CHAPTER II

MATERIAL AND METHODS

MATERIALS

Source of Plant Material

The pyrethrums (*Chrysanthemum cinerariaefolium* Bocc.) Shirayuki I, were used exclusively in this study. The specimens were obtained from the High land Agriculture Project of Kasetsart University, Doi Ang Khang Research Station, Chiang Mai.

Chemical agents

1. Chemical agents for medium preparation

1.1 Callus induction medium

Callus induction medium was basal MS medium (Murashige and Skoog, 1962) with 7% agar, supplemented with 20 levels of 2,4-D and BA recombinations. The medium preparation method was illustrated in Appendix 2-4.

1.2 Cell suspension culture medium

Cell suspension culture medium was MS liquid medium supplemented with 25 levels of 2,4-D and BA, IAA and BA recombinations

2. Surface sterilant

Surface sterilant, 70% ethanol, Clorox (the commercial name of bleach containing 0.525% NaOCl as an active ingredient) and tween-20 (the surfactant).

3. Growth regulators

Growth regulators, 2,4-D, IAA and BA.

4. Staining reagent

Staining reagent, 8-hydroxyquinoline, acetocarmine, and aceto-orcein.

5. Chemical agents for extraction

Chemical agents were for extraction, qualitative and quantitative determination. All analytical reagents (A.R. grade) were used in this study.

5.1 Organic solvent

Organic solvent, petroleum ether, hexane, acetone, ethyl acetate, n-heptane and chloroform.

5.2 TLC quantitative analytical agents

TLC quantitative analytical agents, e.g. methylstearate, a commercial standard pyrethum extract of PBK (provided by Wellcome Company, Thailand) which assayed for 24.8% total pyrethrins were employed. The extract contained 15.1% pyrethrin I and 9.7% pyrethrin II.

Glasswares

1. Glasswares for tissue culture

Glasswares for tissue culture, were 50-125 ml vials, and 25-1,000 ml culture flasks with cover.

2. Glasswares for cell selection

10 ml blunt-end pipettes were used for transferring cells into fresh medium.

3. Glasswares for medium preparation

Glasswares for medium preparation were 50-2,000 ml beakers and 10-1,000 ml graduate cylinders with cover and stirrer.

Miscellaneous

1. Miscellaneous for sterilization

Miscellaneous for sterilization were laminar air flow cabinet, autoclave and millipore filter.

2. Culture room

Culture room was able to control the environmental factors, e.g. temperature, photoperiod and light intensity.

3. Miscelleneous for tissue and cell preparation

Miscelleneous for tissue and cell preparation were surgical blades, forceps, Whatman No.1, nylon mesh (148, 94, 62 and 40 μm).

4. Balance

Coarse and fine balance (Sartorious balance model GMBH COTTINGEN Type 1507 and 1702)

5. Microscope

Nikon FX-35 A Type 104, Nikon Optiphot, Japan

6. Exposure meter

L-330, ROC, Taiwan

7. pH Meter

PHM 883 AUTOCOL

8. Swinging bucket bench top centrifuge.

9. Pipetteman injector.

10. GC apparatus.

11. HPLC apparatus.

METHODS

This experiment was conducted at the Tissue Culture Laboratory, Department of Biochemistry, Faculty of Science, Chulalongkorn University during December, 1986 to December, 1989.

Callus initiation

1. Tissue sterilization

The study on the appropriate conditions of pyrethrum tissue sterilization was the first important step for the tissue culture. Investigations on this process require comparative studies of contamination, death and survival percentage the determination of procedure's efficiency. Various Clorox concentrations and surface sterilization times were available for preparing sterilized plant tissues before starting the callus initiation. The following procedures for surface sterilization and dissection of pyrethrum tissue (Tables 2 and 3) have been found to be convenient and to yield good results.

1.1 Pre-sterilization

Collected healthy plants and separated then into smaller parts, e.g. stems (cut at ca. 1 cm above root),petioles were cut into 2.0 cm long cylinders, and leaf blades removed from the petiole were cut into 1.0 x 1.5 cm squares. For pre-sterilization soaked those pieces in tap water and then separately placed ca.10 pieces in each vials (75 ml) filled with ethanol (70%,30 ml). Keep specimens in the solution for 2-3 min. and subsequently transferred into freshly sterilized vials (75ml).

Table 2 Steps of surface sterilization of Pyrethrum tissues.

