nm.

L = cell path length (cm.)

V_c = volume of sample used for spectrophotometric analysis (ml.)

V_s = volume of solution prepared from extract (ml.)

V_p = volume of solution chromatographed (µl.)

W = wt. of extract used (g.)

M = molecular wt. of ∝-tocopherol = 430

E = molar absorptivity of the complex formed from one mole of ∝-tocopherol[239] = 17550

3.7.4. <u>Tocopherol analysis by omission of the saponification procedure</u>

The extract sample was directly spread as a band on a chromatographic plate and the tocopherol content determined as described in 3.7.3. In equation (xli), $\frac{V_p}{V_s} = 100$ and $G = V_p \times 10^{-3} \times p$ where p = density of the extract sample. Therefore $\frac{V_s}{W}$ is substituted by $\frac{1}{p}$.

The density of extract samples, the mean of duplicate observations, was determined by weighing a known volume in a weighed pipette (50 µl).

3.7.5. <u>Identification of the requisite t.l.c. fractions</u>

A chromatographic plate from the t.l.c. examination of the unsaponifiable fraction of pyrethrum extracts was sprayed with Emmerie-Engel reagent[234] (2,2'-dipyridyl solution followed by freshly prepared ferric chloride solution-see 3.7.3.). Two components (Rf 0.41 and 0.38) in the region of standard α-tocopherol elution (Rf 0.42) showed a positive coloration (red) (see Table 25).

The alcoholic solution, obtained as for spectrophotometric evaluation of the tocopherol content (3.7.3), from several chromatographic plates was concentrated by blowing a stream of nitrogen over the surface of the solution and examined by g.l.c. (on 3% SE30 - see 3.7.1.) and by infra-red spectroscopy (potassium bromide disc). .1.

The chromatogram and spectrum were compared to those of a sample of α-tocopherol.

3.8. Fatty acid analysis (see 2.3.4.2.)

3.8.1. Saponification of extracts and extraction of the fatty acids

The procedure used was based on that reported by Head[126].

An extract sample (Pale Extract concentrate, ex P.M.D.) (2 g.) was refluxed with alcoholic potassium hydroxide (50 ml, 0.5N) under nitrogen for one and a half hours. The contents were then washed into a large flask with distilled water (300 ml.) and the solution boiled to reduce the volume to approximately 150 ml. On cooling to room temperature barium chloride solution (20 ml., 10% solution) was added which precipitated the barium salt of the fatty acids (c.f. the A.O.A.C. procedure[77]).

The precipitate was collected by filtration through a bed of Celite contained in a sintered glass funnel. The filtrate was retreated with barium chloride solution (10 ml.) but no further precipitation occurred. The precipitate and Celite were slurried in hydrochloric acid (10 ml., 2N) and diethyl ether (10 ml.). The glass filter was washed with acid and ether and the washings added to the slurry. This was filtered and washed with a further portion of diethyl ether (10 ml.).

The ether layer, separated from the aqueous layer, was washed twice with 10% potassium hydroxide (2 x 30 ml.) and the combined alkali layers made just acid with hydrochloric acid (10N) causing precipitation of the fatty acids. These were extracted three times with diethyl ether (3 x 25 ml.) and the combined ether extractions were washed until acid-free (to litmus).

The ether solution of fatty acids was dried over magnesium sulphate (> 2 hrs, overnight) then filtered and the filtrate made up to volume in a graduated flask (100 ml.). The yield, determined on an aliquot of this solution, was found to be 15 per cent.

3.8.2. Methylation of the fatty acids

The solvent was removed under reduced pressure from a portion (25 ml.) of the solution prepared in 3.8.1. and 1 per cent sulphuric acid in methanol (20 mls) added. This was refluxed for one hour. On cooling to room temperature distilled water was added (20 ml) and extracted three times with petroleum spirit (b.p. 40 - 60°C) (3 x 10 ml.). The separated aqueous layer was washed twice with petroleum spirit (b.p. 40 - 60°C) (10 ml.) and the combined petroleum spirit fractions were washed with distilled water until acid-free.

The petroleum spirit fraction was dried over magnesium sulphate (> 2 hrs.) then filtered and the filtrate made up to volume in a graduated flask (25 ml.).

3.8.3. G.l.c. examination of the methyl esters

Gas chromatographic analyses of the methyl esters prepared in 3.8.1. and 3.8.2. were carried out on a polar stationary phase (12.5% PEGA) and a non-polar stationary phase (10. Apiezon L). The dimensions of each column were 6 ft by 0.25 in. and 9 ft by 0.25 in. respectively. Operating temperatures were 180°C and 220°C respectively (see Figures 23 and 24).

The equivalent chain lengths (E.C.L.) of the major components in the chromatograms (Table 28) were obtained by reference to the even-numbered fatty acid methyl esters C_{12} to C_{18} (see 3.1.5.).

The concentration of the major components was determined by the addition of methyl myristate solution of known concentration to a portion of the solution as internal standard. From the chromatograms of samples not containing the internal standard the relative proportion of methyl myristate occuring in the sample was determined and a correction then made in subsequent analyses to the area response of the methyl myristate. Peak area evaluation was by planimetry.

3.0.4. Free acidity determination

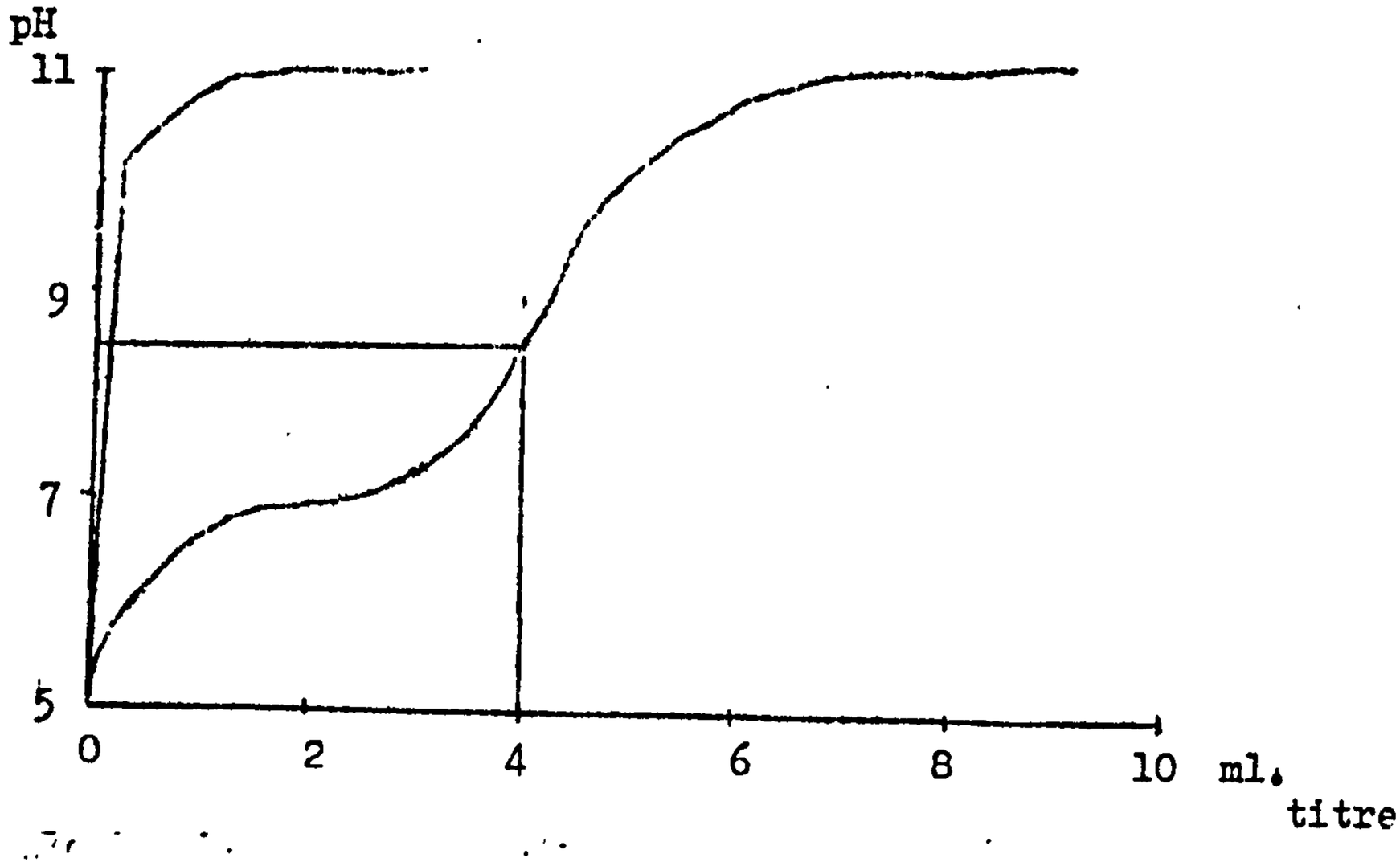
Procedure 1

Refined pyrethrum extract (1 g.) was dissolved in 95 per cent aqueous methanol and made up to volume in a graduated flask (100 ml.). On allowing to stand a few oily drops separated out. An aliquot (50 ml.) of the supernatant liquid was titrated against standard sodium hydroxide (~0.1N). A blank determination was made on the solvent.

Procedure 2

Crude pyrethrum oleoresin (1 g.) was extracted 5 times with 95 per cent aqueous methanol (5 x ~ 20 ml.) and each extraction solution filtered. The combined filtrates were made up to volume in a graduated flask (100 ml.). This was titrated against standard sodium hydroxide (~0.1N). A blank determination was made on the solvent.

Figure 42 Potentiometric titration of pyrethrum extracts against sodium hydroxide



In either procedure the change in pH value of the solution/blank during titration against alkali was monitored by a potentiometer (see Figure 42). The free acidity was calculated from the volume of alkali used at the point of inflection in the pH - titre plot, after correction for the blank at the corresponding pH value (equation(xlii)).

$$\text{Acid value}^{250} \text{ (mg. of KOH to neutralise 1 g.)} = \frac{\text{titre} \times \text{normality} \times \text{Molec. wt. of KOH}}{\text{wt. of sample}}$$

$$\text{Equivalent \% by wt. to stearic acid} = \frac{\text{titre} \times \text{normality} \times \text{molec. wt. of stearic acid}}{\text{Wt. of sample} \times 10} \quad \text{-- (xlii)}$$

3.9. Determination of peroxide values (see 2.3.4.3.)

The procedure used was based on the method reported by Sulley[259].

Analar chloroform (10 ml.) and analar acetic acid (10 ml.) were refluxed in a round bottom flask (100 ml.) fitted with an air condenser (60 cm by 9 mm. i.d.) on the top of which was fitted a water condenser (15 cm long). Steady refluxing close to the top of the water condenser was ensured by the use of a microflame close to the flask. Freshly prepared aqueous potassium iodide solution (~1.3 ml. of a solution of 10 g. in 13 ml.) was added to the top of the column so as not to interrupt the refluxing.

The organic substrate (< 2 g.) was added down the column, temporarily turning of the water to the water condenser to raise the condensing level and thus washing all the sample down into the flask. The refluxing was continued for a further 3 to 5 minutes. The flask was then rapidly cooled under the tap and distilled water added (50 ml.).

The solution was titrated against standard sodium thiosulphate solution with "iodine indicator".

Blank determinations returned zero values (c.f. Sulley[259]).

3.10. Analysis for trace amounts of iron (see 2.3.4.4.)

3.10.1. The α-phenanthroline method

This is a standard procedure for the determination of iron[270,271].

The organic substrate (0.5 to 1 g.) was strongly heated with concentrated sulphuric acid (20 ml.) in a Kjeldahl flask until brown suspension was formed. Concentrated nitric acid (~ 5 ml.) was added dropwise to the solution, allowing it to cool slightly between additions and then heating strongly until brown fumes ceased to be evolved. The solution gradually assumed a straw-colour which did not become paler on further addition of nitric acid and heating. On allowing to cool to room temperature the solution became colourless. The solution was concentrated to about 2 ml. volume by boiling off of excess sulphuric acid and then neutralised (to odour) by the addition of ammonia (Analar). The solution was made just acid by the addition of acetic acid (Analar).

10 per cent hydroxylamine hydrochloride solution (1 ml.) and buffer solution (7.5 ml.) (27 g. of anhydrous sodium acetate in 50 ml. water, 24 ml. acetic acid and diluted to 100 ml.) were added and slightly warmed. On cooling, α-phenanthroline solution (5 ml.) (0.25 g. in 100 ml. hot water and allowed to cool) was added and the solution made up to volume in a graduated flask (25 ml.).

The absorbance of the solution was measured at 510 nm. (wavelength of maximum absorption determined on a recording spectrophotometer) and corrected for that of the complexing-reagents blank. The procedure was repeated using the same volume of reagents but omitting the organic substrate to obtain the true reagent blank.

The corrected absorbance was related to iron content by a calibration graph prepared by dissolving pure iron wire in a small amount of concentrated nitric acid and diluting with distilled water to give a range of iron solutions containing 10^{-5} to 10^{-3} g. per ml. These solutions were reacted with the complexing reagents as described above and their absorbances determined.

3.10.2. Neutron activation analysis

Pyrethrum extracts (1 g.) were sealed in silica ampoules which had been cleaned in boiling nitric acid and washed with distilled water. An ampoule containing a standard solution of iron (~ 50 ug.) was similarly prepared.

Samples and standards were irradiated with thermal neutrons (42 hours at 8 x 10^{12} neutrons per cm^2 per second). After allowing to 'cool' for a few days the ampoules were opened to release pressure and then crushed in polythene bags. The entire contents of each bag were oxidised (sulphuric and nitric acids) to yield a colourless solution (see 3.10.1.).

An iron-carrier (1 drop of saturated ferric chloride solution) was added and the solution made alkaline (sodium hydroxide) to precipitate the iron as ferric hydroxide which was centrifuged off. The precipitate was washed with ammonium chloride solution and dissolved in hydrochloric acid solution. After reprecipitating and washing the precipitate was redissolved in dilute hydrochloric acid (2 ml.).

The radiation emission spectrum of this solution was determined on a γ-ray spectrometer, with a Lithium Drift (Ge(Li)) detector, for 30 minutes and evaluated by a multi-channel pulse height analyser (see Figure 27).

3.11. Rethrin reconstitution

3.11.1. Examinations of purity of components (see 2.4.2.1.).

Portions of the samples of chrysanthemic acid, pyrethric acid and pyrethrolone (supplied by Dr. P.J. Godin) and of allethrolone (supplied by McLaughlin, Gormley, King Company, Minneapolis) were examined by t.l.c. on Kieselgel $HF_{254+366}$ (eluants = ethyl acetate - hexane (1:3) and diethyl ether - benzene (1:19); viewed under ultra-violet light and by spraying with 50% sulphuric acid and heating at 150°C) and by g.l.c. (10% Apiezon L and 12.5% PEGA). Each sample, with the exception of the chrysanthemic acid sample, gave a single spot and peak in the respective chromatograms.

A portion of the chrysanthemic acid (30 mg.) was methylated with 1 per cent sulphuric acid in methanol (20 ml.) as described in 3.8.2. The final solution was evaporated to dryness under reduced pressure and the residue dissolved in a small quantity of carbon tetrachloride (reagent for n.m.r.) (~ 3 ml.).

