CHAPTER III

EXPERIMENTS ON THE EFFECTS OF PHOSPHORUS AND POTASSIUM TO THE YIELD OF CURCUMINOID PIGMENTS AND THE YIELD AND QUALITY OF THE VOLATILE OIL OF CURCUMA LONGA LINN RHIZOMES.

1. Introduction

The purpose of the experiments was to study the effects of phosphorus and potassium added to the plot in the cultivation of C. longa Linn to the yield of the curcuminoid pigments and the yield and quality of the volatile oil produced in the rhizome. The values of turmeric in therapeutics are the high yield and high quality of the volatile oil component, and in commerce is the high yield of the total pigments. Minerals in the soil is one of the important factors affecting the quality of the product, thus the effects of phosphorus and potassium were studied.

The turmeric plants were grown from the seed rhizomes of C. longa Linn of Nakhon Pathom origin previously cultivated in Amphur Hang Dong, Chiang Mai Province. The cultivation experiments were made on the highland of Ban Yang San Lang Village, Amphur Mae-Chaem, Chiang Mai Province where the CARE organization was working on promotion of better life for villagers.

The elements such as phosphorus and potassium were added to the plots in the forms of phosphorus pentaoxide and potassium dioxide respectively. The cultivated rhizomes were harvested after about 9

months of growth, at the stage of drying out of the vegetative parts. The rhizomes were cured and dried and analysed for the general properties and for the yield and quality of the valuable components.

The total pigments and individual curcuminoid compound of the pigment were determined by High Performance Liquid Chromatography (HPLC) under the conditions newly developed in the pharmacognosy laboratories (based partty on the work of Asakawa 1981). The active components of the volatile oil were determined by the gas chromatographic method comparing with the authentic compounds under the same conditions.

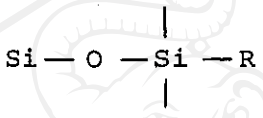
The principles of the two analysis methods are given below:

1.1 Principle of the High Performance Liquid Chromatography (HPLC)[36,37]

1.1.1 The method and advantages. Chromatography in its many forms is widely used as a separative and analytical technique. Liquid chromatography in the form of paper, thin-layer, ion-exchange, and exclusion (gel-permeation and gel-filtration) chromatography had not been able to achieve the success, mainly because of the poor efficiencies and the long analysis times arising from the low mobile phase flow rates. But HPLC had already shown the potential advantage, interms of column efficiencies and speed of analysis, of liquid chromatography. So, the advantages of HPLC over other forms of liquid chromatography may be summarized thus: (a) the HPLC column can be used many times without regeneration; (b) the resolution achieved on such columns for exceeds that of the older methods;

(c) the technique is less dependent on the operator's skill and reproducibility is greatly improved; (d) the instrumentation of HPLC lends itself to automation and quantitation; (e) analysis times are generally much shorter.

The use of stationary phases are chemically bonded stationary phases that were prepared from silica by reacting the surface silanol groups with organochlorosilane or alkoxysilane to give a linkage which is hydrolytically stable.



The R group may be a hydrocarbon (e.g C_8 or C_{18}) or a hydrocarbon with a polar terminal group (e.g R-CN, $R-NH_2$). The bonded phases have virtually eliminated the use of coated stationary phases. The term normal phase HPLC originally referred to a system with a polar liquid stationary phase, e.g. water, glycol or β , β' -oxydipropionitrile, which the mobile phase is relatively non-polar, e.g. hexane, benzene, or chloroform. This mode of operation was used to separate polar compounds which would be distributed preferentially in the polar stationary phase. It the stationary phase is non-polar, e.g. a hydrocarbon and the mobile phase is polar e.g water, the technique is referred to as reverse phase liquid chromatography. Normal phase refers to a system where the stationary phase is more polar than the mobile phase and reverse phase where the stationary phase is less polar than the mobile phase. Since the majority of separations are

now carried out in the reversed phase mode. Some classes of compounds are best separated by normal phase HPLC and some by reverse phase HPLC. Examples include: Normal phase HPLC-plasticizers, dyes, pesticides, steroids, anilines, alkaloids, glycols, alcohols, phenols, aromatics, and metal complexes. Reverse phase HPLC-alcohols, aromatics, anthraquinones alkaloids, oligomers, antibiotics, barbiturates, steroids, chlorinated pesticides, and vitamins.