Step of sterilization	Chlorox (NaOCl 0.525%) concentration (%)	Time (min.) 1	2	3	4	5
1	0	A1	A2	A3	A4	A5
	10	A6	A7	A8	A9	A10
	15	A11	A12	A13	A14	A15
	20	A16	A17	A18	A19	A20
2	Further, immerse in 5% NaOCl for 15 min.					
3	Tissues were rinsed twice in sterile water prior step on further					

Table 3 Tissue preparation prior to callus establishment

Plant parts	Size (cm)	Procedures
Stem	0.5	Peeled of epidermis and further dissect into size
Petiole	0.5	Removed leaf blade before dissected into size
Leaf blade	0.5 x 0.5	Cut strips across the leaf blade and made into size

All dissected tissues each placed with one of its flat surfaces in contact with the medium, MS agarized Medium containing 2,4-D (1.0 mg/l) recombined with BA (3.0 mg/l) under 2,000 lux of fluorescent illumination about 8-hour dark period and maintained at the temperature range 25 $\pm$ 2 °C

1.2 Surface sterilization

Starting from this step, the rest of the experiment have to be carried out aseptically. 30 ml each of the four Chlorox concentrations (0, 10, 15 and 20%) was poured in vials. Added 2-3 drops of tween-20, the wet agent, into each vial. Shaked vigorously on the rotary shaker (100 rpm) for a limited time (1, 2, 3, 4 and 5 mins.) and subsequently, transferred these explants into the vials containing 5% NaOCl. All vials were then shaken for 15 mins at 60 rpm and finally rinsed all explants in sterile water twice.

1.3 Explants dissection

Tissue preparation prior to the next step, callus establishment, was conducted in laminar flow. The sterilized explants from 1.2 were placed onto freshly sterilized petri dish and cut into sizes. Dissection method is illustrated in Table 3.

1.4 Characteristic observation.

Callus characteristics after 7 days of cultivation, percentage of each treatments was observed and calculated as follows:-

$$\text{Contamination (\%)} = \frac{\text{Contaminated tissues} \times 100}{\text{Total no. of experimental tissue}}$$

$$\text{Death (\%)} = \frac{\text{Death tissue} \times 100}{\text{Total no. of experimental tissue}}$$

$$\text{Survival (\%)} = \frac{\text{Survival tissue} \times 100}{\text{Total no. of experimental tissue}}$$

where the total number of experimental tissue was 30 and each explant was available for one culture flask.

2. Callus culture establishment

Two major aspects were studied. The first was a medium constituent requires for cultivation, e.g. organic constituent, salt and carbon source concentrations. The second was physical factors such as culture light intensity, dark period and temperature.

2.1 Medium constituent

Since 2,4-D and BA had been found to be effective for callus cultivation (Zieg,1983; Kueh,1985) therefore various the concentrations of both 2,4-D and BA were used in this study and the medium constituents are shown in Table 4.

2.1.1 Procedures

Transferred separately each sterilized explants into each culture vial containing callus induction medium supplemented with 2,4-D and BA in various concentrations (ca. 30 replicates/treatment) (Table 4). Explants were cultivated in a culture room with fluorescent illumination of 2,000 lux for an 8-hour dark period and maintained at temperature of $25\pm2^{o}C$.

2.1.2 Characteristic observation

Photograph records were taken after 8 weeks of an inoculation. Then the comparative studies and the determination of the efficiency of each treatment were carried out by these equations (Figure 7).

$$\text{Percentage of callusing} = \frac{\text{Total number of calli formation} \times 100}{\text{Total number of Experimental explants}}$$

$$\text{Score signified size of callus} = \frac{\text{Total score}}{10}$$

Measurement of growth and development of calli were carried out and recorded after 6 weeks of inoculation by mean of Growth Index (GI). GI could be calculated via the equation below:-

$$GI = \frac{\text{Final wet weight - initial wet weight}}{\text{Initial wet weight}}$$

2.2 Salt requirement

Two MS salt concentrations, full strength and half strength were employed in this study.

2.2.1 Procedure

Trimmed sterilized explants into shape (Table 3). Separately transferred them into culture flasks containing half strength and full strength MS medium supplemented with 2,4-D (1.0 mg/l) and BA (3.0 mg/l). All vials were incubated in culture room with 2,000 lux of fluorescent illumination for an 8-hour dark period and maintained at the temperature range of $25\pm2^{o}C$ which conditions were found to be satisfied for this investigation.