The solution was analysed by g.l.c. on a 12.5 per cent PEGA column (6 ft by 0.25 in.), temperature programmed from 80° to 190°C at 2° per minute with a carrier-gas flow rate of 30 ml. per minute. (see Figure 33). The assigned peaks were methyl chrysanthemate (122°), methyl methoxy ester of chrysanthemic acid (131°), as known impurity in acid sample (140°) chrysanthemic acid (168°) and pyrethric acid (187°).

N.m.r. analysis of the products of methylation was carried out in the carbon tetrachloride solution with added tetramethyl silane as internal standard (Figure 34).

3.11.2. Acid Chloride method of reconstitution[32,291] (see 2.4.2.2. and 2.1.2.5.)

The chrysanthemum acid (2 millimoles) was treated with excess thionyl chloride (3 millimoles) and refluxed in dry benzene (15 ml.) to yield the acid chloride. Excess reagents and volatile byproducts (hydrochloric acid and sulphur dioxide) were removed at the pump. For the formation of the pyrethrins, the rethrolone (pyrethrolone) was obtained as its monohydrate but was regenerated by stirring overnight in a vacuum dessicator at reduced pressure (10^{-3} mm.). The rethrolone (2 millimoles) and pyridine (0.5 ml.) were dissolved in dry benzene (7 ml.) and the solution slowly added at 0°C to the acid chloride. The mixture was then allowed to warm to room temperature and left to stand for 40 hours.

The mixture was washed with hydrochloric acid (3 x 5 ml., 2N) 10% sodium carbonate solution (3 x 5 ml.) and finally with water till neutral to litmus. The benzene fraction was then eluted down a silica column (10cm. by 1 cm.) with benzene and the fraction collected was concentrated under reduced pressure to approximately 10 ml. This was stored at -5°C until required.

The product was shown to be a single component by t.l.c. (Kieselgel $HF_{254+366}$; eluant = ethyl acetate - hexane (1:3)) and by g.l.c. (10% Apiezon L and 1% N.P.G.S.). The Rf values and relative retention times were checked against those of the corresponding component in a refined extract and the infra-red spectrum (potassium bromide disc) of prepared pyrethrins were correlated with those published by Elliott [196].

3.11.3: Reaction of D.C.C.I., pyrethrolone monohydrate and pyrethric acid (2.4.2.3.)

Pyrethrolone monohydrate solution (196 mg. (1 millimole) dissolved in chloroform: 1,4-dioxan (1:4) (4 ml.)) was mixed with pyrethric acid solution (209 mg. (1 millimole) dissolved in chloroform (2 ml.)) and D.C.C.I. solution (512 mg. (2 millimole) dissolved in chloroform (2 ml.)) added. The mixture was well shaken until a white precipitate appeared (2 to 5 minutes) and was then left to stand for 48 hours in the dark.

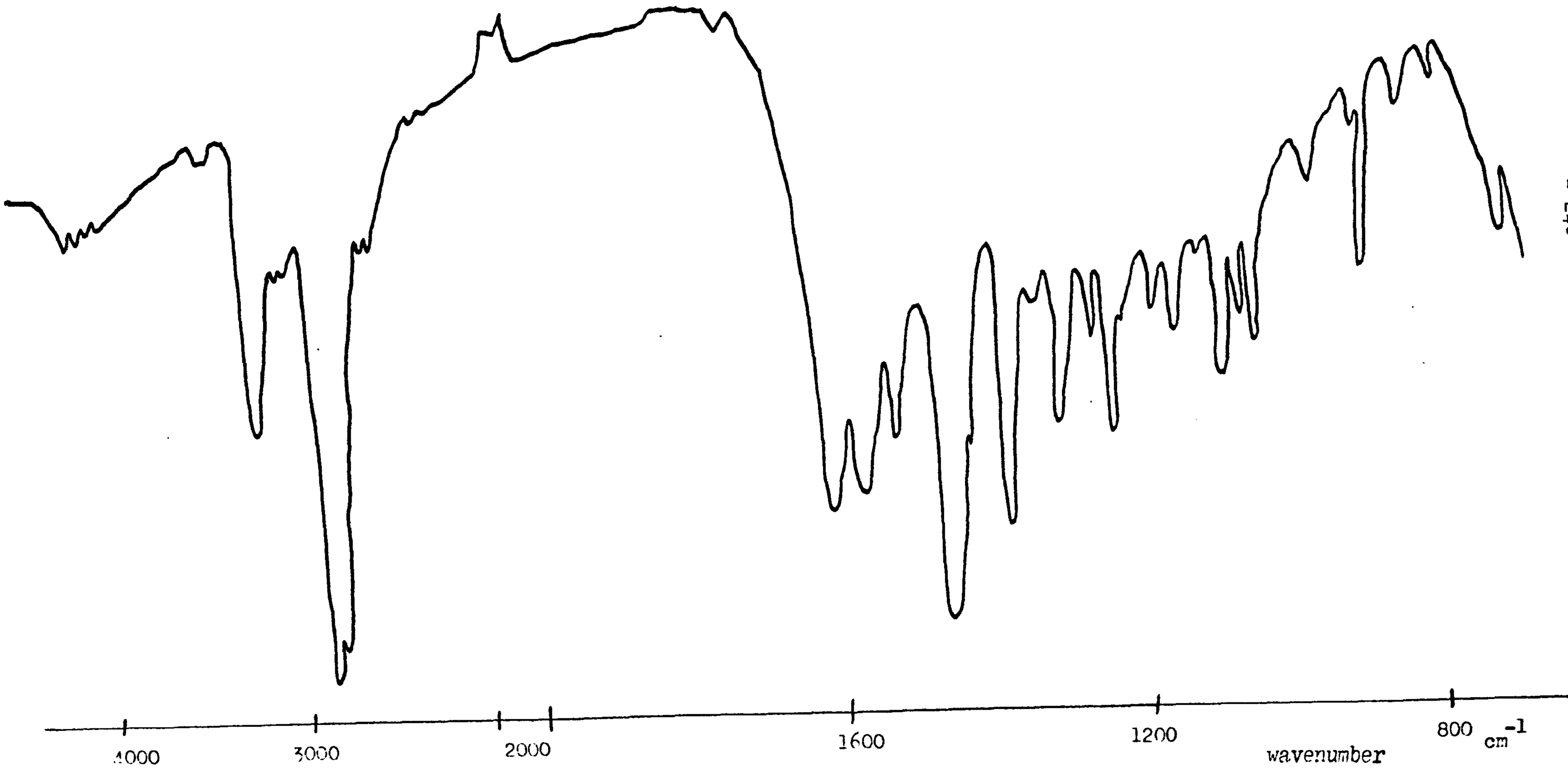
The reaction mixture was washed four times with water (4 x 10 ml.) to remove the 1,4-dioxan and filtered.

3.11.3.1. Identification of dicyclohexyl urea by product

The precipitate of the reaction in 3.11.3. was washed several times with chloroform. Partial solution of a portion of the precipitate was achieved in ethanol. T.l.c. on Kieselgel $HF_{254+366}$ (eluant = ethyl acetate - hexane (1:1)) produced a single spot (Rf = 0.1) viewed under ultra-violet light and by spraying with 50% sulphuric acid and heating at 150°C) corresponding to that of an authenticated sample. G.l.c. examination on 10% Apiezon L produced similar confirmation although the component peak was broad and tailed.

The infra-red spectrum (Nujol mull) (Figure 43) of the reaction product was identical to that of the authenticated sample and exhibited absorption bands at 3260 cm^{-1} (N-H stretch of a secondary amide), 1620 and 1580 cm^{-1} (Amide I band of secondary amide), and 1530 cm^{-1} (Amide II band of secondary amide).

Figure 43 Infra-red spectrum of dicyclonexyl urea.



The melting point (uncorrected) of the reaction product was 227°C and that of the authenticated compound to be 220°C. (Literature values[303] quote 227 - 220°C).

3.11.3.2. Examination for pyrethrin II

The filtrate of the reaction (from 3.11.3.) was examined by t.l.c. (Kieselgel $HF_{254+366}$; eluant = ethyl acetate - hexane (1:3)) showed the presence of unreacted acid (Rf 0.22) and alcohol/D.C.C.I. (Rf 0.06) with a major component (Rf 0.34) corresponding to that of the rethrins II fraction of pyrethrum extracts. (Viewed by ultra-violet light (wavelength 254 nm.) and by eluting at 150°C after spraying with 50% sulphuric acid).

The component eluting with Rf 0.34 was removed from the plate (eluted from the silica by ethanol) and examined spectrophotometrically. The ultra-violet spectrum (in ethanol) showed an absorption maximum at 233 nm. (c.f. rethrins II). Major absorption bands in the infra-red spectrum (potassium bromide disc) were observed at 2920 and 2851 cm^{-1} (C-H stretch), 1440 and 1385 cm^{-1} (C-H bend), 1650 cm^{-1} (C=C stretch), 1710 cm^{-1} (C=O stretching) but the overall spectrum showed only slight similarity to that published by Elliott[196].

The filtrate was also examined by g.l.c. on 1 per cent N.P.G.S. and 10% Apiezon L columns (3 ft. by 0.25 in. and 6 ft. by 0.25 in. respectively) at 190° and 240°C respectively. The presence of the reagents was confirmed and a major component (E.C.L. = 16.0 on Apiezon L, with reference to methyl esters of even numbered fatty acids C_{12} to C_{18} (see 3.1.5.3) was noted in both chromatograms. A minor component (elution time equivalent to that of pyrethrin II in chromatograms of refined pyrethrum extracts) was also observed.

3.11.4. Reaction of D.C.C.I., allethrolone and chrysanthemic acid

Allethrolone solution (152 mg. (1 millimole) dissolved in 2 ml. chloroform was mixed with chrysanthemic acid solution (168 mg. (1 millimole) (Supplied by Roussel U.C.L.A.F. Roumanville, France) in 2 ml. chloroform) and D.C.C.I. solution (206 mg (1 millimole) in 2 ml. chloroform) added. In some reactions methyl myristate solution (60 mg. in 2 ml. chloroform) was added as internal standard for g.l.c. The mixture was well shaken until a white precipitate formed (2 to 5 minutes).

G.l.c. examination of the reaction mixture was performed on a 10% Apiezon L column at 210°C with a carrier-gas flow rate of 30 ml. per minute (see Figure 35). The major product of the reaction was found to have an E.C.L. value of 15.7. A minor component was shown to correspond to allethrin I, determined by comparison with a commercially prepared sample, and this was confirmed by g.l.c. on 1% N.P.G.S. (see 3.11.3.2.). The quantity of products was determined by reference to the response of methyl myristate added as internal standard. Daily analyses were carried out on the reaction mixture (and a heated (40°C in a water bath) sample) to study the change in the relative concentrations of the constituents.

3.11.5. Reaction of D.C.C.I. and chrysanthemic acid

Each of three reaction mixtures were prepared as in 3.11.4. with the omission of one of the three reactants. G.l.c. examination of these solutions was carried out on 10% Apiezon L, as described in 3.11.4., which demonstrated that a reaction occurred only between D.C.C.I. and chrysanthemic acid. This

latter reaction yielded a white precipitate and the gas chromatogram was similar to Figure 35 with the omission of allethrolone and allethrin I assigned peaks.

T.l.c. examinations were carried out on Kieselgel $HF_{254+366}$ stationary phase with ethyl acetate - hexane (1:3) as mobile phase. Spots at Rf 0.0, 0.52 and 0.63 were observed for the reaction mixture and 0.0 and 0.52 for cyclohexyl urea and chrysanthemic acid respectively. The component eluting to Rf 0.63 was separated by t.l.c. as a band from the other constituents removed from the plate and extracted from the silica with chloroform.

The solution of this product was examined by g.l.c. on 10% Apiezon L, as described in 3.11.4.

The mass spectrum of the major product of the D.C.C.I. - chrysanthemic acid reaction (Figure 36) was obtained by g.l.c. of the t.l.c. separated product (see above) on 10% Apiezon L stationary phase at 230°C with the column outlet connected to a single-beam mass spectrometer. The carrier-gas was helium (30 ml. per minute) and the temperature of the heated line was 220°C. The ion current was 70 eV.

3.11.6. Reaction of chrysanthemoyl chloride and cyclohexylamine

Cyclohexylamine (100 mg. (~1 millimole) was added dropwise onto chrysanthemoyl chloride (supplied by P.M.B.) (300 mg. (~ 1 millimole) in chloroform (10 ml.) at 0°C with constant shaking. Gaseous hydrogen chloride was partially removed at the pump and then the mixture was washed with distilled water (3 x 10 ml.), 10% sodium bicarbonate solution (3 x 10 ml.) and distilled water till neutral to litmus.

The chloroform solution was then concentrated by blowing a stream of nitrogen over the surface and then examined by g.l.c. (see 3.11.4.) and by t.l.c. (see 3.11.5.) for comparison with the product of the D.C.C.I. - chrysanthemic acid reaction. The infra-red spectrum of the products of these reactions were obtained as potassium bromide discs (Figures 38 and 39). Major absorption bands were noted at 3280 cm^{-1} (N-H stretch of secondary amide), 2920 and 2851 cm^{-1} (C-H stretch) 1440 and 1385 cm^{-1} (C-H bend), 1640 cm^{-1} (Amide I band) and 1550 cm^{-1} (Amide II band).

4. SUMMARY.

4. Summary

The stability of refined pyrethrum extract concentrates was investigated and found to be more pronounced than had been generally concluded from previous observations. An induction period, in respect of rethrin degradation, of at least six months was observed for Pale Extract concentrates which would appear to be sufficient to achieve air transportation of such extracts and of storage prior to their use.

More highly refined extracts (achieved by nitromethane extraction of oleoresin) showed even greater stability than Pale Extracts over long periods (two years), indicating that the removal of some of the extraneous matter in Pale Extracts enhanced rethrin stability.

The detrimental effect of this non-rethrin material was further indicated by the more rapid consumption of the antioxidant, B.H.T., in Pale Extracts compared to Nitromethane Extracts.

The extraneous material in Pale Extracts was shown to include the tocopherols, a group of antioxidants which are ubiquitous in vegetable extracts. These undoubtedly confer a certain degree of stability to pyrethrum extracts in addition to that shown to be achieved by the B.H.T.

Other non-rethrin constituents were shown to include a relatively large proportion of unsaturated fatty acid material which is known to be susceptible to autoxidative degradation. This would undoubtedly contribute to the long term instability of the rethrin in pyrethrum extracts.

The study of pro-oxidant material likely to cause loss of pyrethrum extract stability included the determination of the peroxide content and the extent of trace iron contamination. The peroxide content of freshly prepared extracts was shown to be less than one milliequivalent per kilogram. The high free acidity of both crude and refined commercial extracts (equivalent to 6.9 and 5.6 per cent, stearic acid respectively) indicated the possibility of metal ion contamination by reaction with metallic parts of the processing plant. In the samples examined however the iron content was shown to be no greater than 0.2 p.p.m. which is of the order of that found naturally, but it is not improbable that a reduction in iron concentration would lead to greater pyrethrum extract stability.