1.1.2 Apparatus. A schematic diagram of a HPLC is shown in Figure 6. The unit consists of a high pressure pumping system to force the liquid mobile phase through the column packed with a small particle size stationary phase, a presaturator, an injector, the column, and an on-line highly sensitive detector system.

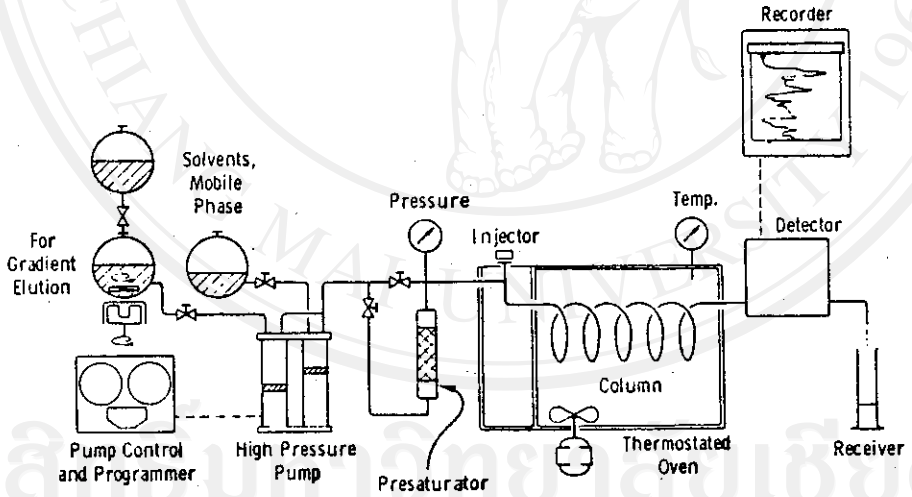


Figure 6: A schematic diagram of a High-Performance Liquid Chromatography.

As in all chromatographic techniques, the sample is introduced into the system through the injector; the components in the sample are fractionated during their passage through the column. The detector system senses these components as they elute from the column and generates a signal proportional to the amount of the solutes passing through the system. For analytical separations the solvent reservoir should be of about 1 dm^3 capacity. Before the solvent is used it should be degassed. Degassing is required to remove dissolved gasses (in particular oxygen) which may react with either the mobile or the stationary phase. Ultrasonic degassing is cheap and convenient but not very effective. The pumping systems have been one of the main factors in HPLC and the pump is a critically important component. The pumps in general use are piston-type pumps to drive the mobile solvent. With mechanical piston pumps, the "flow rate" is set to the desired value; and the pressure is observed as a dependent variable. In HPLC, the technique does to improve the separation by changing the flow rate of the solvent. A major development in high efficiency HPLC has been the development of sensitive, on-line detector systems there are two basic types of detector for use in HPLC involving: (a) The differential measurement of a property common to both the sample and the mobile phase; (b) The measurement of a property that is specific to the sample, either with or without the removal of the mobile phase before detection. The first types of detector, also known as bulk property detectors, are the differential refractometer, conductivity, and dielectric constant

detectors. Solute property detectors which do not require removal of the mobile phase before detection include the ultraviolet absorption, polarographic and radioactivity detectors, while the moving wire flame ionization detector (FID) and electron capture (EC) detector both require removal of the mobile phase before detection. UV detector that is based on the absorption of ultraviolet light, is the most widely used. It is therefore not universal in application, but the great many substances do absorb UV radiation including all substances having π -bonding electrons and also those with unshared (non-bonded) electrons, e.g. olefins, aromatics, and compounds containing $\begin{matrix} | & | \\ C{=}O, & C{=}S, \end{matrix}$ -N=O, and -N=N. The radiation source used in many UV detectors is a low-pressure mercury vapour lamp. The predominant line in the spectrum is at 254 nm and other lines are filtered out to give monochromatic light at this wavelength.

1.1.3 Data handling. The data handling in chromatography now ranges from a simple pen recorder to complicated computing integrators and computerized data handling systems. Chart speeds generally used are a range from 0.5 to 30 cm/sec. In order to obtain precise quantitative data and obtain maximum benefit from computerized data handling, several factors must be controlled. The injector, column, detector and data system must operate reproducibly, and the solvent flow rate and column temperature must remain stable during the run. There are several methods for determining peak areas or heights as follows.