2.2.2 Characteristic observation

Photograph records carried out at 8 weeks after and inoculation. Then the calculation of sign signifies the intensity of callusing and GI which were used for the comparative study on the efficiency of each process were carried out.

Table 4 Interaction of 2,4-D and BA effecting on pyrethrum callus initiation

2,4-D Concentration (mg/l)	BA Concentration (mg/l)			
	0.0	1.0	2.0	3.0
0.0	B1	B2	B3	B4
0.5	B5	B6	B7	B8
1.0	B9	B10	B11	B12
1.5	B13	B14	B15	B16
2.0	B17	B18	B19	B20

All explants were cultured on full strength MS agarized medium containing various concentration of growth regulators as above illustration under 2,000 lux of fluolescent illumination with an 8-hour dark period and maintained at a temperature range of 25 ± 2 °C

Table 5 Interaction of 2,4-D and BA effecting on pyrethrum cell culture

2,4-D Concentration (mg/l)	BA Concentration (mg/l)				
	0.0	1.0	2.0	3.0	4.0
0.00	C1	C2	C3	C4	C5
0.25	C6	C7	C8	C9	C10
0.50	C11	C12	C13	C14	C15
0.75	C16	C17	C18	C19	C20
1.00	C21	C22	C23	C24	C25

0.5 g Leaf calli were cultured in 25 ml full strength MS liquid medium containing various concentration of growth regulators as above illustration under 2,000 lux of continuous fluolescent illumination with an 8-hour dark period and maintained at temperature range of 25 ± 2 °C on a rotary shaker (100 rpm)

Table 6 Interaction of IAA and BA effecting on pyrethrum cell culture

IAA Concentration (mg/l)	BA Concentration (mg/l)				
	0.0	1.0	2.0	3.0	4.0
0.00	D1	D2	D3	D4	D5
1.00	D6	D7	D8	D9	D10
2.00	D11	D12	D13	D14	D15
3.00	D16	D17	D18	D19	D20
4.00	D21	D22	D23	D24	D25

0.5 g Leaf calli were cultured in 25 ml full strength MS liquid medium containing various concentration as above illustration under 2,000 lux of continuous fluolescent illumination with an 8-hour dark period and maintained at temperature range of 25 ± 2 °C on a rotary shaker (100 rpm)

Figure 7 Degree signifies size of leaf derived callus.

0 = ca. 0.02-0.04 gm

1 = ca. 0.05-0.07 gm

2 = ca. 0.08-0.10 gm

3 = ca. 0.11-0.13 gm

4 = ca. 0.14 --> gm

2.3 The environmental factors

The environmental factors that affect the callus culture are light and temperature.

2.3.1 Light

In this study, attempts to investigate the appropriate conditions for pyrethrum callus initiation had been conducted in two main categories; illumination of fluorescent light (2,000 lux) for an 8 hour dark period and a continuous dark condition.

2.3.1.1 Procedures

Sterilized pyrethrum explants were transferred to the freshly sterilized petri dish and immediately cut into sizes (Table 3) followed by transferring each piece to vial containing the callus induction medium supplemented with 2,4-D (1.0 mg/l) and BA (3.0 mg/l). The specimen (ca. 30 replicates/treatment) were separately maintained under the illumination of fluorescent light (2,000 lux) for an 8-hour dark period and continuous dark condition at the same temperature of 25±2°C in the culture room.

2.3.1.2 Characteristic observation.

Same as 2.2.2

2.3.2 Temperature.

The temperature range of 25±2°C and room temperature of 30±2°C were chosen during an to investigation which is a more optimistic condition for pyrethrum callus culture.

2.3.2.1 Procedures

Aseptically cut sterilized explants into sizes and transferred to vial containing the callus induction medium supplemented with 2,4-D (1.0 mg/l) and BA (3.0 mg/l). Specimens were cultured separately under the temperature range of 25±2°C and room temperature. The illumination of fluorescent light (2,000 lux) for an 8-hour dark period was performed.

2.3.2.2 Characteristic observation

Same as 2.2.2

Establishment and development of suspension culture

The objective of this study is to investigate the available information on nutrition and environmental factors affecting the establishment and development of suspension cultures.