The inclusion of small quantities of iron (2 p.p.m.) or of peroxides (20 milliequivalents per kilogram) was shown to significantly reduce the stability of extracts in terms of peroxide accumulation and antioxidant consumption and such changes in extract composition would undoubtedly lead to instability in respect of the rethrins as indicated in the examination of B.H.T.-free samples.

In the course of this work a new method of rethrin analysis was developed based on the gas chromatographic examination of the extracts containing allethrin I, a commercially available analogue of the rethrins, as internal standard. This method demonstrated serious inaccuracies in all of the current methods of rethrin analysis available.

Gas chromatographic analysis of B.H.T. levels in pyrethrum extracts was shown to be possible down to 0.1 per cent of the involatile content of such extracts and was used to monitor changes in the concentration of this component.

A computerised procedure for the evaluation of gas liquid chromatograms was developed and a computer program written. Evaluation by this technique was shown to improve the accuracy of chromatogram interpretation and is preferred to commercially available instruments because of the degree of manual interpretation still afforded to the operator.

Neutron activation analysis, which is free from the vagaries of contaminated solvents, was applied to the determination of iron in pyrethrum extracts and shown to be generally superior to chemical methods of analysis.

The possibility of a novel procedure for the reconstitution of the rethrins was investigated which utilised the condensing agent N,N'-dicyclo-hexylcarbodiimide (D.C.C.I.). Rethrins were observed to be formed in approximately 2 per cent yield at room temperature. The major product (identified as the N-cyclohexyl chrysanthemumamide) was found to be formed by the reaction of the chrysanthemum acid and the reagent.