1.1.3.1 Peak Height. The peak height is measured as the distance from the baseline to the peak maximum. Baseline drift is compensated by interpolation of the baseline between start and finish. Peak heights should not be used when peaks are visibly distorted or when the column is overloaded, or for shoulders.

1.1.3.2 Height times Width at Half-Height. Normal chromatographic peaks often approximate a triangle, and the area can be calculated by the triangle formular. The width at half height is used rather than the width at baseline to reduce errors due to tailing and adsorption. The technique is relatively rapid and simple. It requires only four operations: draw the base line; determine the half-height, and measure the height and the width at half-height. The technique should be applied only to symmetrical peaks or peaks which have similar shapes. The area measured is less than the true area, but is proportional to sample size, provided the peaks are not distorted badly. The precision depended upon the height to width at half height ratio. A range from approximately 2 to 10 should be strived for. The width can be increased by using a faster recorder chart speed.

1.1.3.3 Triangulation. This technique is also based on the fact that chromatographic peaks approximate triangles. The triangulation technique requires that tangent lines be drawn along the sides of the peak. The height is measured from the baseline to the intersection of the tangent lines. The width measurement is taken, as the intersection of the two tangent lines with the baseline.

The area is calculated by the triangulation formula. This technique has all the limitations of the previous technique plus the added complexity of drawing the tangent lines. A slight error in placement of the tangent lines can have a profound effect on the height measurement. A height to width at half-height ratio of approximately one is best. This technique is not recommended since it is susceptible to operator error and shows no improvement in accuracy over the previous method.

1.1.3.4 Planimetry. A planimeter is a mechanical device used to measure the peak area by tracing the perimeter of the peak. The area is measured digitally on a dial. The precision and accuracy of this method are dependent on the device to use and requires considerable operator skill. The planimeter technique, which perhaps no more precise than the triangulation technique, is more accurate, particularly with skewed peaks, because the ture peak area is measured. Precision can be improved by starting the trace at a position of low sensitivity and by tracing each peak several times and taking an average. Repetitive traces make an already time consuming technique even more lengthy.

1.1.3.5 Cut and Weigh. This is another perimeter technique which requires cutting out the chromatographic peak and weighing the paper on an analytical balance. The accuracy of the method depends on the care used in cutting and on the constancy of the weight of the chart paper. The inaccuracy of the cutting can be

minimized by keeping the ratio of height to width at half height in the range 1 to 10. Destroying the chromatographic data can be avoided by previously copying the chromatogram and cutting the peak out of the copy. The homogeneity of the paper, moisture content, and the weight of the paper are important factors. This method is time consuming, but superior to triangulation techniques for irregularly shaped peaks.

1.1.3.6 DISC® Integrator. The DISC® integrator is a popular method in liquid chromatography. It provides a degree of automation at a relatively low price, but accuracy is limited by the performance of the recorder and independent of peak shape. The main disadvantage of the DISC® integrator is that the peak must not be allowed to go off scale.

1.1.3.7 Digital Integrator. Electronic integration offers the liquid chromatography highly precise and automatic conversion of the chromatographic signal into numerical form. This technique eliminates errors from the recorder since the detector out put is sent directly to the integrator. The electronic integrator is a device which automatically measures the peak area and retention times. Digital integrators usually give better accuracy and precision than the chromatograph. Sharp peaks are most easily handled. Digital integrators were very expensive for the average laboratory instruments.

1.1.3.8 Computers. The digital integrator does an excellent job of peak area measurement, but does not do any compo-

sition calculations or data interpretation. Microprocessors in computer technology have greatly lowered the cost of computers and due to distinct advantages. They have become very popular. There are several approaches being used, but two types have become very popular: Dedicated computer is a single computer attached to the liquid chromatography and multichannel dedicated computer has several chromatographs on-line to a dedicated computer. The single greatest advantage of on-line computer techniques is that manual measurements and calculations are no longer performed by the operator, thus increasing the convenience, precision, and accuracy. At the end of a chromatographic separation, the results are immediately available.