1. Free cell and aggregates of cell formation.

The study on factors influencing the formation of free cell and aggregates of cell formation had been concentrated in this investigation. The principal limitation on the direct initiation of free cell and aggregates of cell formation is probably the requirements of media concentration and culture conditions.

1.1 Media formulation.

Salt requirement of cell culture suspension was also the first step of the investigation. MS liquid medium had already been

proved to yield the best results. Further organic constituents supplementation was used to promote the free cell formation.

1.1.1 Procedures

Placed aseptically friable callus on the freshly sterilized petri dish and cut to cubes, not longer than 2 cm^3. Transferred 3 pieces of callus into Erlenmeyer flask (125 ml)containing 50 ml suspension culture medium supplemented with growth regulators at various concentrations (Tables 5 and 6). Further, keep the specimens (4 culture flasks/treatment) on a rotary shaker (100 rpm) in the culture room under continuous fluorescent illumination (2,000 lux) and maintained at a temperature range of $25\pm2^{o}C$.

1.1.2 Charateristic observation

Photograph records were obtained after 10 weeks of inoculation. Scoring of the intensity of cell formation (Figure 8) together with measuring the cell growth and development were carried out by means of GI which was calculated as follow:-

$$GI = \frac{\text{Final cell count number - Initial cell count number}}{\text{Final cell count number}}$$

This category is also evaluated by means of the growth and development of suspension cell in a liquid MS medium (GI). The four replicates of each concentration were accepted with satisfactory.

1.2 Environmental factors

Both light and temperature were important factors affecting the growth and development of suspension cells.

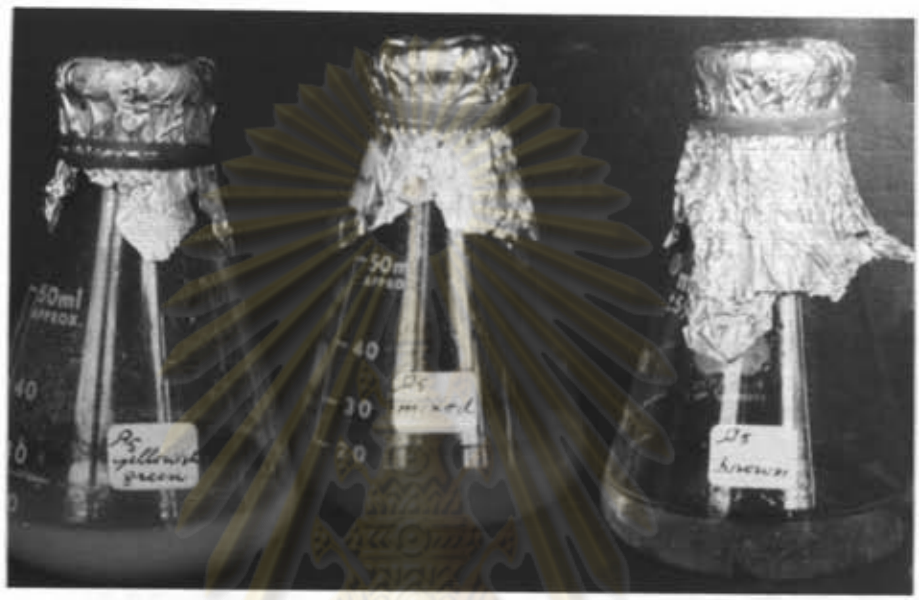


Figure 8 Signifies Intensity of Cell Formation

A	High response	ca. cell count no.	$> 10^6$	cell/ml
B	Moderate	" "	$10^5 - 10^6$	"
C	No response	" "	$< 10^5$	"

1.2.1 Light

The characteristics of radiation which influence the plant development is quite common and also those which affect plant tissue in culture. These aspects are generally classified as an intensity, spectral quality, and the length of the daily exposure period.

1.2.1.1 Procedures

Transferred 3 pieces of callus into Erlenmeyer flask (1,000 ml) containing 200 ml cell suspension culture medium supplemented with 2,4-D (0.25 mg/l) and BA (3.0 mg/l). Subsequently placed all vials separately (3 Erlenmeyer flasks/treatment) in the culture room under 2,000 lux continuouse fluorescent illumination and continuous dark condition and maintained at the temperature of $25\pm2^{o}C$ on the rotary shaker (100 rpm).