APPENDIX.

```
DIGIT;

"BEGIN"
"COMMENT" COMPUTATION OF PEAK AREAS FROM DIGITISED OUTPUT;
"INTEGER" B,C,G,I,J,K,L,M,N,P,Q,R,T,X,Y,Z,TOTAL,PRESOLV,MAX;
"REAL" TIME,SAMPLERATE,RUN;
"INTEGER" "ARRAY"A,TITLE[1:500];
"BOOLEAN" ERROR;

"BOOLEAN" "PROCEDURE" KEY(N);
 "VALUE" N;  "INTEGER" N;
"BEGIN" "BOOLEAN" Z;
"CODE"
%DECS$N
%LD$N
%MTOK
%LD:L$1
%SML
%ST$N
%CTOM
%AND$N
%SUB$N
%ST$Z
;
KEY:=Z
"END" OF PROCEDURE KEY;

"INTEGER" "PROCEDURE" DATA;
"COMMENT" TO RECALL DIGITAL DATA STORED ON MAGNETIC TAPE.
          (T IS THE DATA-COUNT, N IS THE BLOCK-COUNT
           SET T AND N TO ZERO PRIOR TO FIRST CALL);
   "BEGIN"
"IF" T=500*N "THEN"
   "BEGIN"
L:= "IF" (TOTAL-T)>500 "THEN" 500 "ELSE" (TOTAL-T);
MTREAD(2,A,1,L);  N:=N+1;
   "END";
T:=T+1;
DATA:=A[T-500*(N-1)];
   "END" OF PROCEDURE DATA;

"BOOLEAN" "PROCEDURE" TYPERROR;
"COMMENT" FOR CORRECTIONS TO CONTROL-TELETYPED DATA;
   "BEGIN"
"IF" "NOT" KEY(3) "THEN" ERROR:="FALSE" "ELSE"
   "BEGIN"
"PRINT" ''L'IF TYPING ERROR TYPE T ELSE F --- `;
"READ" READER(3), ERROR;
   "END";
TYPERROR:=ERROR;
   "END" OF PROCEDURE TYPERROR;

PUNCH(3);  SPECIAL(1);  SAMELINE;
RUN:=TIME:=0;  MAX:=255;

   "BEGIN"
"COMMENT" SEGMENT;
"COMMENT" READING OF DIGITISED DATA AND STORAGE ON MAGNETIC TAPE;
```

```
"INTEGER" "PROCEDURE" READING;
"COMMENT" TO CHECK, AFTER ALIGNMENT, THE COMPLEMENTARY NATURE
          OF THE TWO VALUES CONSTITUTING ONE READING (I.E. = 255);
   "BEGIN"
"INTEGER" "PROCEDURE" VALUE;
"COMMENT" TO CONVERT EACH VALUE ON THE
          BINARY DATA TAPE TO ITS DECIMAL EQUIVALENT;
   "BEGIN"
   "INTEGER" DATA;
   "CODE"
   %ISUM¢2
   %JS:L¢4
   %ISUM¢2
   %JS:L¢8
   %ST¢DATA
   ;
   VALUE := DATA
   "END" OF VALUE;

K:=0:   Q:=VALUE;
STEP1:
P:=Q:   Q:=VALUE;
"IF" N=0 "AND" T=1 "AND" Q>P "THEN" "GOTO" STEP1;
"IF" P+Q "NE" 255 "THEN"
   "BEGIN"
K:=K+1;
"IF" P=0 "AND" Q=0 "AND" K<20
"OR" N=0 "AND" T=1 "AND" K<20
"OR" K=1 "THEN" "GOTO" STEP1;
Q:="IF" K=20 "THEN" -2 "ELSE" -1;
   "END";
READING:=Q;
   "END" OF PROCEDURE READING;

MTDEST(2,'DIGDAT',"FALSE");  N:=T:=0;
"PRINT"''L'PLEASE LOAD BINARY DATA TAPE --- THANKYOU';  WAIT;

NEWBLOCK:
"FOR" T:=1 "STEP" 1 "UNTIL" 500 "DO"
   "BEGIN"
A[T]:=READING;
J:= "IF" T=1 "THEN" 500 "ELSE" T-1;
"IF" A[T]=-2 "THEN" "GOTO" TAPEEND;
"IF" A[T]=-1 "THEN" "PRINT"''L'COMP. CHECK FALSE -
',500*N+T-1,' SUBSTITUTED FOR ',500*N+T;
"IF" A[T]=-1 "THEN" A[T]:=A[J];
"IF" (A[T]<MAX*0.45 "OR" A[T]>MAX*0.55) "AND"
MAX-A[T]"LE"A[J]+1 "AND" MAX-A[T]"GE"A[J]-1 "THEN" A[T]:=MAX-A[T];
   "END";
TAPEEND:
"IF" T>1 "THEN" MTWRITE(2,A,1,T-1);  N:=N+1;
"IF" T>500 "THEN" "GOTO" NEWBLOCK "ELSE" MTCLOSE(2,0);
TOTAL:=500*N+T-501;  "PRINT" ''L'',TOTAL,' DATA.';
   "END" OF DIGITISED DATA READING;

SECTION A:
J:=RUN;  RUN:=J+1;
"PRINT" PUNCH(3),ALIGNED(2,0),''L'SECTION A -- RUN ',RUN; WAIT;
"IF" "NOT" KEY(1) "AND" "NOT" KEY(4) "AND" "NOT" KEY(5)
"THEN" "GOTO" DIGITEND;
```

```
MTREWIND(2);  MTSEEK(2);
T:=N:=P:=0;  J:=DATA;

RETYP1:
I:="IF" KEY(3) "THEN" 3 "ELSE" 1;
READFR(I);  PUNCH(3);
"IF" TIME"LE"0 "THEN"
   "BEGIN"
"IF" KEY(3) "THEN" "PRINT" ''L`TYPE 'Q`TITLE'U` AND
TIME UP SAMPLERATE NO. (-1,-2,-3,-4,-5) 'L``;
J:=1;  INSTRING(TITLE,J);
"READ" TIME;
"IF" TYPERROR "THEN"
   "BEGIN"
TIME:=0; "GOTO" RETYP1;
   "END";
"FOR" J:=1 "WHILE" P<MAX "AND"
T<("IF" TOTAL<500 "THEN" TOTAL "ELSE" 500)
"DO" P:=DATA;
PRESOLV:="IF" P<MAX "THEN" 0 "ELSE" T-1;
SAMPLERATE:="IF" TIME>0 "THEN" (TOTAL-PRESOLV)/(TIME*60)
"ELSE" "IF" TIME=-1 "THEN" 0.290 "ELSE" "IF" TIME=-2 "THEN" 0.490
"ELSE" "IF" TIME=-3 "THEN" 0.794 "ELSE" "IF" TIME=-4 "THEN" 1.349
"ELSE" "IF" TIME=-5 "THEN" 2.213 "ELSE" 0;
"IF" SAMPLERATE=0 "THEN" TIME:=0 "ELSE"
"IF" TIME<0 "THEN" TIME:=(TOTAL-PRESOLV)/(SAMPLERATE*60);
   "END";

"IF" KEY(4) "THEN" "GOTO" LIST;
"IF" KEY(5) "THEN" "GOTO" PLOT;

DATAPROCESSING:
   "BEGIN"
"COMMENT" SEGMENT;
"INTEGER" C,SUBC,NOB,PIN;
"INTEGER" "ARRAY"
PI,START,STARTIME,PKHT,RTIME,END,ENDTIME,AREA[1:200];
"BOOLEAN" SEEKMAY,SEEKEND;
"IF" TIME=0 "THEN"
   "BEGIN"
"PRINT" ''L`CHROMATOGRAM TIME-LENGTH = ZERO MINS.`;
"IF" KEY(3) "THEN" "GOTO" RETYP1 "ELSE" "GOTO" SECTION A;
   "END";
"IF" KEY(3) "THEN" "PRINT" ''L`TYPE IN RESOLUTION VALUE (SECS.) --
AND NO. OF BASELINES -- `
"ELSE" "PRINT" ''L`THIS STAGE MAY TAKE SEVERAL MINS.
PLEASE ALLOW UPTO 3 MINS. IF NECESSARY -- THANKYOU.`;
"READ" C,NOR;
"IF" C>200 "THEN" C:=200;  C:=C*SAMPLERATE;
"IF" C<10 "THEN" C:=10;  SUBC:=C/5;
"IF" NOR<1 "THEN" NOR:=0;
Q:="IF" NOR<1 "THEN" 1 "ELSE" NOB;

PUNCH(4);
"IF" RUN>1 "THEN" "PRINT" ''F``;
"PRINT" ''S10``;  J:=1;  OUTSTRING(TITLE,J);
"PRINT" ''L3S10`SUPPLEMENTARY OUTPUT.'L3S2``,TOTAL,' SAMPLES    `;
"IF" PRESOLV>0 "THEN"
"PRINT" '(',TOTAL-PRESOLV,' FROM SOLVENT FRONT.)`;
```

```
"PRINT" ''L3S2'TIMELENGTH OF CHROMATOGRAM = ',ALIGNED(3,1),TIME,
' MINS.SAMPLING RATE = ',FREEPOINT(3),SAMPLERATE,
' COUNTS PER SEC.'L3S2'RESOLUTION = ',DIGITS(3),C,' SAMPLES   = ',
ALIGNED(3,0),C/SAMPLERATE,' SECS.';
"IF" NOB>0 "THEN" "PRINT" ''L2S2'BASELINE SECTIONS :';

   "BEGIN"
"COMMENT" PEAK ANALYSIS BLOCK;
"REAL" B1,B2;
"INTEGER" "ARRAY" BR[1:NOB+1,1:9];
"REAL" "ARRAY" BASE[1:NOB+1,1:2];
"BOOLEAN" "ARRAY" BENT[1:NOB+1];

"PROCEDURE" ORDER(X,Y);
"INTEGER" X,Y;
   "BEGIN"
"IF" X>Y "THEN"
   "BEGIN"
K:=X;  X:=Y;  Y:=K;
"PRINT" DIGITS(3),
''L'REF. PT.',J,' AND',J+1,' OF BASELINE',Q,' ORDERED';
   "END";
   "END" OF ORDER;

PUNCH(3);
X:=Y:=1;
"FOR" J:=1 "STEP" 1 "UNTIL" 9 "DO" BR[NOB+1,J]:=TOTAL+1;
"FOR" Q:=1 "STEP" 1 "UNTIL" NOB "DO"
   "BEGIN"
"COMMENT" READING AND CHECKING OF BASELINE PARAMETERS;
RETYP2:
"IF" KEY(3) "THEN" "PRINT" DIGITS(3),
''L'TYPE IN REF. PTS. (6,T/F,IF T - 3)'L'FOR BASELINE ',Q,' -- ';
"FOR" J:=1 "STEP" 1 "UNTIL" 6 "DO"
   "BEGIN"
"READ" B1;  BR[Q,J]:=B1*60*SAMPLERATE +PRESOLV;
   "END";
"READ" BENT[Q];
"IF" BENT[Q] "THEN"
   "BEGIN"
"FOR" J:=7,8,9 "DO"
   "BEGIN"
"READ" B1;  BR[Q,J]:=B1*60*SAMPLERATE+PRESOLV;
   "END";
   "END"
"ELSE"
   "BEGIN"
BR[Q,7]:=BR[Q,8]:=BR[Q,4];  BR[Q,9]:=BR[Q,6];
   "END";
"IF" BR[Q,2]=BR[Q,3] "THEN" BR[Q,3]:=BR[Q,3]+1;
"FOR" J:=1,2,1,3,2,1,5,7 "DO" ORDER(BR[Q,J],BR[Q,J+1]);
"IF" BENT[Q] "AND" BR[Q,7]=BR[Q,6] "THEN" BR[Q,7]:=BR[Q,7]+1;
"IF" BENT[Q] "AND" BR[Q,8]=BR[Q,5] "THEN" BR[Q,8]:=BR[Q,8]-1;
"IF" BR[Q,9]<BR[Q,6] "AND" BR[Q,9]>BR[Q,5] "THEN" BR[Q,9]:=BR[Q,6];
"FOR" J:=1 "STEP" 1 "UNTIL" 9 "DO"
"IF" BR[Q,J]>TOTAL "THEN" BR[Q,J]:=TOTAL;
"IF" Q>1 "THEN"
   "BEGIN"
M:="IF" BR[Q-1,4]>BR[Q-1,8] "THEN" BR[Q-1,4] "ELSE" BR[Q-1,8];
"FOR" J:=1,7 "DO"
```

```
"IF" BR[Q,J]"LE" M "THEN"
   "BEGIN"
"PRINT" DIGITS(3),''L'OVERLAP OF BASELINE SECTIONS',Q-1,' AND',Q;
BR[Q,J]:=M+1;
   "END";
   "END";
"IF" TYPERROR "THEN" "GOTO" RETYP2;
"IF" BR[Q,2]-BR[Q,1]"GE"X "THEN" X:=BR[Q,2]-BR[Q,1]+1;
"IF" BR[Q,8]-BR[Q,7]"GE"Y "AND" BR[Q,9]<BR[Q,5]
"THEN" Y:=BR[Q,8]-BR[Q,7]+1;
BASE[Q,1]:=1000000;
BASE[Q,2]:="IF" BR[Q,9]<BR[Q,5] "THEN" -1000000 "ELSE" 1000000;
   "END"OF BASELINE PARAMETER CHECKING;

   "BEGIN"
"COMMENT" DETECTION BLOCK;
"INTEGER" GRAD,MNX,MNXT,X1,X2,X3,Y1,Y2,Y3;
"INTEGER" "ARRAY" DO[1:C],REG1[1:X],REG2[1:Y];

"PROCEDURE" MINMAX;
"COMMENT" DETERMINATION OF HIGHEST/LOWEST READINGS
          BETWEEN SET POINTS IN RESOLUTION ARRAY,
          TOTAL NO. OF SUCH VALUES AND TIME-POSITION OF FIRST;
   "BEGIN"
G:="IF" C+MNXT-T>1 "THEN" C+MNXT-T "ELSE" 1;
MNX:=DO[G]; MNXT:=T-C+G;  M:=0;
"FOR" J:=G+1 "STEP" 1 "UNTIL" C "DO"
   "BEGIN"
"IF" DO[J]=MNX "THEN" M:=M+1;
"IF" SEEKMAX "AND" DO[J]>MNX
"OR" "NOT" SEEKMAX "AND" DO[J]<MNX "THEN"
   "BEGIN"
MNX:=DO[J];  MNXT:=T-C+J;  M:=0;
   "END";
   "END";
   "END" OF PROCEDURE MINMAX;

"PROCEDURE" MARKSTART;
"COMMENT" TO RECORD START/END OF THE SEGMENT;
   "BEGIN"
P:=P+1;
START[P]:=MNX;  STARTIME[P]:=MNXT;  AREA[P]:=0;
"FOR" J:=(C+1-(T-STARTIME[P])) "STEP" 1 "UNTIL" C "DO"
AREA[P]:=AREA[P]+DO[J];
"IF" P>1 "THEN"
   "BEGIN"
END[P-1]:=START[P];  ENDTIME[P-1]:=STARTIME[P];
AREA[P-1]:=AREA[P-1]-AREA[P];
   "END";
   "END" OF PROCEDURE MARKSTART;

PUNCH(4);
Q:=1;  P:=K:=0;  T:=MNXT:=PRESOLV;  SEEKMAX:=SEEKEND:="FALSE";

SEEK:
"IF" "NOT" KEY(1) "THEN" "GOTO" SECTION A;
"IF" KEY(6) "THEN" "PRINT" PUNCH(3),DIGITS(5),''L'',T,' (',
ALIGNED(3,1),(T-PRESOLV)/(SAMPLERATE*60),')';
"IF" "NOT" KEY(1) "OR" T=TOTAL "OR" P=200
"THEN" "GOTO" END OF SEARCH;
```

```
"FOR" G:=1 "STEP" 1 "UNTIL" C-1 "DO" DO[G]:=DO[G+1];  DO[C]:=DATA;

"COMMENT" FITTING OF BASELINE DURING ANALYSIS;
"IF" T"GE"BR[Q,1] "AND" T"LE"BR[Q,2] "THEN"
REG1[T-BR[Q,1]+1]:=DO[C];
"IF" T"GE"BR[Q,3] "AND" T"LE"BR[Q,4] "THEN"
   "BEGIN"
B2:=-1000000;
"FOR" J:=1 "STEP" 1 "UNTIL" BR[Q,2]-BR[Q,1]+1 "DO"
   "BEGIN"
B1:=(DO[C]-REG1[J])/(T-BR[Q,1]-J+1);
"IF" B1>B2 "THEN"
   "BEGIN"
B2:=B1;  X1:=REG1[J];  Y1:=BR[Q,1]+J-1;
   "END";
   "END";
"IF" B2<BASE[Q,1] "THEN"
   "BEGIN"
BASE[Q,1]:=B2;  X2:=X1;  Y2:=Y1;  X3:=DO[C];  Y3:=T;
   "END";
"IF" T=BR[Q,4] "THEN"
   "BEGIN"
"PRINT" DIGITS(3),''L2S11`SECTION `,Q,' FROM `,
ALIGNED(3,1),(Y2-PRESOLV)/(SAMPLERATE*60),' MINS. TO `,
(Y3-PRESOLV)/(SAMPLERATE*60),' MINS.(`,DIGITS(5),Y2,' [`,DIGITS(2),
X2,'] - `,DIGITS(5),Y3,' [`,DIGITS(2),X3,'] )`,FREEPOINT(4);
BR[Q,1]:=Y2;  BR[Q,2]:=X2;  BR[Q,3]:=Y3;
Y1:="IF" BR[Q,9]<BR[Q,5] "THEN" BR[Q,5] "ELSE" BR[Q,6];
X1:=BASE[Q,1]*(Y1-PR[Q,1])+BR[Q,2];
"IF" BR[Q,9]<BR[Q,5] "THEN"
   "BEGIN"
"FOR" J:=1 "STEP" 1 "UNTIL" BR[Q,8]-BR[Q,7]+1 "DO"
   "BEGIN"
B1:=(X1-REG2[J])/(Y1-BR[Q,7]-J+1);
"IF" B1>BASE[Q,2] "THEN"
   "BEGIN"
BASE[Q,2]:=B1;  X2:=REG2[J];  Y2:=BR[Q,7]+J-1;
   "END";
   "END";
   "END";
   "END";
   "END";

"IF" T"GE"BR[Q,7] "AND" T"LE"BR[Q,8] "THEN"
   "BEGIN"
"IF" BR[Q,9]>BR[Q,6] "THEN"
   "BEGIN"
B1:=(DO[C]-X1)/(T-Y1);
"IF" B1<BASE[Q,2]."THEN"
   "BEGIN"
BASE[Q,2]:=B1;  X2:=DO[C];  Y2:=T;
   "END";
   "END"
"ELSE" REG2[T-BR[Q,7]+1]:=DO[C];
   "END";

"IF" T"GE"BR[Q,4] "AND" T"GE"BR[Q,8] "THEN"
   "BEGIN"
"IF" BENT[Q] "THEN"
   "BEGIN"
```

```
"PRINT" ALIGNED(3,1),
''L2S23'CHANGING AT ',(Y1-PRESOLV)/(SAMPLERATE*60),' MINS. TO ',
(Y2-PRESOLV)/(SAMPLERATE*60),' MINS. (',DIGITS(5),Y1,' [',DIGITS(2),
X1,'] - ',DIGITS(5),Y2,' [',DIGITS(2),X2,'] )',FREEPOINT(4);
BR[Q,7]:=Y2;  BR[Q,8]:=X2;
   "END";
Q:=Q+1;
   "END";
"COMMENT" END OF BASELINE FITTING;

"COMMENT" DETECTION OF PEAKS/SEGMENTS DURING ANALYSIS;
"IF" P>0 "THEN" AREA[P]:=AREA[P]+DO[C];