1.2 Principle of the Gas Chromatography (G.C.)[36,37]

1.2.1 The method and application. The basis for gas chromatographic separation is the distribution of a sample between two phases. One of these phases is a stationary bed of large surface area, and the other phase is a gas which percolates through the stationry bed. Gas chromatography is a technique for separating volatile substances by percolating a gas stream over a stationary phase. If the stationary phase is a solid, it is called gas solid chromatography (G.S.C). The basis for separation depends upon the adsorptive properties of the column packing. Common column packings used are silica gel, molecular sieve, and charcoal. If the stationary phase is liquid, it is called Gas Liquid Chromatography (G.L.C.) The liquid is spread as a thin film over an inert solid and the basis

for separation is the partitioning of the sample in and out of this liquid film. It is used to analyzed gases, liquids and solids. In gas liquid chromatography the components to be separated are carried through the column by an inert gas (carrier gas). The sample mixture is partitioned between the carrier gas and a non volatile solvent (stationary phase) supported on an inert size-graded solid (solid support). The solvent selectively retards the sample components, according to their distribution coefficient, until they form separate bands in the carrier gas. These component bands leave the column in the gas stream and are recorded as a function of time by a detector.

The retention time is that time from injection to the peak maxima. This property is characteristic of the sample and the liquid phase at a given temperature. With proper flow and temperature control, it can be used to identify each peak. Several compounds can have identical or close retention times, but each compound has only one retention time. This retention time is not influenced by the presence of other components. The area produced for each peak is proportional to that peak's concentration. This can be used to determine the exact concentration of each component. Accuracy attainable with gas chromatography depends upon technique, detector, integration method, and sample concentration. Interpretation of the data obtained is usually rapid and straight forward.

1.2.2 Apparatus. A schematic drawing of a gas chromatographic system is shown as Figure 7.,page 60.

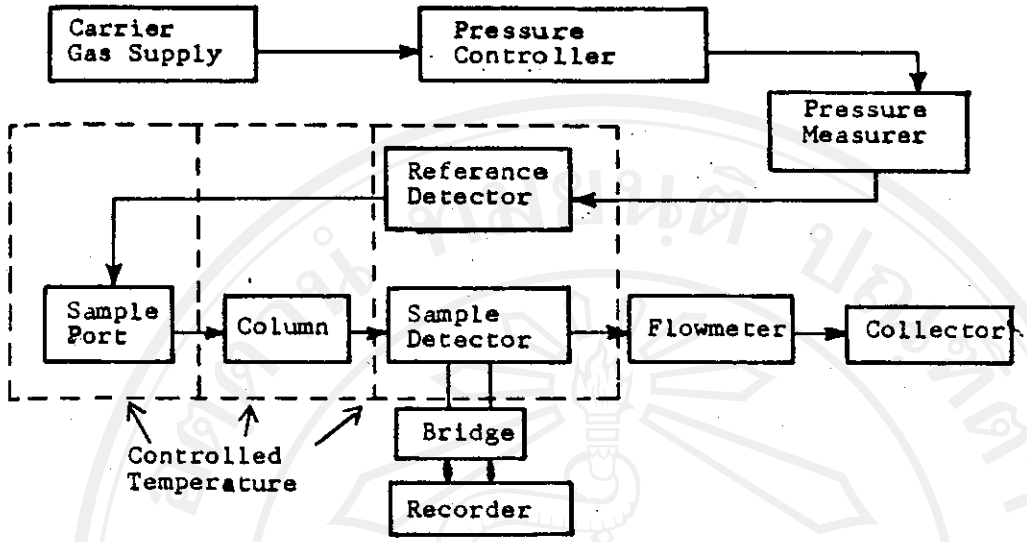


Figure 7: A schematic drawing of a gas chromatographic system.

1.2.3 Data handling. The data handling in gas chromatography is the same as those described in HPLC method.(see section 1.1.3)

The general properties of the rhizomes were determined through the ether-soluble extractives, both volatile and non-volatile the total ash and the acid-insoluble ash contents, and percentage of loss on drying as for the volatile oil-containing vegetative drugs general method of analysis.

2. Experimental part

2.1 Apparatus and chemicals

2.1.1 Apparatus

2.1.1.1 Gas chromatograph Perkin-Elmer, model Sigma 3B with dual flame ionization detector, equipped with glass column diameter x length 1/6 inch x 6 ft packed with 5% SE-30 on 60-80 mesh chromosorb HMDS.