1.2.1.2 Characteristic observation

These characters were recorded after 10 weeks of an inoculation.

$$GI = \frac{\text{Final wet weight - Initial wet weight}}{\text{Initial wet weight}}$$

1.2.2 Temperature

a) It has been reported that temperature affected the growth and development of suspension culture.

b) Two different temperature, $25^{o}C$ and $30^{o}C$, were used to investigate an appropriate condition for pyrethrum

suspension culture.

1.2.2.1 Procedures

Trimmed aseptically friable callus to 2 cm^3 on the sterilized petri dish. Subsequently transferred 3 pieces of callus into a culture flask(1,000 ml) containing 200 ml suspension culture medium supplemented with 2,4-D (0.25 mg/l)and BA (3.0 mg/l) and placed separately (3 culture flasks/treatment). Preparation of inoculum was the same as 1.2.1 but the temperature for cultivation was changed to two conditions, 25^{o}C and 30^{o}C.

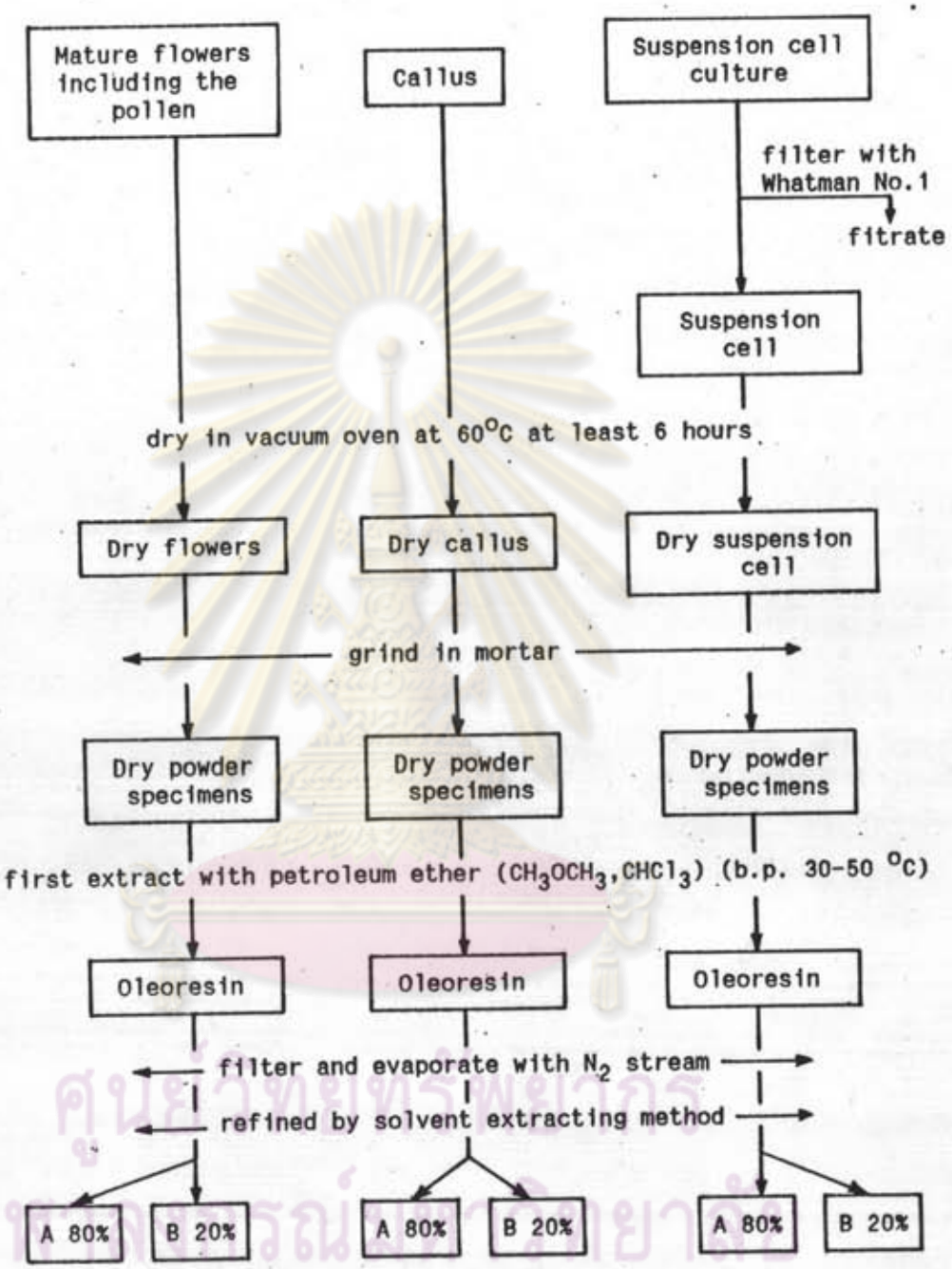
1.2.2.2 Characteristic observation.