"IF" SEEKMAX "AND" SEEKEND "AND" DO[C]=MNX "THEN" M:=M+1;
"IF" T<MNXT+C-SURC "THEN" "GOTO" SEEK;
GRAD:=DO[C]-DO[SUBC+1];

"IF" P=0 "AND" GRAD>0
"OR" SEEKMAX "AND" "NOT" SEEKEND "AND" GRAD"LE"0
"OR" SEEKEND "AND" "NOT" SEEKMAX "AND" GRAD"GE"0
"OR" SEEKMAX "AND" SEEKEND "AND" GRAD"NE"0
"THEN" K:=K+1 "ELSE" K:=0;
"IF" K"LE"SURC "THEN" "GOTO" SEEK;

"IF" SEEKMAX "AND"  "NOT" SEEKEND "THEN"
   "BEGIN"
MINMAX;
"IF" DO[C]"NE" MNX "AND" DO[C-1]"NE" MNX "THEN"
   "BEGIN"
"COMMENT" A DEFINED PEAK-TOP;
MNXT:=MNXT+M/2;  PKHT[P]:=MNX;  RTIME[P]:=MNXT;  SEEKMAX:="FALSE";
   "END";
SEEKEND:="TRUE";  "GOTO" SEEK;
   "END";

"IF" SEEKEND "AND" "NOT" SEEKMAX "THEN"
   "BEGIN"
MINMAX;
"IF" DO[C]"NE"MNX "AND" DO[C-1]"NE" MNX "THEN"
   "BEGIN"
"COMMENT" A DEFINED PEAK-VALLEY;
MNXT:=MNXT+M/2;  SEEKEND:="FALSE";
   "END";
MARKSTART;  SEEKMAX:="TRUE";
"IF" DO[C]=MNX "OR" DO[C-1]=MNX "THEN" MINMAX;
"GOTO" SEEK;
   "END":

"IF" SEEKMAX "AND" SEEKEND "THEN"
   "BEGIN"
"COMMENT" END OF A PLATEAU;
MNXT:=MNXT+M/2;  PKHT[P]:=MNX;  RTIME[P]:=MNXT;  SEEKMAX:="FALSE";
   "END":

"IF" GRAD>0 "THEN"
   "BEGIN"
"COMMENT" AN UPWARD TREND;
MINMAX;  MNXT:=MNXT+M;  MARKSTART;
SEEKEND:="FALSE";  SEEKMAX:="TRUE";
   "END";
"GOTO" SEEK;
```

```
END OF SEARCH:
"IF" SEEKEND "THEN"
   "BEGIN"
"IF" SEEKMAX "THEN"
   "BEGIN"
PKHT[P]:=MNX;  RTIME[P]:=MNXT+M/2;  SEEKMAX:="FALSE";
   "END";
MINMAX;  MNXT:=MNXT+M;  MARKSTART;
   "END";
"IF" SEEKMAX "OR" SEEKEND "THEN" P:=P-1;

   "BEGIN"
"COMMENT" BASELINE CORRECTION TO SEGMENT AREAS;
"INTEGER" "ARRAY" SC,EC,AC[1:P];
PIN:=0;
"IF" NOB<1 "THEN" "PRINT" ''L2S23'NO BASELINE CONSTRUCTED.'
"ELSE" "PRINT" ''L3S11'FOR CORRECTION OF SEGMENTS WITHIN THE RANGE';
"FOR" Q:=1 "STEP" 1 "UNTIL" NOB "DO"
   "BEGIN"
"IF" "NOT" BENT[Q] "THEN"
   "BEGIN"
X3:=BR[Q,5];  Y3:=K:=BR[Q,6];  B1:=B2:=BASE[Q,1];
X1:=X2:=BR[Q,2];  Y1:=Y2:=BR[Q,1];
   "END"
"ELSE"
   "BEGIN"
"IF" BR[Q,9]<BR[Q,5] "THEN"
   "BEGIN"
X3:=BR[Q,9];  Y3:=BR[Q,6];   B1:=BASE[Q,2];  B2:=BASE[Q,1];
X1:=BR[Q,8];  Y1:=BR[Q,7];  X2:=BR[Q,2];  Y2:=BR[Q,1];  K:=BR[Q,5];
   "END"
"ELSE"
   "BEGIN"
X3:=BR[Q,5];  Y3:=BR[Q,9];  B1:=BASE[Q,1];  B2:=BASE[Q,2];
X1:=BR[Q,2];  Y1:=BR[Q,1];  X2:=BR[Q,8];  Y2:=BR[Q,7];  K:=BR[Q,6];
   "END";
   "END";
C:=B1*(K-Y1)+X1;
"FOR" N:=X3,Y3 "DO"
   "BEGIN"
M:=1;
"FOR" J:=2 "STEP" 1 "UNTIL" P+1 "DO"
"IF" ABS(N-STARTIME[J])<ABS(N-STARTIME[M]) "THEN" M:=J;
"IF" N=X3 "THEN" L:=M;
   "END";
"FOR" J:=L "STEP" 1 "UNTIL" M-1 "DO"
   "BEGIN"
"IF" STARTIME[J]<K "THEN" SC[J]:=B1*(STARTIME[J]-Y1)+X1
                   "ELSE" SC[J]:=B2*(STARTIME[J]-Y2)+X2;
"IF" ENDTIME[J]<K  "THEN" EC[J]:=B1*(ENDTIME[J]-Y1)+X1
                   "ELSE" EC[J]:=B2*(ENDTIME[J]-Y2)+X2;
"IF" STARTIME[J]<K "AND" ENDTIME[J]>K "THEN"
AC[J]:=(SC[J]+C)*(K-STARTIME[J])/2 + (EC[J]+C)*(ENDTIME[J]-K)/2
 "ELSE"AC[J]:=(SC[J]+EC[J])*(ENDTIME[J]-STARTIME[J])/2;
AREA[J]:=AREA[J]-AC[J];  PIN:=PIN+1;  PI[PIN]:=J;
   "END";
"PRINT" ALIGNED(3,1),
''L2S23'',(STARTIME[L]-PRESOLV)/(SAMPLERATE*60),' MINS. TO ',
(STARTIME[M]-PPESOLV)/(SAMPLERATE*60),' MINS. (',
```

```
DIGITS(5),STARTIME[L];
"IF" M=L
"THEN" "PRINT"' *** SINGLE-POINT RANGE - DATA INCORRECT *** )'
"ELSE" "PRINT" ' [',DIGITS(2),SC[L],'] - ',
DIGITS(5),ENDTIME[M-1],' [',DIGITS(2),EC[M-1],']',
DIGITS(3),' = SEGMENTS ',L,' TO',M-1,')'L'';
   "END";
   "END" OF BASELINE CORRECTION TO SEGMENT AREAS;

"PRINT" ''L2'',T,' SAMPLES PROCESSED.'S10'',P,' SEGMENTS RECORDED.';
"IF" P"LE"0 "THEN"
   "BEGIN"
"PRINT" ''F'',PUNCH(3),
''L'',DIGITS(3),P,' SEGMENTS RECORDED.
PLEASE SET KEY(4).
AT NEXT SECTION CANCEL ALL KEYS AND
IGNORE OTHER INSTRUCTIONS FOR THIS DATA RUN';
WAIT;
"GOTO" LIST;
   "END";

"PRINT"''S10'',PIN,' SEGMENTS CORRECTED.';
"IF" PIN=0 "THEN"
   "BEGIN"
PIN:=P;
"FOR" J:=1 "STEP" 1 "UNTIL" P "DO" PI[J]:=J;
   "END";
   "END" OF DETECTION BLOCK;

   "BEGIN"
"COMMENT" TO ALLOW FOR SEGMENT-GROUPING, COMPONENT LABELLING
          AND REL. RESP. FACTOR ASSIGNMENT;
"INTEGER" GP,LC,SP;
"REAL" WST,CST,WS,CS;
"BOOLEAN" GROUPED,FACTORED,LABELLED;
"INTEGER" "ARRAY" CMPD[1:20*PIN];
"REAL" "ARRAY" RF[1:PIN];

"PROCEDURE" SPACE(X,Y);
"INTEGER" X,Y;
   "BEGIN"
"INTEGER" J;
"FOR" J:=X "STEP" 1 "UNTIL" Y "DO" "PRINT" ' ';
   "END" OF PROCEDURE SPACE;

"PROCEDURE" OUTSEM(A,I,N);
   "VALUE" N;
   "INTEGER" "ARRAY" A;
   "INTEGER" I,N;
"BEGIN" "INTEGER" "ARRAY" B[1:1];
        "INTEGER" TEMP,MM,NN,C,WORD,CHAR,ADDRESS,K,IN;

        IN:=0;
        WORD:=0;  CHAR:=1;  K:=I;
        "CODE"
        %GETA$A
        %INDEX$K
        %STR$ADDRESS
        %JOF$L
        $$L;
```

```
          K:=0;
LOOP:     "CODE"
          %LDR$WORD
          %ADDR$ADDRESS
          %LD:M$0
          %LDK$CHAR
          %SMLC
          %AND:L$63
          %ST$C;
          CHAR:=CHAR+1;
          "IF" CHAR>4 "THEN"
          "BEGIN" CHAR:=1;  WORD:=WORD+1;
          "END";
          "IF" C>61 "THEN"
          "BEGIN" IN:=63-C;  "GOTO" LOOP
          "END";
          C:=C+64*IN;
          "IF" C=96 "THEN" "GOTO" FIN;
          "IF" K"GE" N "THEN" "GOTO" LOOP;

          MM:=2;  NN:=3;
            "IF"C>63"THEN"
            "BEGIN" MM:=3; NN:=2; C:=C-64
            "END";
          "CODE"
          %LD:L$62
          %LDK$MM
          %SMLC
          %ADD:L$2111
          %ST$TEMP
          %LD$C
          %LDK$NN
          %SMLC
          %ADD$TEMP
          %ST$C
          %JOF$NOFLO
          $$NOFLO;
          B[1]:=C;
          MM:=1;
          OUTSTRING(B,MM);
          K:=K+1;
          "GOTO" LOOP;
FIN:      "IF" CHAR>1 "THEN" WORD:=WORD+1;
          I:=I+WORD;
          "IF" K<N "THEN" "FOR" K:=K+1 "WHILE" K<N+1 "DO" "PRINT" ' `;
    "END" OF PROCEDURE OUTSEM;

GROUPED:=FACTORED:=LABELLED:="FALSE";  GP:=PIN;  LC:=3;

SUBSECTION B:
RUN:=RUN+0.1;
"PRINT" PUNCH(3),ALIGNED(3,1),''L`SECTION B  --  RUN  `,RUN;  WAIT;
I:="IF" KEY(3) "THEN" 3 "ELSE" 1;
READER(I);  PUNCH(3);
"IF" "NOT" KEY(1) "THEN" "GOTO" SECTION A;

RETYP3:
"IF" KEY(2) "THEN"
   "BEGIN"
```

```
"COMMENT" TO GROUP THE SEGMENTS DETECTED INTO THE PEAKS OF INTEREST;
"IF" KEY(3) "THEN" "PRINT" ''L`TYPE IN NO. OF GROUPED PKS. ---`;
"READ"GP;
"IF" GP"LE"0 "THEN"
   "BEGIN"
"PRINT" ''L`NO. OF GROUPED PEAKS = ZERO`;  GP:=PIN;
"IF" KEY(3) "THEN" "GOTO" RETYP3 "ELSE" "GOTO" RETYP4;
   "END";
   "BEGIN"
"INTEGER" "ARRAY" RP[1:GP,1:2];
"IF" KEY(3) "THEN" "PRINT" ''L`TYPE IN PK. GROUP REF. PTS. ---'L'';
"FOR" Q:=1 "STEP" 1 "UNTIL"GP "DO"
"FOR" J:=1,2 "DO"
   "BEGIN"
"READ" B1;
RP[Q,J]:=B1*60*SAMPLERATE + PRESOLV*(ABS(B1)/B1);
"IF" ABS(RP[Q,J])>TOTAL "THEN"
   "BEGIN"
"PRINT" ''L`REF. PT. BEYOND CHROMATOGRAM TIME-LENGTH`;
"IF" KEY(3) "THEN" "GOTO" RETYP3;
RP[Q,J]:=TOTAL*(ABS(B1)/B1);
   "END";
   "END";
"IF" GP>PIN "THEN"
   "BEGIN"
"PRINT" DIGITS(3),''L'',GP,' PEAKS REQUESTED,
ONLY `,PIN,' SEGMENTS CORRECTED FOR BASELINE.`;
GP:=PIN;
"IF" "NOT" KEY(3) "THEN" "GOTO" RETYP4;
   "END";
"IF" TYPERROR "THEN" "GOTO" RETYP3;

"FOR" Q:=1 "STEP" 1 "UNTIL"GP "DO"
   "BEGIN"
"FOR" N:=RP[Q,1],RP[Q,2] "DO"
"IF" N<0 "THEN"
   "BEGIN"
N:=-N;  L:=M:=1;
"FOR" J:=1 "STEP" 1 "UNTIL" NOR "DO"
"FOR" K:=1,3,7 "DO"
"IF" ABS(N-BR[J,K])<ABS(N-BR[L,M]) "THEN"
   "BEGIN"
L:=J;  M:=K;
   "END";
"IF" N=-RP[Q,1] "THEN" RP[Q,1]:=BR[L,M] "ELSE" RP[Q,2]:=BR[L,M];
   "END";
"FOR" N:=RP[Q,1],RP[Q,2] "DO"
   "BEGIN"
M:=1;
"FOR" J:=2 "STEP" 1 "UNTIL" P+1 "DO"
"IF" ABS(N-STARTIME[J])<ABS(N-STARTIME[M]) "THEN" M:=J;
"IF" N=RP[Q,1] "THEN" L:=M;
   "END";
"IF" "NOT" GROUPED "THEN" "PRINT" PUNCH(4),DIGITS(3),
''L2S29`GROUPED PEAK`,Q,'  COMPRISES OF`,M-L,DIGITS(5),
' DETECTED SEGMENTS (SAMPLE-POINT`,STARTIME[L],' TO`,STARTIME[M],')`;
START[Q]:=START[L];   STARTIME[Q]:=STARTIME[L];
END[Q]:=START[M];       ENDTIME[Q]:=STARTIME[M];
PKHT[Q]:=PKHT[L];     RTIME[Q]:=RTIME[L];  AREA[Q]:=AREA[L];
N:=1;
```

```
"FOR" J:=L+1 "STEP" 1 "UNTIL" M-1 "DO"
   "BEGIN"
AREA[Q]:=AREA[Q]+AREA[J];
"IF" PKHT[J]>PKHT[Q] "THEN"
   "BEGIN"
PKHT[Q]:=PKHT[J];  RTIME[Q]:=RTIME[J];  N:=1;
   "END";
"IF" PKHT[J]=PKHT[Q] "THEN"
   "BEGIN"
RTIME[Q]:=RTIME[Q]+RTIME[J];  N:=N+1;
   "END";
   "END";
RTIME[Q]:=RTIME[Q]/N;  PI[Q]:=0;
"IF" STARTIME[Q]=ENDTIME[Q] "THEN"
   "BEGIN"
"PRINT" PUNCH(3),''L'ERROR IN PK. GROUP REF. PTS.';
"IF" KEY(3) "THEN" "GOTO" RETYP3;
RTIME[Q]:=STARTIME[Q];  AREA[Q]:=0;
   "END";
   "END";
   "END";
GROUPED:="TRUE";  PIN:=GP;
   "END" OF SEGMENT GROUPING;

RETYP4:
"IF" KEY(4) "OR" KEY(6) "THEN"
   "BEGIN"
"IF" KEY(6) "THEN"
   "BEGIN"
"IF" KEY(3) "THEN"
"PRINT" ''L'TYPE CHARACTER-LENGTH OF LONGEST NAME (MAX 60). 'L'';
"READ"LC;
"IF" LC<7 "THEN" LC:=7;  "IF" LC>60 "THEN" LC:=60;
   "END";
"FOR" Q:=1 "STEP" 1 "UNTIL" GP "DO"
   "BEGIN"
"IF" KEY(6) "THEN"
   "BEGIN"
"IF" KEY(3) "THEN" "PRINT" DIGITS(3),''LS11Q'NAME'U' ('',Q,' )  ';
LABELLED:="TRUE";  K:=20*Q-19;  INSTRING(CMPD,K);
   "END";
"IF" KEY(4) "THEN"
   "BEGIN"
"IF" KEY(3)
"THEN" "PRINT" DIGITS(3),''L'REL. RESP. FACTOR ('',Q,' )  ';
"READ"RF[Q];  FACTORED:="TRUE";
   "END";
   "END";
"IF" TYPERROR "THEN" "GOTO"  RETYP4;
   "END";

RETYP5:
M:=0;  "IF" "NOT" FACTORED "THEN" "GOTO" HEADINGS;
"IF" KEY(3) "THEN" "PRINT" ''L'TYPE M-VALUE --- ';
"COMMENT" TO ALLOW CHOICE OF FORMAT
M = 0 FOR ABSOLUTE AREAS.
M = 1 FOR NORMALISATION.
M = 2 FOR STANDARDISATION REL. TO INTERNAL STANDARD.
M = 3  "          "      "    "     "        "      OF KNOWN WT.
                                                    AND CONCN. ;
```

```
"READ" M;
"IF" M<1 "OR" M>3 "THEN"
   "BEGIN"
"PRINT" ''L'ERROR IN M-VALUE,';
"IF" KEY(3) "THEN" "GOTO" RETYP5 "ELSE" M:=1;
   "END";
"IF" KEY(3) "THEN" "PRINT" ''L'TYPE NO. OF PKS. OF INTEREST --- ';
"READ" PIN;
"IF" PIN>GP "THEN"
   "BEGIN"
"PRINT" ''L'PKS.OF INTEREST > GROUPED PKS.';
PIN:=GP;  M:=1;
   "END";
"IF" KEY(3) "THEN"
   "BEGIN"
"IF" PIN<GP "THEN"
"PRINT" ''L'TYPE GROUP-PK NOS. OF PKS OF INTEREST - ';
"IF" M>1 "THEN" "PRINT" ''L'TYPE INTERNAL STANDARD PK. NO. --- ';
"IF" M>2 "THEN" "PRINT"
''L'TYPE - WT. (GMS.) AND % CONCN. OF ADDED STANDARD SOLN.
        WT. (GMS.) AND % CONCN. OF SAMPLE SOLN. 'L'';
   "END":
"FOR" J:=1 "STEP" 1 "UNTIL" PIN "DO"
   "BEGIN"
"IF" PIN=GP "THEN" PI[J]:=J "ELSE" "READ" PI[J];
"IF" PI[J]>GP "THEN"
   "BEGIN"
"PRINT"''L'GROUP PK. NO. OF PK. OF INTEREST