2.1.1.2 High Performance Liquid Chromatograph model 6000 A, Waters Association with UV Model 440 absorbance detector, equipped with metallic column diameter x length 3.9 mm. x 30 cm. packed with 10 μ Bondapak®

2.1.1.3 Other laboratory apparatus included:

Muffle furnace, Thermolyne Corporation, Subsidiary of Sybron Corporation, Iowa, U.S.A.

Drying oven, gravity convection type, Precision Scientific

Moisture balance, Mettler LP 12

Automatic shaker, Lab-Line Instrument Inc.

Millipore filter pore size 0.5 μm,Millipore Corporation

pH meter, PHM 61 Laboratory PH meter, Radiometer, Copenhagen

Mill, Arthur H.Thomas Co,Phila, U.S.A.

Extraction apparatus, Soxhlet 45/50, Pyrex,Corning

glass works, Corning New York.

Ultrasonic bath Brasonic® 221, Waters Association.

2.1.2 Chemicals

Acetone Analar®, BDH Chemicals Ltd.

Acetonenitrile HPLC grade, H & W

Benzene 'Baker Analyzed® Reagent

Diethylether, Riedel-Dehainag, Seelzer-Hannover.

Double distilled water

Ethanol absolute, R.P.Normapur®

Formic acid, A.R., Fluka

Hydrochloric acid, Pronalys. May & Baker Ltd.

Methanol "Pronalys" May & Baker Ltd.

ar-Turmerone from faculty of Science, Chulalongkorn University.

Anhydrous sodium sulphate, AR,Riedel

Curcumin Fluka AG,Buchs SG.

Kieselgel 60 (35-70 mesh Astm), E.Merck,Darmstadt.

Potassium sulphate fertilizer containing 50% K_2O; Yip In Soy Co.Ltd.

Tripple superphosphate fertilizer containing 46% P_2O_5; Yip In Soy Co.Ltd.

2.2 Cultivation experiments

2.2.1 Site of experiments

Site: Hilly area at Ban Yang San Lang, Tambon Tha Pha, Amphur Mae Chaem, ChiangMai Province.

Altitude: About 1500 meters above the average sea level

Slope: 20-25%

Average rainfall: About 67.35 millimeters

2.2.2 Date of experiments

From July of 1983 to March of 1984

2.2.3 Soil condition and preparation. The land at the site of the experiments was formerly a rice and corn field. The soil was a well-drained rich loam. The soil analysis made by the Soil Department, Faculty of Agriculture, Chiang Mai University gave the following data.

PH 6.33

Organic matter content 2.76%

Available phosphorus content 14.00 ppm

Available potassium content 435.42 ppm

A plot of the dimensions 6 m x 12 m was raised 6 inches above ground to prevent soaking of the underground part of the plants during the heavy rain as well as to prevent explosure of the root at the time. The plot was divided into 9 beds of the area 0.66 m x 12 m each as shown in Figure 8. No other preparations were needed as the soil was rich enough for growing plants.