Same as 1.2.1.2

2. Batch Culture on Platform Shaker

Extraction of Pyrethrins

Two extraction methods for the pyrethrum flowers are currently in use. One is percolation with the petroleum ether which is a batch system. The other is the continuous flow system which is a counter current extraction. This investigation has developed a procedure for the pyrethrins extraction from both pyrethrum dried flowers and dried culture cells, including with the pre-purification of the crude oleoresin before starting the pyrethrins analysis. The preparation of samples were illustrated in Figure 9.



A - various fatty acid : oleic, linoleic and palmetic acid
- vegetable extractives

B insecticidal principle

Figure 9 The process for preparing refined pyrethrum extract.

1. Selection of the organic solvent for pyrethrin extraction

1.1 Procedures.

Dried flower, callus and suspension cell powder were accurately weighed and extracted in the Elenmayer flasks (125 ml) with all three volatile organic solvents, CH_3OCH_3, $CHCl_3$, petroleum ether (b.p. 30-60^{o}C) (powder:solvent = 1:10 w/v) by shaking for at least 7 hours on a mechanical shaker.

The mixture was then filtered and the solvent was removed by evaporation on a stream bath with a stream of N_2. The residue was transferred to a small screw cap vial for diluting up to 1,000 μl

Eventually, 10 ul of the solution was injected into the GC column and analysed under appropriate conditions (see GC analysis).

1.2 Characteristic observation

The calculation of the peak area ratio of pyrethrin I to methylsterate obtained from GC analysis was used for a comparative study of the effectency of solvent for pyrethrins extraction.

2. Screening for extracting procedure.

2.1 Procedures

The specimens obtained from the sample preparation illustrated in Figure 9 were extracted with petroleum ether (b.p.30-60^{o}C)(powder:solvent = 1:10 w/v) by three extracting procedures e.g. 1) extracting in a Soxhlet apparatus for at least 7 hrs, 2) shaking

vigoriouly in the Erlenmayer flask at the temperature of 25, and 40°C , and 3) immersing in a Erlenmayer flask at the temperature of 4°C.

2.2 Characteristic observation.

The ratio of pyrethrin I to methylstearate peak area obtained from GC analysis was calculated to define the procedure's efficiency.

Pyrethrins Analysis

1. Preparation of TLC spraying reagent

1.1 p-Anisaldehyde sulfuric Acid

1.1.1 Spray reagent

1 ml conc. sulphuric acid was added to solution of 0.5 ml anisaldehyde in 50 ml acetic acid. Freshly prepared for use.

1.1.2 Treatment after spraying.

Heated at 100-105°C until the spots attain maximum colour intensity. The pink backgrounds could be bleached by exposure to steam (water bath).

1.2 Antimony (III) chloride (Carr-Price Reagent).

1.2.1 Spray reagent

25 g antimony (III) chloride were dissolved in 75 g chloroform. Generally a saturated solution of the antimony (III) chloride in chloroform or carbon tetrachloride was used.

1.2.2 Treatment after spraying.

The TLC plates were heated at 100°C for 10 mins. The chromatogram was also inspected under long wave UV light.

2. Preparation of the GC standard solution

Two external standards, a commercial standard of pyrethrum extract from PBK (Pyrethrum Board of Kenya) and world pyrethrum extract provided by Dainihon Joju Kigu Company (Japan) and one internal standard, methyl stearate, had been employed in the quantitative determination of pyrethrins by GC. These following descriptions shows the preparation procedures of those three standards.

2.1 Preparation of external standardsolution

Both pyrethrum extracts were weighted accurately and redissolved in acetone for making up concentrations of 3.02, 2.7, 2.4, 2.1, 1.8, 1.5, 1.2, 0.9, 0.6 and 0 mg/ml. Further, separately injected the solution into GC under these following conditions.