IS OUTSIDE RANGE OF GROUPED PKS.';
PIN:=GP;  M:=1;  J:=0;
   "END";
   "END";
"IF" M>1 "THEN"
   "BEGIN"
"READ" SP;
"FOR" J:=1 "STEP" 1 "UNTIL" PIN "DO"
"IF" SP=PI[J] "THEN" ERROR:="TRUE";
"IF" "NOT" ERROR "THEN"
   "BEGIN"
"PRINT" ''L'STANDARD PEAK OUTSIDE RANGE OF PEAKS OF INTEREST.';
M:=1;
   "END";
   "END";
"IF" M=1 "THEN"
   "BEGIN"
SP:=P+1;  AREA[SP]:=0;
"FOR" J:=1 "STEP" 1 "UNTIL" PIN "DO"
AREA[SP]:=AREA[SP]+RF[PI[J]]*AREA[PI[J]];
   "END";
"IF" M>2 "THEN" "READ" WST,CST,WS,CS;
"IF" TYPERROR "THEN" "GOTO" RETYP5;

HEADINGS:
PUNCH(4);
C:="IF" "NOT" FACTORED "AND" "NOT" GROUPED "THEN" 50"ELSE"
"IF" M=0 "THEN" 36"ELSE" "IF" M=1 "THEN" 62"ELSE"
"IF" M=2 "THEN" 66 "ELSE" 72;
B:="IF" LC>9 "THEN" LC "ELSE" 9;
G:=66-(B+C)"DIV"2;
B:="IF" LC>9 "THEN" ((LC-9)"DIV"2+1) "ELSE" 1;
```

```
C:="IF" LC>9 "THEN" (G+LC-9) "ELSE" G;
"PRINT" ''F'';  SPACE(1,G);  J:=1;  OUTSTRING(TITLE,J);
"PRINT" ''L2S5B'ANALYSIS RESULTS 'L4'';  SPACE(B,C);
"PRINT" 'COMPONENT';  SPACE(1,B+3);
"IF" "NOT" FACTORED "AND" "NOT" GROUPED "THEN"
"PRINT" ' SEGMENT'S4'' "ELSE"   "PRINT" 'PEAK-SPAN'S3'';
"IF"PRESOLV>0 "THEN" "PRINT" 'REL.R' "ELSE" "PRINT" ''S'RET.';
"PRINT" 'TIME'S3'ARS.AREA';
"IF" "NOT" FACTORED "AND" "NOT" GROUPED "THEN"
"PRINT" ''S3'AREA : SPAN';
"IF" M>0 "THEN" "PRINT" ''S4'REL.R.F.';
"IF" M=1 "THEN" "PRINT" ''S7'% COMP.';
"IF" M=2 "THEN" "PRINT" ''S2'REL. TO STANDARD';
"IF" M=3 "THEN" "PRINT" ''S5'WT.'S7'% OF';
"PRINT" ''L'';  SPACE(1,C+12);
"IF" "NOT" FACTORED "AND" "NOT" GROUPED "THEN" "PRINT"
''S3'SPAN'S9'MINS.'S18'RATIO' "ELSE" "PRINT" ''S4'MINS.'S7'MINS.';
"IF" M=1 "OR" M=2 "THEN" "PRINT" ''S32'W/W';
"IF" M=3 "THEN" "PRINT" ''S28'GMS.'S3'SAMPLE SOLN.';

PRINTOUT:
B:="IF" LC>9 "THEN" 1 "ELSE" ((9-LC)"DIV"2+1);
C:="IF" LC>9 "THEN" G "ELSE" (G-LC+9);
"FOR" Q:=1 "STEP" 1 "UNTIL" PIN "DO"
   "BEGIN"
J:= PI[Q];
"IF" J=SP "THEN" Z:=J;
"PRINT" ''L2'';  SPACE(B,C);
"IF" "NOT" LABELLED "THEN" "PRINT" DIGITS(3),Q,''S5'' "ELSE"
   "BEGIN"
K:=20*J-19;  OUTSFM(CMPD,K,LC);  SPACE(1,B+1);
   "END":
"IF" "NOT" FACTORED "AND" "NOT" GROUPED "THEN"
"PRINT" DIGITS(5),STARTIME[J],'-',ENDTIME[J]  "ELSE"
"PRINT" ALIGNED(3,1),(STARTIME[J]-PRESOLV)/(SAMPLERATE*60),
'-',(ENDTIME[J]-PRESOLV)/(SAMPLERATE*60);
"PRINT" ALIGNED(3,1),''S5'',(RTIME[J]-PRESOLV)/(SAMPLERATE*60),
''S4'',DIGITS(8),AREA[J];
"IF" "NOT" FACTORED "AND" "NOT" GROUPED "THEN"
"PRINT" ''S6'',ALIGNED(4,1),AREA[J]/(ENDTIME[J]-STARTIME[J]);
"IF" M>0 "THEN" "PRINT" ''S5'',ALIGNED(3,3),RF[J];
"IF" M=1 "THEN" "PRINT" ''S7'',ALIGNED(3,1),AREA[J]*RF[J]/AREA[SP]*100;
"IF" M=2 "THEN" "PRINT" ''S6'',ALIGNED(4,3),
(AREA[J]*RF[J])/(AREA[SP]*RF[SP]);
"IF" M=3 "THEN"
   "BEGIN"
 "PRINT" ''S4'',FREEPOINT(5),
(AREA[J]*RF[J])/(AREA[SP]*RF[SP])*WST*CST/100;
"IF" J"NE"SP "THEN" "PRINT" ''S6'',FREEPOINT(3),
(AREA[J]*RF[J])/(AREA[SP]*RF[SP])*(WST*CST)/(WS*CS)*100
"ELSE" "PRINT" ''S7'-';
   "END";
   "END" OF PRINTOUT BLOCK;

"IF" M=1 "THEN" "PRINT" ''L4S58'AREAS NORMALISED.';
"IF" M>1 "THEN"
   "BEGIN"
"PRINT" ''L4S56'AREAS STANDARDISED -'L2S55' REL. TO ';
"IF" "NOT" LABELLED "THEN" "PRINT" ALIGNED(3,1),
'COMPONENT OF RTIME ',(RTIME[SP]-PRESOLV)/(SAMPLERATE*60),' MINS.'
```

```
 "ELSE"
   "BEGIN"
K:=20*7-19:  OUTSTRING(CMPD,K);
   "END";
   "END":
"IF" M=3 "THEN" "PRINT" FREEPOINT(5),
''L5S40'WT. OF ADDED INTERNAL STANDARD SOLN. = ',WST,' GMS.',
''L2S37'CONCN. OF ADDED INTERNAL STANDARD SOLN. = ',CST,' %',
''L2S57'WT. OF SAMPLE SOLN. = ',WS,' GMS.',
''L2S54'CONCN. OF SAMPLE SOLN. = ',CS,' %';
"GOTO" SUBSECTION B;
   "END" OF PROVISION FOR LABELLING AND RESP FACTOR ASSIGNMENT;
   "END" OF PEAK ANALYSIS BLOCK;
   "END" OF DATAPROCESSING BLOCK;

PLOT:
   "BEGIN"
"COMMENT" SEGMENT;
"REAL" DIR,DD,LENGTH,FORESTEP,SIDESTEP,SUBSTEP;
"INTEGER" "ARRAY" B[1:4];
"IF" KEY(3) "THEN" "PRINT" PUNCH(3),
''L'TYPE IN DIMENSIONS OF PLOT (INCHES)
(DEFLECTION AXIS, TIME AXIS)
(+VE DEFLECTION FOR L->R / -VE FOR R->L) 'L'';
"READ" DIR,LENGTH;
"IF" DIR=0 "OR" ABS(DIR)>12 "THEN" DIR:=5;
"IF" LENGTH"LE"0 "OR" LENGTH>36 "THEN" LENGTH:=9;
SIDESTEP:=DIR*200/MAX;  FORESTEP:=LENGTH*200/(TOTAL-PRESOLV);
"IF" KEY(6) "AND" KEY(3) "AND" TIME"NE"0 "THEN" "PRINT" PUNCH(3),
''L'TYPE IN NO. OF NAMED PEAKS -- ';
"IF" KEY(6) "AND" TIME"NE"0 "THEN" "READ" X "ELSE" X:=0;
"IF" X<0 "THEN" X:=0;

   "BEGIN"
"INTEGER" "ARRAY" NT[1:X+1],NAME[1:6*X+6];
"FOR" Q:=1 "STEP" 1 "UNTIL" X "DO"
   "BEGIN"
"IF" KEY(3) "THEN" "PRINT" PUNCH(3),DIGITS(3),
''L'TYPE IN NAME AND TIME (MINS.) OF COMPONENT',Q,' -- ';
Y:=6*Q-5;  INSTRING(NAME,Y);
"READ" SUBSTEP;  NT[Q]:=SUBSTEP*60*SAMPLERATE + PRESOLV;
"IF" Q>1 "THEN"
   "BEGIN"
"IF" NT[Q]-NT[Q-1]<30/FORESTEP
"THEN" NT[Q]:=NT[Q-1]-ABS(DIR)/DIR*30/FORESTEP;
   "END";
   "END";
"IF" TYPERROR "THEN" "GOTO" PLOT;
C:=M:=R:=T:=0;  DD:=0;

PUNCH(5);
J:="IF" DIR>0 "THEN" 100 "ELSE" 2400;
L:="IF" DIR>0 "THEN" 0 "ELSE" 2;
Z:=4;
SETORIGIN(J,0);  WAY(L,Z);
"FOR" K:=1 "STEP" 1 "UNTIL" ("IF" KEY(2) "THEN" 4 "ELSE" 1 ) "DO"
B[K]:=DATA;
MOVEPEN(B[1]*SIDESTEP,0);  DRAWLINE(B[1]*SIDESTEP,0);
"FOR" J:=1 "WHILE" T<TOTAL "DO"
   "BEGIN"
```

```
"IF" "NOT" KEY(5) "THEN" "GOTO" SECTION A;
"FOR" K:=2 "STEP" 1 "UNTIL" ("IF" KEY(2) "THEN" 4 "ELSE" 1 ) "DO"
B[K-1]:=B[K];
B[K-1]:=DATA;
"FOR" Q:=1 "STEP" 1 "UNTIL" X "DO"
"IF" T=NT[Q] "THEN"
   "BEGIN"
MOVEPEN(B[K-1]*SIDESTEP+100*ABS(DIR)/DIR, T*FORESTEP);
Y:=6*Q-5:  OUTSTRING(NAME,Y);  MOVEPEN(DD,M*FORESTEP);
   "END";

"IF" "NOT" KEY(2) "THEN"
   "BEGIN"
DD:=B[1]*SIDESTEP;  M:=T;
DRAWLINE(DD,M*FORESTEP);
   "END"
"ELSE"
   "BEGIN"
C:=C+1:
"IF" T<TOTAL "AND"
(B[2]-B[1]=1 "AND" B[3]"LE"B[1] "AND" B[4]"LE"B[1] "OR"
B[1]-B[2]=1 "AND" B[3]"GE"B[1] "AND" B[4]"GE"B[1] "OR"
B[1]=B[2]) "THEN" B[2]:=B[1]
"ELSE"
   "BEGIN"
"IF" B[2]-B[1]=1 "AND" B[3]"LE"B[1]
"OR" B[1]-B[2]=1 "AND" B[3]"GE"B[1] "THEN" B[3]:=B[2];
P:=B[1];  Q:="IF" T=TOTAL "THEN" B[4] "ELSE" B[2];
L:="IF" T=TOTAL "THEN" T "ELSE"
"IF" ABS(P-Q)=1 "THEN" ENTIER(T-3-(C-1)/2) "ELSE" T-3;
SUBSTEP:=SIDESTEP*R/(L-M);  DD:=SIDESTEP*(P-R);
"FOR" K:=M+1 "STEP" 1 "UNTIL" L "DO"
   "BEGIN"
DD:=DD+SUBSTEP;  DRAWLINE(DD,K*FORESTEP);
   "END";
M:=L;  C:=0;  R:="IF" ABS(P-Q)>1 "THEN" Q "ELSE" Q-P;
   "END";
   "END";
   "END";
   "END":

L:="IF" DIR>0 "THEN" 3 "ELSE" 1;
WAY(L,7);
T:="IF" DIR<0 "THEN" 200 "ELSE" TOTAL*FORESTEP-200;
MOVEPEN(MAX*SIDESTEP+2,T);  J:=1;  OUTSTRING(TITLE,J);
R:=ABS(DIR)/DIR;

"IF" TIME>0 "THEN"
   "BEGIN"
I:="IF" TIME<10 "THEN" 1 "ELSE" "IF" TIME<20 "THEN" 2
"ELSE" "IF" TIME<50 "THEN" 5 "ELSE" 10;
"FOR" Q:=1 "WHILE" TIME/I>LENGTH "DO" I:=2*I;
"FOR" K:=0 "STEP" I "UNTIL" TIME "DO"
   "BEGIN"
T:=K*(TOTAL-PRESOLV)/TIME + PRESOLV;
MOVEPEN(0,T*FORESTEP);  DRAWLINE(-10*R,T*FORESTEP);
L:="IF" K<10 "THEN" 1 "ELSE" "IF" K<100 "THEN" 2 "ELSE"3;
MOVEPEN(-6*(Z+3)*R,T*FORESTEP+2.5*Z*L*R); "PRINT" DIGITS(L),K;
   "END";
MOVEPEN(-12*(Z+2)*R,TOTAL*FORESTEP+12.5*Z*(R-1));  "PRINT" 'MINS.';
```

```
    "END";

MOVEPEN(0,TOTAL*FORESTEP+200);
"GOTO" SECTION A;
    "END" OF PLOT;

LIST:
    "BEGIN"
"COMMENT" SEGMENT;
"INTEGER" "ARRAY" B[1:20];
I:="IF" KEY(6) "THEN" 1 "ELSE" 4;
PUNCH(I);
"IF" RUN>1 "THEN" "PRINT" ''F``;
T:=0;  J:=1;
"PRINT" ''S9``;  OUTSTRING(TITLE,J);
"PRINT"''L2S9`LIST OF DIGITAL OUTPUT'S9`TOTAL DATA = `,TOTAL,''LR40``;
"FOR" I:=I "WHILE" T<TOTAL "DO"
    "BEGIN"
"FOR" M:=1 "STEP" 1 "UNTIL" 10 "DO"
    "BEGIN"
"FOR" K:=1 "STEP" 1 "UNTIL" 5 "DO"
    "BEGIN"
"PRINT" ''L``;
"FOR" J:=1 "STEP" 1 "UNTIL" 20 "DO"
    "BEGIN"
B[J]:=DATA;  "PRINT" DIGITS(6),B[J];
"IF" T=TOTAL "OR" "NOT" KEY(4) "THEN"
    "BEGIN"
"IF" I=1 "THEN" "PRINT" ''LR20`-1'LR20HR60``;
"GOTO" SECTION A;
   "END";
    "END";
    "END";
"IF" "NOT" KEY(6) "THEN" "PRINT" ' **(`,DIGITS(5),T,' )`;
    "END";
"PRINT" ''F``;
    "END";
    "END" OF LIST;

DIGITEND:
    "END" OF PROGRAM DIGIT;
```

```
        SUPPLEMENTARY DATA & KEY SETTINGS FOR PROGRAM DIGIT.
AT MESSAGE "SECTION A"
**********************
NO KEYS - ENDS PROGRAM.
KEY(1) FOR PEAK-DETECTION & BASELINE CORRECTION.
KEY(4) FOR LIST OF DIGITAL DATA- WITH KEY(6) IF REQUIRED ON PUNCH TAPE.
KEY(5) FOR PLOT OF DIGITAL DATA, KEY(2) IF SMOOTHING REQUIRED
                                AND KEY(6) FOR NAMING..

SUPPLEMENTARY DATA REQUIRED :-
IF TIMELENGTH=0 OR HAS NOT YET BEEN ASSIGNED THEN
     - 'TITLE',
     - TIMELENGTH (MINS.) OR SAMPLERSPEED NO. (-1,-2,-3,-4,-5),

KEY 4 - NO FURTHER SUPPLEMENTARY DATA REQUIRED.
     RETURNS TO SECTION A AT END OF OPERATION.

KEY 5 - DIMENSIONS OF PLOT (INCHES)(DEFLECTION AXIS, TIME AXIS),
     - KEY 6 - NO. OF PEAKS TO BE NAMED,
               - 'NAME' AND ELUTION TIME (MINS.) -- FOR EACH PEAK.
     RETURNS TO SECTION A AT END OF OPERATION.

KEY 1 - RESOLUTION VALUE (SECS.), NO. OF BASELINES,
     - BASELINE REF. PTS. (MINS.),          )
     - EXTENSION LIMITS (MINS.),            )  FOR
     - T/F (BENT OR NOT BENT),              )  EACH
     - IF T - BASELINE REF. PTS. (MINS.))  BASE-
            - EXTENSION LIMIT (MINS.),  )  LINE
     - AT MESSAGE "SECTION B"
       **********************
   - KEY 1 NOT SET - RETURNS TO SECTION A.
   - KEY 2 FOR PK. GROUPING/RE-GROUPING.
   - KEY 4 FOR REL. RESP. FACTOR ASSIGNMENT/RE-ASSIGNMENT.
   - KEY 6 FOR COMPONENT LABELLING/RE-LABELLING.
   - KEY 2 - NO. OF PEAK GROUPS,
           - PK. GROUP REF. PTS. (MINS.)
             (IF -VE, NEAREST BASELINE REF. PT. USED),
   - KEY 6 - CHARACTER LENGTH OF LONGEST NAME (MAX. 60),
           - 'COMPONENT NAME',    )  FOR EACH
   - KEY 4 - REL. RESP. FACTOR,   )  GROUPED PEAK.
   - IF REL. RESP. FACTOR ASSIGNED -
           - M-VALUE (0,1,2,3,),
                     (0 FOR ABSOLUTE AREAS ONLY.
                      1 FOR NORMALISATION.
                      2 FOR STANDARDISATION REL.
                      TO INTERNAL STANDARD PEAK.
                      3 FOR STANDARDISATION WITH
                      STANDARD OF KNOWN WT. & CONCN.)
             - NO. OF PEAKS OF INTEREST,
             - GROUP PK. NOS. OF PKS. OF INTEREST
               (OMIT IF ALL PKS. ARE OF INTEREST),
             - IF M>1 - GROUP PK. NO. OF INTERNAL STANDARD PK.,
             - IF M>2 - WT. (GMS.) & % CONCN. OF INT. STAND. SOLN.,
                      - "    "    " "   "    "  SAMPLE SOLN.,
   RETURNS TO SECTION B AT END OF OPERATION.
```