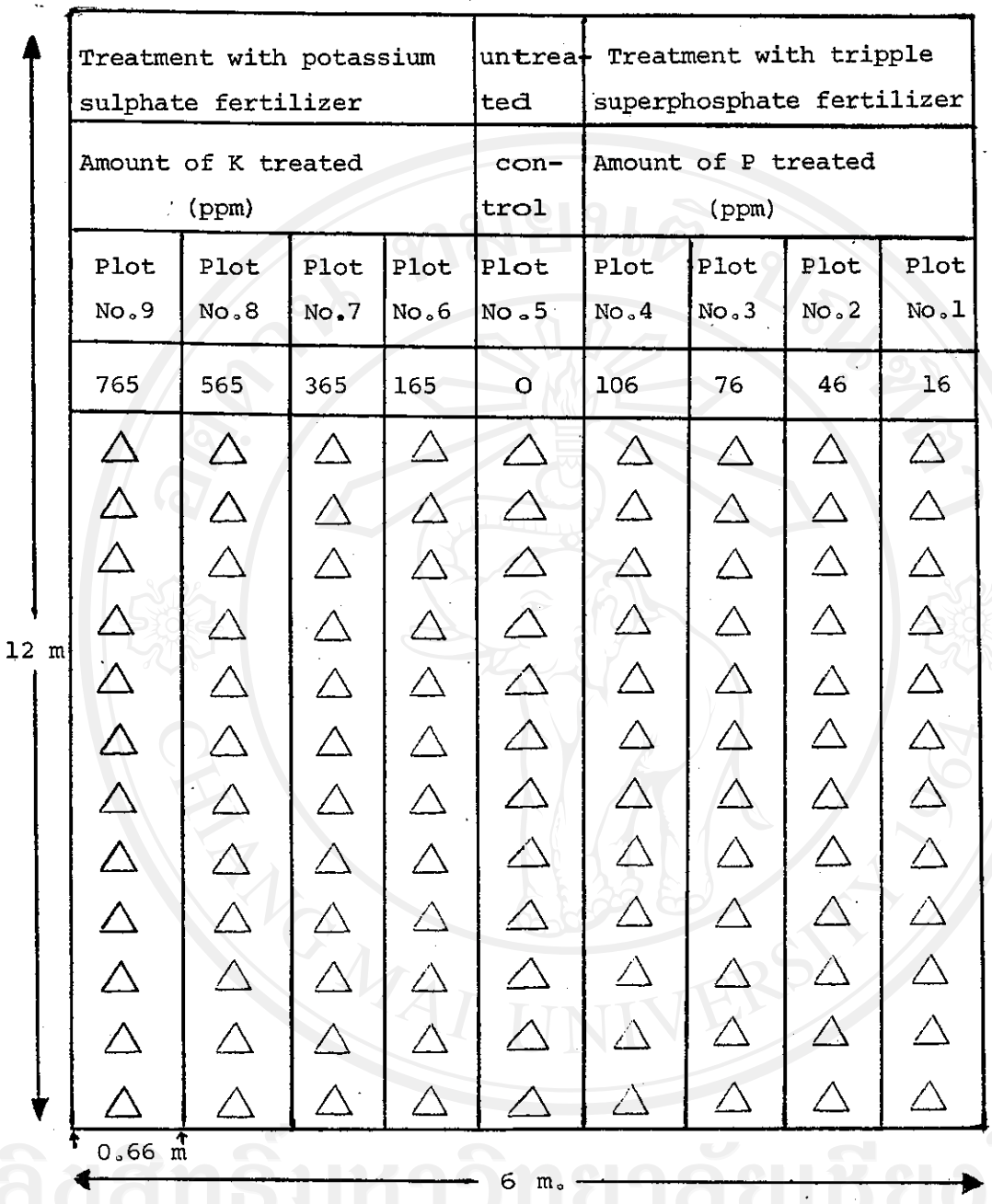


Figure 8 : A diagram showing of divided plots for cultivation of curcuma and treatment with fertilizers

Control: containing 14 ppm of phosphorus and 435 ppm of potassium

△ : Curcuma plant

2.2.4 Planting experiments

2.2.4.1 Curcuma longa Linn rhizomes for propagation. Seeded rhizomes were put underground in a store place about one month. before the beginning of the experiments. They were the Nakhon Pathom variety previously grown and propagated in Amphur Hang Dong of Chiang Mai Province for one season.

2.2.4.2 Planting, The finger parts of the seed rhizomes were used for propagation. They were removed from the store place in July of 1983 and put in the prepared beds in rows of 50 cm. apart and 50 cm intervals in each row. The fingers were covered with 3-5 cm layer thickness of the soil and pressed firm. Light irrigation from a shower pot was given at the beginning on the dry days. Too much water was avoided to prevent rottening of the seed rhizomes. The bud appeared on each finger within 30 days after putting in the soil, and the first leaf within 20-50 days.

2.2.4.3 Care and management, C.longa Linn was an easy plant to manage. It grow well if the soil was rich and there was sufficient rainfall. After the first leaf appeared, the other leaves of rather bright green colour followed rapidly. The size of the leaf was large, of approximately 13 cm x 35 cm. The leaf number varied from four to six and the leaves could well shadow the ground that only one weeding was required in the first 30 days period after emergence of the plant. No weeding was necessary ever since.