Chromatographic conditions :

Column : 2 m x 3.175 mm, stainless steel columns packed with 3%OV-17 on 80-100 mesh Chromosorb WHP.

Carrier gas (N_2) (50 ml/min.)

Hydrogen (H_2) (40 ml/min.)

Detector : Flame ionization detector

Operating condition :

Temperature

Column	205°C
Injector port	230°C
Detector	230°C
Sensitivity	10^{-10} AUFS

Before operation, conditioned column at 225°C for 3 hrs with N_2 flow 50 ml/min.

2.2 Preparation of internal standard solution

25 mg of methyl stearate was weighted accurately and then dissolved in 50 mlacetone to make the concentration of 2,500 µg/µl in a small screw cap vial and consequently stored at 4°C. Prior to GC analysis, added before time injection with the external standard at a ratio of 1:1.

3. Standard calibrating curve

Peak areas of external standard and internal standard were measured individually for each concentration. The standard calibration curve was obtained by plotting of an external standard's peak area correlating with internal standard's peak area against weight of the pyrethrins content (µg/g dry wt.)

4. Qualitative analysis of pyrethrins by TLC

TLC was performed on silica gel HF (BDH; 250 mM wet thickness for qualitative TLC) coated air-dried, activated plates using these various developing solvents :

1.	benzene/petroleum ether 60-80°C	70 : 30
2.	benzene/petroleum ether 60-80°C	75 : 25
3.	petroleum ether 30-60°C/ethylacetate	80 : 20
4.	petroleum ether 30-60°C/ethylacetate	90 : 10
5.	n-hexane/ethylacetate	75 : 25
6.	n-hexane/ethylacetate	85 : 15
7.	n-hexane/ethylacetate	90 : 10
8.	n-hexane/n-heptane/ethylacetate	48 : 40 : 12

The spots of crude fraction from each pyrethrum extracts were dissolved in acetone and applied separately 1 cm^3 above the edge of the activated plates along with the references (PBK standard and World Pyrethrum standard). Further, developed the plates in the organic solvent sytem referred above at the ambient temperature. Finally, plates were sprayed with specific spray reagent mentioned in subsections 1.1 and 1.2.

5. Quantitative analysis of pyrethrins by GC

5.1. Instrumental method

Stainless column 2 m x 3.175 mm i.d. packed with 3% OV-17 (phenylmethyl silicone) on 80-100 mesh Chromosorb WHP were employed exclusively in this study. The column was flushed successively with N_2 : H_2 (5 : 4) as the carrier gas (30 ml/min.) for 4 hours at the 215°C column temperature which was 10°C rising from general operating temperature of 205°C.

6.2. GC operation and calculation of results

Two pyrethrum extracts of known pyrethrins content as determined by the PBK and Dainihon Joju Kigu Company method of analysis were employed as standards. Solutions of the standards and the unknown were prepared in acetone containing between 0.3 to 3.02 mg pyrethrins/ml solvent and trace were run using 2 ul injection for each analysis.

These following variable factors were tried to evaluate the most appropriate performing conditions.

- Temperature (operated between 150-225^{o}C)
- Carrier gas flow rate

The peak area of each constituent was calculated by triangulation and the total area response for "Pyrethrin I" was determined. These were then applied to the "Pyrethrin I" and "Pyrethrin II" responses of the unknown, given directly amounts present in the original solution.

6. Quantitative determination of pyrethrins by HPLC

6.1 Instrumental method

The octadodecylsulfate (ODS) spherisorb column 5 um particle size, 15 cm x 0.45 cm i.d. were flushed successively with methanol, chloroform and hexane prior to use in the mobile phase. Columns were equilibrated with the eluent by pumping through several column volumes of solvent before an application of the first sample. All the experiments were performed at room temperature (25$\pm$1^{o}C)

6.2 HPLC operating method and calculation of results

In course of quantitative analysis of pyrethrins by HPLC, pyrethrins content of refined *in vitro* shoot, callus and suspension cells extracts were determined. Standard and unknown solution were prepared in methanol containing between 0.3 to 3.2 mg. pyrethrins/1,000 ul solvent, and trace were run using 20 ul injection for each analysis. A mixture of methanol and water (3:1) and the flow rate used ranged from 0.5-1.5 ml/min were employed in this study. The results were precisely calculated by computer via video chromatography control center model PU4895 LC interfece.