POST-GRADUATE STUDIES

Practical Course in Mass Spectronetry, Kingston Polytechnic, 1960 - 69

Spectroscopic Methods on Organic Metallic Chemistry, Kingston Polytechnic, 1969

Algol Programming, Kingston Polytechnic, 1969

Introduction to Gas Chromatography, Kingston Polytechnic, 1969 - 70

Quantitative Gas Chromatography, Kingston Polytechnic, 1970

Recent Advances in Chromatography, Kingston Polytechnic, 1970

Antioxidants and Antioxidant Behaviour, Hatfield Polytechnic, 1970

Symposium of Mass Spectrometry, Kingston Polytechnic, 1970

Modern Industrial Inorganic Chemistry, Kingston Polytechnic, 1971

Automation in Analytical Chemistry, Manchester University, 1971

A number of lectures organised by the Chemical Society and Royal Institute of Chemistry, London, 1963 - 72.

Research colloquia and individual special lectures were attended and a research colloquium was given.

References

1. S.N. Muturi, J.E. Parlevliet and J.G. Brewer, Pyrethrum Post, 10 (1), 24 and 28, (1969).
2. J.E. Parlevliet, Pyrethrum Post, 10 (3), 20, (1970); 10 (4), 10, (1970).
3. S.W. Head, Pyrethrum Post, 7 (2), 3, (1963).
4. S.W. Head, Pyrethrum Post, 8 (4), 32, (1966).
5. C.B. Gnadinger, "Pyrethrum Flowers", McGill Lithograph Co., Minneapolis, Minn., U.S.A., 1st Edition, 1933; 2nd Edition, 1936.
6. G.D. Glynne Jones and S.W. Head, Pyrethrum Post, 8 (1), 11, (1965).
7. H.P. Smart, Pyrethrum Post, 6 (3), 3, (1962).
8. D.M. Hatcher, Pyrethrum Post, 7 (2), 28, (1963).
9. D.R. Maciver, Pyrethrum Post, 7 (2), 22, (1963); 7 (3), 7, (1964); 7 (3), 15, (1964).
10. J.E. Lee, Pyrethrum Post, 9 (2), 18, (1967); 10 (2), 9, (1969).
11. A. Ecclestein and R.B. Taylor, Pyrethrum Post, 6 (2), 43, (1961).
12. S.W. Head, Pyrethrum Post, 10 (2), 17, (1969).
13. L.O. Hopkins, Pyrethrum Post, 7 (2), 41, (1964).
14. S.W. Head, Private communication.
15. S. Prasad and R. Janwalz, Chem. and Ind., 756, (1969); 650, (1970).
16. W.F. Barthel, F.L. Haller and F.B. La Forge, Soap. Sanit. Chem., 20, 121, (1944).

17. H. Staudinger and L. Ruzocka, Helv. Chim. Acta., 7, 177 and 377, (1924).

18. F.B. La Forge and W.B. Barthel, J. Org. Chem., 9, 242, (1944).

19. P.J.Godin, R.J. Sleeman, M. Snarey and E.M. Thain, Chem. and Ind., 371, (1964).

20. P.S. Beevor, P.J. Godin and M. Snarey, Chem. and Ind., 1342, (1965); reprinted Pyrethrum Post, 8 (2), 29, (1965).

21. P.J. Godin, R.S. Sleeman, M. Snarey and E.M. Thain, J. Chem. Soc., 332, (1966).

22. S.H. Harper, Pyrethrum Post, 2 (1), 20, (1950).

23. S.W. Head, Pyrethrum Post, 9 (2), 3, (1967).

24. S.W. Head, Pyrethrum Post, 8 (4), 3, (1966).

25. S.W. Head, Pyrethrum Post, 9 (1), 12, (1967).

26. M. Elliott, Pyrethrum Post, 2 (3), 18, (1951).

27. W.F. Barthel, World Rev. Pest Control, 6 (2), 54, (1967).

28. P.J. Godin, Pyrethrum Post, 9 (4), 17, (1968).

29. A.H. McIntosh, Ann. Appl. Biol., 34, 586, (1947); 36, 535, (1949).

30. Reference 27, p. 59.

31. G.D. Glynne Jones and P.R. Chadwick, Pyrethrum Post, 6 (3), 27, (1962).

32. D.J. Broadbent and J.D. Hagarty, Pyrethrum Post, 10 (1), 17, (1969).

33. R.M. Sawicki and E.M. Thain, J. Sci. Food Agric., 12, 137, (1961).

34. M. Elliott, P.H. Needham and C. Potter, J. Sci. Food Agric., 20, 561, (1969).

35. G.D. Glynne Jones and N.K. Sylvester, Pyrethrum Post, 8 (4), 38, (1966).

36. N.K. Sylvester and A.J.S. Weaving, Pyrethrum Post, 9 (1), 31, (1967); 9 (2), 8, (1967).

37. R.H. Wright and D.J. Burton, Pyrethrum Post, 10 (2), 14, (1969).

38. J.P. Brooke, Pyrethrum Post, 6 (1), 14, (1961).

39. R.E. Blackith, Pyrethrum Post, 3 (2), 20, (1953).

40. P.R. Chadwick, Pyrethrum Post, 5 (3), 22, (1960); 6 (2), 30, (1961); 7 (1), 11, (1963).

41. L.O. Hopkins and D.R. Maciver, Pyrethrum Post, 8 (2), 3, (1965).

42. H.L. Haller, E.R. McGovern, L.D. Goodhue and W.N. Sullivan, J. Org. Chem., 7, 183, (1942).

43. C. Eagleson, Soap. Sanit. Chem., 18 (12), 125, (1942).

44. E.A. Parkin, Pyrethrum Post, 8 (2), 21, (1965).

45. M. Beroza, J. Amer. Oil Chemists' Soc., 31, 302, (1954).

46. H. Wachs, Science, 105, 530, (1947).

47. P.J. Godin, J.H. Stevenson and R.M. Sawicki, J. Econ. Entomol. 58, 548, (1965).

48. P.R. Chadwick, Pyrethrum Post, 7 (1), 25, (1963).

49. I. Yamamoto and J.E. Casida, J.Econ. Entomol., 59 (6), 1542, (1966).

50. I. Yamamoto, E.C. Kimmel and J.E. Casida, J. Agric. Food Chem., 17 (6), 1227, (1969).

51. J.E. Casida, J.L. Engel, E.G. Essac, F.X. Kamienski and S. Kawatsuka, Science, 153, 1130, (1966).

52. R.W. Brown, Bull. ent. Soc. Amer., 7 (1), 6, (1961).

53. B.C. Fine, Pyrethrum Post, 7 (2), 18, (1963).

54. D.W. Jolly and C.E. Waterhouse, Huntingdon Research Centre Report, (1962).

55. J.C. Malone and N.C. Brown, Pyrethrum Post, 9 (3), 3, (1968).

56. J.B. Moore, Pyrethrum Post, 7 (4), 15, (1964).

57. L.O. Hopkins and S.W. Head, Pyrethrum Post, 7 (4), 9, (1964).

58. B.T. Croll, Wat. Treat. Exam., 18, 220, (1969)

59. P.H. McLellan, Pyrethrum Post, 7 (4), 23, (1964).

60. J.F. Casida, E.C. Kimmel, M. Elliott and N.F. Janes, Nature, 230, 326, (1971).

61. J.T. Martin and K.H.C. Hester, Brit. J. Derm. Syph., 53, 127, (1941).

62. K.A. Lord and C.G. Johnson, Brit. J. Derm.Syph., 59, 367, (1947).

63. A. Zucker, Annals of Allergy, 23, (July 1965).

64. M. Elliott, Science Journal, 3 (3), 61, (1967).

65. M. Elliott, Chem. and Ind., 776, (1969).

66. M. Elliott, Bull. Hlth. Org., 44, 315, (1970).

67. P.E. Berteau, J.E. Casida and T. Narahishi, Science, 161, 1151, (1968).

68. M. Elliott, J. Sci. Food Agric., 5, 505, (1954).

69. Anon, Chem. and Eng., 32, (Jan. 1971).

70. M. Elliott, A.W.Farnham, N.F. Janes, P.H. Needham and B.C. Pearson, Nature, 213, 493, (1967).

71. F. Barlow, M. Elliott, A.W. Farnham, A.B. Hadaway, N.F. Janes, P.H. Needham and J.C. Wickham, Pest. Sci., 2, 115, (1971).

72. H.A. Scil, Soap, 10 (5), 89 (1934).

73. W. Mitchell, F.H. Tresadem and S.A. Wood, Analyst, 73, 484, (1948).

74. F. Wilcoxon, Contrib. Bryce Thompson Inst., 8, 175, (1936).

75. D.A. Holaday, Ind. Eng. Chem., 10, 5, (1938).

76. G.T. Bray, S.H, Harper, K.A. Lord, F. Major and F.H. Tresadern, J. Soc. Chem. Ind., 66, 275, (1947).

77. 'Official Methods of Analysis of the A.O.A.C.', Association of Official Agricultural Chemists, Washington D.C., U.S.A. 10th Edition, 1965, p. 50.
9th Edition, 1960, o. 41.
8th Edition, 1955, p. 68.

78. 'Determination of Pyrethrins', A printed leaflet published by the Pyrethrum Board of Kenya, Nakuru, 1954.

79. 'Determination of Pyrethrins', A printed leaflet published by the Tanganyika Extract Company Limited, 1954.

80. 'British Pharmaceutical Codex 1954', The Pharmaceutical Press, London, p. 31.

81. 'British Veterinary Eodex 1953', Supplement 1959, The Pharmaceutical Press, London, p. 53.

82. W. Mitchell, J. Sci. Food Agric., 4, 246, (1953).

83. W. Mitchell and F.H. Tresadern, J. Sci. Food Agric., 6, 465 (1955).

84. D.B. McClellan, J. Assoc. Offic. Agric; Chemists, 46, 664, (1963).

85. J.N. Hogsett, A.W. Kacey and J.B. Johnson, Analyt. Chem., 25, 1207, (1953).

86. H. Wachs and A.V. Hanley, Soap Chem. Spec., 43 (10), 78, (1967); reprinted Pyrethrum Post, 9 (3), 23, (1968).

87. H. Wachs and A.V. Hanley, Soap Chem. Spec., 45 (5), 107, (1969).

88. H.L. Haller and F. Acree, Ind. Eng. Chem., Anal. Ed., 7, 343, (1935).

89. R.H. Cundiff and P.C. Markunas, Analyt. Chem., 33, 1028, (1961).

90. W. Mitchell, J.H.N. Byrne and F.H. Tresadern, Analyst, 88, 538, (1963).

91. J.H.N. Byrne, W. Mitchell and F.H. Tresadern, Analyst, 90, 362, (1965).

92. N.G. McTaggart, E. Thornton and A.D. Harford, Pyrethrum Post, 4 (4), 12, (1958).

93. V.A. Beckley, Pyrethrum Post, 2 (1), 23, (1950).

94. A.J. Shukis, D. Christi and H. Wachs, Soap Sanit. Chem., 27 (11), 124, (1951).

95. J. Ward and G. Newham, Pyrethrum Post, 6 (3), 34, (1962).

96. B.P. Moore, J. Sci. Food Agric., 5, 500, (1954).

97. N. Green and M.S. Schoeter, Analyt. Chem., 27, 1261, (1955).

98. B.P. Moore, J.Sci. Food Agric., 7, 740, (1956).

99. H.J. Smith, J. Sci. Food Agric., 10, 260, (1959) and 11, 172, (1960).

100. S.W. Head, Soap Chem. Spec., 39 (10), 97, (1963); Pyrethrum Post, 7 (4), 7, (1964).

101. S.W. Head, J. Sci. Food Agric., 15, 390, (1964).

102. C.B. Gnadinger and C.S. Corl, J. Amer. Chem. Soc., 51, 1054, (1929).

103. K.A. Lord, Nature, 165, 567, (1950).

104. A.A. Schreiber and D.B. McClellan, Analyt. Chem., 26, 604, (1954).

105. L.W. Levy and H. Molina, Pyrethrum Post, 4 (2), 22, (1957).

106. H.L. Williams, W.E. Dale and J.P. Sweeney, J. Assoc. Offic. Agric. Chemists, 39, 872, (1956).

107. L.W. Levy and R.J. Estrada, J. Agric. Food. Chem., 2, 629, (1954).

108. N.C. Brown, R.F. Phipers and M.C. Wood, Pyrethrum Post, 4 (1), 24, (1956).

109. L. Donegan, J.N. Morrison and D.J. Webley, Pyrethrum Post, 11(1), 36, (1971).

110. L.W. Levy and Usubilliga, Biol. inform. cient. nachs., 9, 190, (1956); CA, 51, 622e.

111. L.W. Levy and D. Geller, Pyrethrum Post, 5 (1), 12, (1959).

112. N.C. Brown, D.T. Hollinshead, R.F. Phipers and M.C. Wood, Pyrethrum Post, 4 (1), 30, (1956).

113. N.C. Brown, D.T. Hollinshead, R.F. Phipers and M.C. Wood, Pyrethrum Post, 4 (2), 13, (1957).

114. N.C. Brown and M.C. Wood, Pyrethrum Post, 6 (4), 11, (1962).

115. L. Donegan, P.J. Godin and E.M. Thain, Chem. and Ind., 1420, (1962).

116. H.F. Beckman, P.T. Allen and P.J. Berkenhotter, J. Gas Chromatog., 1 (8), 21, (1963).

117. B.J. Gudzinowicz, Analyt. Chem., 37 (8), 6068, (1965).

118. S.W. Head, Pyrethrum Post, 7 (4), 12, (1964).

119. B.T. Croll, communication to S.W. Head, reported Pyrethrum Post, 8 (4), 3, (1966).

120. A. Bevenue, Y. Kawano and F. Delano, J. Chromatog., 50 (1), 49, (1970), reprinted, Pyrethrum Post., 11 (1), 41, (1971).

121. H.F. Beckman and P.T. Allen, Adv. Chem., 53, 51, (1966).

122. S.W. Head, Pyrethrum Post, 9 (4), 31, (1968).

123. A. Brierley and N.C. Brown, Soap Chem. Spec., 38 (10), 105, (1962).

124. A. Brierley and N.C. Brown, Soap Chem. Spec., 40 (5), 149, (1964).

125. J. Ripert, Ann. Fals., Paris, 27, 580, (1934).

126. S.W. Head, J. Agric. Food Chem., 16 (5), 762, (1968).

127. F.B. La Forge and H.L. Haller, J. Org. Chem., 2, 56, (1937).

128. F.B. La Forge and F. Acree, Soap, 17 (1), 95, (1941).

129. N.C. Brown, R.F. Phipers and K.G. Singleton, Pyrethrum Post, 3 (3), 3, (1954).

130. N.C. Hodgson, J. Sci. Food Agric., 8, 347, (1957); reprinted Pyrethrum Post, 4 (3), 13, (1957).

131. Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry, Analyst, 89, 689, (1964).

132. N.C. Brown and R.F. Phipers, Pyrethrum Post, 3 (4), 23, (1955).

133. H.L. Haller and F.B. La Forge, J. Org. Chem., 1, 38, (1936).

134. M. Elliott, P.H. Needham and R.M. Sawicki, Report Rothamsted exp. Sta., 137, (1957).

135. A.J. Muro, Pyrethrum Post, 6 (2), 25, (1961).

136. A.A. Goldberg, S.W. Head and P. Johnston, J. Sci. Food Agric., 16 (2), 104, (1965).

137. D.R. Maciver, Pyrethrum Post, 6 (4), 16, (1962).

138. A.A. Goldberg, S.W. Head and P. Johnston, J. Sci. Food Agric., 16 (1), 43, (1965).

139. F. Tattersfield and J.T. Martin, J. Agric. Sci., 22, 396, (1932); 24, 598, (1934).

140. Y. Katsuda et al., Botyo-Kaguka, 20, 15, (1955); 21, 139, (1956); CA., 55, 703e.

141. A. Campbell and W. Mitchell, J. Sci. Food Agric., 1, 137, (1950).

142. S.K. Freeman, Soap Chem. Spec., 32 (2), 131; (3), 150, (1956).

143. N.C. Brown, D.T. Hollinshead, R.F. Phipers and M.C. Wood, Soap Chem. Spec., 33 (9), 87; (10), 91, (1957).

144. G.D. Glynne Jones, Ann. Appl. Biol., 48 (2), 352, (1960).

145. S.W. Head, N.K. Sylvester and S.K. Challinor, Pyrethrum Post, 9 (3), 14, (1968).

146. Y.L. Chen and J.E. Casida, J. Agric. Food Chem., 17 (2), 208, (1969).

147. M.M. Nasir, J. Sci. Food Agric., 4, 374, (1953).

148. A.E. Gillan and T.F. West, J. Chem. Soc., 49, (1944).

149. D.R. Maciver, Pyrethrum Post, 8 (4), 23, (1966).

150. A. Bell and G.S. Kido, J. Agric. Food.Chem., 4 (4), 340, (1956).

151. S.W. Head and G.D. Glynne Jones, Pyrethrum Post, 8 (1) 14, (1965).

152. J.P. Brooke, Pyrethrum Post, 9 (1), 18, (1967).

153. P.A. Keane, Pyrethrum Post, 10 (3), 17, (1970).

154. J.B. Moore, Pyrethrum Post, 11 (1), 2, (1971).

155. J.P. Brooke and P.H. Lonax, Food Processing and Marketing, Jan, (1967), reprinted Pyrethrum Post, 9 (1), 36, (1967).

156. J.P. Brooke, Pyrethrum Post, 6 (1), 14, (1961).

157. Editorial, Pyrethrum Post, 9 (2), 2, (1967).

158. Anon, Pyrethrum Post, 8 (4), 42, (1966).

159. D.J. Webley, Pyrethrum Post, 9 (4), 4, (1968).

160. R. Winney and D.J. Webley, Pyrethrum Post, 10 (1), 44, (1969).

161. G. Pattenden, Pyrethrum Post, 10 (4), 2, (1970).

162. T.A. Gough and E.A. Walker, Analyst, 95, 1, (1970).

163. I.G. McWilliam, J. Chromatog., 6, 110, (1961).

164. L.S. Ettre, Gas Chromatography 1962, Editors N. Brenner, J.E. Callan and M.D. Weiss, Academic Press (N.Y.), 1962, p. 307 and 541.

165. L.S. Ettre, J. Chromatog., 8, 525, (1962).

166. F.J. Kabot and L.S. Ettre, J. Gas Chromatog., 1, 7, (1963).

167. R.G. Ackman and J.C. Sipos, J. Chromatog., 16, 248, (1964).

168. R.G. Ackman and J.C. Sipos, J. Amer. Oil Chemists' Soc., 41, 377, (1964).

169. G. Perkins, R.E. Laramy and L.D. Lively, Analyt. Chem., 35, 360, (1963).

170. W.A. Dietz, J. of Gas Chromatog., 5, 68, (1967).

171. D.R. Deans, Chromatographia, 1, 187, (1968).

172. M. Elliott, Chem. and Ind., 1142, (1960).

173. J. Ward, Chem. and Ind., 586, (1953).

174. M. Elliott, J. Chem. Soc., 888, (1964).

175. S.W. Head, Pyrethrum Post, 10 (3), 27, (1970).

176. Anon., Analyt. Chem., 33, 480, (1961).

177. R.S. Evans and R.P.W. Scott, Nature, 190, 710, (1961).

178. L. Mikkelson, J. Gas Chromatog., 5, 601, (1967).

179. H.W. Johnson, Adv. Chromatog., 5, 175, (1968).

180. T.A. Gough and E.A. Walker, J. Chromatog., 45, 14, (1969).

181. R.P.W. Scott and D.W. Grant, Analyst, 89, 179, (1964).

182. J.M. Gill and H.M. McNair, Aerograph Research Notes, Internat. Edit., Fall 1965.

183. N. Emanuel and Y.N. Lyaskovskaya, "The Inhibition of Fat Oxidation Processes", pub. Pergamon, 1967, translated from Russian 1961.

184. "Autoxidation and Antioxidants", Vol. 1, Edit. W.O. Lundberg, pub. Interscience, 1961.

185. C.S. Foote, Science, 162, 963, (1968).

186. G. Scott, "Atmospheric Oxidation and Antioxidants", pub. Elsevier, 1965.

187. H.C. Bailey, Industrial Chemist, May 1962.

188. K.U. Ingold, Chem. Rev. 61, 563, (1961).

189. Reference 184, pp 83 and 90.

190. Reference 183, pp 12.

191. Reference 186, pp 82.

192. Reference 188, pp 564.

193. Reference 188, pp 566.

194. Reference 186, pp 41 and 88.

195. Reference 188, pp 573.

196. J.L. Bolland and P. ten Have, Trans. Faraday Soc., 43, 201, (1947); and Discuss. Faraday Soc., 2, 252, (1947).

197. A.F. Bickel and C.E. Kooyman, J. Chem. Soc., 2215, (1956).

198. G.S. Hammond, C.E. Boozer, C.E. Hamilton and J.N. Sen, J. Amer. Chem. Soc., 77, 3238, (1955).

199. Reference 184, pp 138.

200. Reference 186, pp 188.

201. Reference 186, pp 198.

202. C.B. Gnadinger, C.S. Corl and C.A. Clark, Soap, 11, 95, (1935).

203. G.D. Glynne Jones, private communication.

204. Reference 186, pp 156.

205. C. Anglin, J.H. Mahon and R.A. Chapman, J. Agric. Food Chem., 4, 1018, (1956).

206. C.R. Spalkowski and J.B. Garber, J. Agric. Food Chem., 10, 490, (1962).

207. M.R. Sahasrabudhe, J. of Assoc. Offic. Agric. Chemists., 47, 888, (1964).

208. N.J. Alicino, H.C. Klein, J.J. Quatone and T.K. Choy, J. Agric. Food Chem., 11, 496, (1963).

209. S. Ishikawa and G. Katsui, J. Vitaminol., 12, 112, (1966).

210. G. Janicek and J. Davidek, Qual. Plant Mater. Veg., 16, 292, (1968).

211. T.K. Choy, J.J. Quatrone and N.J. Alicino, J. Chromatog., 12, 171, (1963).

212. D.M. Takahashi, J. of Assoc. Offic. Agric. Chemists., 48, 694, (1965).

213. W.A.B. Thomson, J. of Chromatog., 19, 599, (1965).

214. B. Braithwaite and G.E. Penketh, Analyt. Chem., 36, 185, (1964).

215. J.P. Wolff and F. Andiau, Bull. Soc. Chim. France, 2662, (1964).

216. H.S. Olcott and H.A. Mattill, J. Amer. Chem. Soc., 58, 1627 and 2204, (1936).

217. P. Karrer and H. Fritzsche, Helv. Chim. Acta., 21, 1234, (1938).

218. J.F. Pennock, F.W. Hemming and J.D. Kerr, Biochem. Biophys. Res. Comm., 17, 542, (1964).

219. V.N. Krukovsky and J.N. Loosly, J. Dairy Sci., 35, 843, (1952).

220. Reference 183, pp 266.

221. H.T. Slover, J. Lehman and R.J. Valis, J. Amer. Oil Chemists' Soc., 46, 417, (1969).

222. T.P. Hilditch and J.J.Sleightholme, J. Soc. Chem. Ind., 51, 397, (1932).

223. R.W. Riemenschneider and W.C. Ault, Food Industry, 16, 892, (1954).

224. C.E. Swift, W.G. Rose and G.S. Jamieson, Oil and Soap, 19, 176, (1942).

225. G.D. Oliver, W.S. Singleton and A.E. Bailey, Oil and Soap, 21, 188, (1944).

226. B.M. Parkhurst, W.A. Skinner and P.A. Sturm, J. Amer. Oil Chemists' Soc., 45, 641, (1968).

227. H.S. Olcott and H.A. Mattill, Chem. Rev., 29, 257, (1941).

228. C. Golumbic, J. Amer. Chem. Soc., 64, 2337, (1942); and Oil and Soap, 20, 105, (1943).

229. E.H. Gruger and A.L. Tappel, Lipids, 5 (3), 326 and 331, (1970).

230. V.L. Frampton, W.A. Skinner, P. Cambour and P.S. Bailey, J. Amer. Chem. Soc., 82, 4632, (1960).

231. D. McHale and J. Green, Chem. and Ind., 982, (1963).

232. A.S. Csallany, M. Chui and H.H. Draper, Lipids, 5, (1), 63, (1970).

233. F.W. Knapp and A.L. Tappel, J. Amer. Oil Chemists' Soc., 38, 151, (1961).

234. A. Emmerie and C. Engel, Rec. Trav. Chim., 58, 283, (1939).

235. D.A. Libby and A.J. Sheppard, J. Assoc. Offic. Agric. Chemists, 47, 371, (1964).

236. H.C. Pillsbury, A.J. Sheppard and D.A. Libby, J. Assoc. Offic. Agric. Chemists, 50, 809, (1967).

237. A.J. Sheppard, W.D. Hubbard and A.R. Prosser, J. Assoc. Offic. Agric. Chemists, 52, 442, (1969).

238. H.T. Slover, J. Lehman and R.J. Valis, J. Amer. Oil Chemists' Soc., 46, 417, (1969).

239. Analytical Methods Committee (Vitamin E Panel), Analyst, 84, 356, (1959).

240. J. Tosic and T. Moore, J. Biochem., 37, 498, (1945).

241. K.J. Whittle and J.F. Pennock, Analyst, 92, 423, (1967).

242. G.G. Shone, J. Sci. Food Agric., 13, 315, (1962).

243. C. Lea and R. Ward, J. Sci. Food Agric., 10, 537, (1959).

244. G.G. Shone, private communication.

245. D. Swern, "Fatty Acids", pub. Markley, part 2, 2nd Edition, 1961, p. 1387.

246. T.W. Hammonds and G.G. Shone, Analyst, 91, 455, (1966).

247. J.L. Bolland, Trans. Faraday Soc., 44, 669, (1948).

248. F.D. Gunstone and T.P. Hilditch, J. Chem. Soc., 1022, (1946).

249. T.P. Hilditch, Nature, 166, 588, (1950).

250. Reference 183, pp 39.

251. R.W. Riemenschneider, Oil and Soap, 20, 169, (1943).

252. C.O. Willits, C. Ricciuti, H.B. Knight and D. Swern, Analyt. Chem., 24, 785, (1952).

253. J.L. Bolland, Trans. Faraday Soc., 46, 358, (1950).

254. C. Ricciuti, C.O. Willits, C.L. Ogg, S.G. Morris and R.W. Riemenschneider, J. Amer. Oil Chemists' Soc., 31, 456, (1954).

255. E.G.E. Hawkins, "Organic Peroxides", pub. Spon Ltd., London, 1961.

256. R.M. Johnson and I.W. Siddiqi, "The Determination of Organic Peroxides", Monographs in Organic Functional Group Analysis, Vol. 4, Pergamon Press, 1970.

257. Reference 183, pp 16.

258. J.H. Skellon and E.D. Willes, Analyst, 73, 78, (1948).

259. B.D. Sulley, Analyst, 79, 86, (1954).

260. C.H. Lea, J. Soc. Chem. Ind., 64, 106, (1945).

261. W. Kern and H. Willersin, Angew. Chem., 67, 573, (1955).

262. C.E.H. Bawn, Discuss. Faraday Soc., 14, 181 and 240, (1953).

263. A.J. Chalk and J.E. Smith, Trans. Faraday Soc., 53, 1214 and 1235, (1957).

264. S.G. Morris, J.S. Meyers, M.L. Kip and R.W. Riemenschneider, J. Amer. Oil. Chemists' Soc., 27, 105, (1950).

265. J.W. Stull, E.D. Herreid and P.H. Tracy, J. Dairy Sci., 34, 187, (1951).

266. J.C. Cowan, J. Amer. Oil Chemists' Soc., 43, 300A, (1966).

267. G.R. Ames, W.D. Raymond and J.B. Ward, J. Sci. Food Agric., 11, 194, (1960).

268. G.H. Denison, Ind. Eng. Chem., 36, 477, (1944).

269. C.F. Prutton, D.R. Frey, D. Turnbull and G. Dlouhy, Ind. Eng. Chem., 37, 90, (1945).

270. J.W. Price and W.C. Coppins, "The Analysis of Tin-Base Bearing Metals", paper published by the Tin Research Institute, Middlesex, 1954.

271, "Organic Reagents for Metals", Vol. 1, Edited by W.C. Johnson, pub. Hopkin and Williams Ltd., 5th Edition, 1955, pp 124.

272. T.T. Gorsuch, "The Destruction of Organic Matter", Pergamon Press, 1970.

273. Reference 272, pp 123.

274. R.C. Koch, "Activation Analysis Handbook", Academic Press, 1960.

275. D.F.C. Morris, Chem. and Process Engineering, June, 1960.

276. M. Austreng, L.R.I.C. project, Kingston Polytechnic, Kingston upon Thames, 1972.

277. R.A.G. Marshall, Analyst, 96, 675, (1971).

278. L.J. Filer, K.F. Mattil and H.E. Longenecker, Oil and Soap, 21, 289, (1944).

279. W.O. Lundberg, W.B. Docksader and H.O. Halvorson, J. Amer. Oil Chemists' Soc., 24, 89, (1947).

280. S.W. Head, Ph.D. Thesis, 1968.

281. M. Elliott, Chem. and Ind., 685, (1958).

282. M. Elliott, J. Chem. Soc., 5225, (1964).

283. D.R. Maciver, Pyrethrum Post, 9 (4), 41, (1968).

284. L. Crombie, S.H. Harper, R.E. Steadman and D. Thompson, J. Chem. Soc., 2445, (1951).

285. I.G.M. Campbell and S.H. Harper, J. Chem. Soc., 283, (1945)

286. F.B. La Forge, W.A. Gersdorff, N. Green and M.S. Schecter, J. Org. Chem., 17, 381, (1952).

287. J. Miyamoto, Y. Sato and K. Awano, Agr. Biol. Chem. (Tokyo), 33, 1095, (1969).

288. B. Goffinet and A. Locatelli, French Patent, 1536458, (1964)

289. L. Crombie, A.J.B. Edgar, S.H. Harper, M.W. Lowe and D. Thompson, J. Chem. Soc., 3552, (1950).

290. R.M. Sawicki, M. Elliott, J.C. Gower, M.Snarey, E.M. Thain, J. Sci. Food Agric., 13, 172, (1962)

291. M. Elliott and N.F. Janes, Chem. and Ind., 270, (1969)

292. S.H. Harper, Pyrethrum Post, 1 (4), 10, (1949)

293. A.F. Bramwell, L. Crombie, P. Hemesley, G. Pattenden, M. Elliott and N.F. Janes, Tetrahedron, 25, 1727, (1969)

294. A.T. Moore, N.H. Rydon and M.J. Smithers, J. Chem. Soc., 2349, (1966).

295. C. Alexandre and F. Rouessac, Tet. Letters, 1011, (1970)

296. M. Elliott, J. Appl. Chem., 11, 19, (1961)

297. T.A. King and H.M. Paisley, J. Chem. Soc., 870, (1969)

298. J.H. Beynon, R.A. Saunders and A.E. Williams, "The Mass Spectra of Organic Molecules", pub. Elsevier, 1968.

299. H. Budzikiewicz, C. Djerassi and D.H. Williams, "Mass Spectra of Organic Compounds", pub. Holden-Day Inc., 1967.

300. C.B. Barrett, M.S.J. Dallas and F.B. Padley, Chem. and Ind., 1050, (1962)

301. E. Stahl, Arch. Pharm., 293, 531, (1960)

302. D. Furmanec, A.E. Schilling and B.B. Brown, Pyrethrum Post, 10 (1), 21, (1969)

303. A.F. MacKay, N.R.R. Park and S.J. Viron, J. Amer. Chem. Soc., 72, 3659, (1950)