2.2.4.4 Fertilizer experiments. In October 1983, the third month after planting, the prepared fertilizers were given.

Phosphorus was given in the form of tripple superphosphate containing 46% of P_2O_5. Potassium was given in the form of potassium sulphate containing 50% K_2O. The calculated quantity of each compound equivalent to the required weight of phosphorus or potassium was mixed thoroughly with soil to produce the specified concentration of the element. The soil around each plant, 10 cm from the stem, was turned up and the prepared mixture of the fertilizer element was applied. The soil around the plant was mixed well with the fertilizer and then damped with water from a shower plot. Supposing, the presence of 800 ppm of potassium in the soil was sufficient for the cultivation of curcuma. The available potassium in soil sample was found to be 435 ppm. The extra amounts of potassium sulfate fertilizer added to reach 800 ppm of potassium could be calculated.

Example of calculation.

available K in the sample soil = 435 ppm

general loamy soil, weight of soil = 320,000 kg/Rai

1 Rai = 1,600 m^2

∴ Soil 10^6 kg have K = 435 kg

Soil 320,000 kg have K = $\frac{435 \times 320,000}{10^6}$ = 139.2 kg/Rai

Addition K to 800 ppm

∴ Soil 10^6 kg have K = 800kg

Soil 320,000 kg have K = $\frac{800 \times 320,000}{10^6}$ = 256 kg/Rai

∴ K was used to add into the soil = 256-139.2 = 116.8 kg/Rai

= 0.073 kg/m^2

Each plot has the 0.66 x 12 = 7.92 m^2 areas

∴ addition of K = 0.073 x 7.92 = 0.578 kg/7.92 m^2

But K_2O = K x 1.2

∴ Addition K in the form of K_2O = 0.578 x 1.2 = 0.6936 kg/7.92 m^2

Potassium sulphate fertilizer containing 50% K_2O

K_2O 50 kg receive from potassium sulphate = 100 kg

K_2O 0.6936 " ——— " = $\frac{100 \times 0.6936}{50}$

= 1.387 kg

Therefore, addition potassium sulphate 1.387 kg/7.92 m^2 equal K 800 ppm.

Table 10: The data of the amount of fertilizers applied

Treatment number	Amount of Phosphorus applied (ppm)	Amount of Potassium applied (ppm)
1	16	-
2	46	-
3	76	-
4	106	-
5	control	control
6	-	165
7	-	365
8	-	565
9	-	765

Control: containing 14 ppm of phosphorus and 435 ppm of potassium

2.2.5 Harvesting. Curcuma longa Linn plants grew well through the rainy season to the average height of 1,500 meters. After full growth the plants flowered. With the approach of the cool season in November the plants began to wither. When the whole vegetative part withered, the harvest time came. The soil around the plants was dug with a small iron hoe and the whole clumps of the rhizomes, complete with the fingers and rootlets, were carefully lifted out. The adhering soil was removed and the rootlets were cut off, and the clump of rhizome washed clean. The rhizomes obtained were separated into the bulb part and the finger part for the curing process to prevent budding afterwards.

2.3 Analysis

2.3.1 Preparation of the sample for analysis. The bulbs and fingers were cured according to the process described in section 2.2.2, Chapter II. Then the cured samples were ground (2.2.3, p 39) to obtain the sample powder passing the sieve diameter 1 mm.

2.3.2 General analysis. The criteria of general properties of the samples were determine as follows:

Table 11: The criteria of general properties of the samples

Criteria	Method of analysis described in details in:		
	Chapter	heading	page
Loss on drying	II	2.3.1	40
The volatile oil content	II	2.3.2	40
The total ash content	II	2.3.3.1	40
The acid-insoluble ash content	II	2.3.3.2	40
The volatile,and non-volatile ether-soluble extractives	II	2.3.4	41

Triplicate analyses were made for each sample, and the average was taken as the result which was shown in the part "Results and discussion". (Table 14, page 78)

2.3.3 Determination of the curcuminoid pigment content

2.3.3.1 The method employed and the conditions of analysis. The method employed in the determination of each curcuminoid compound as well as the total pigment content was the High Performance Liquid Chromatography (HPLC method), with the following specified conditions:

Column: diameter x length 3.9 mm x 30 cm, packed with Nucleosil C_{18}, particle size 10 μ

Elution solvent: Acetonitrile-water-formic acid 45:75:2

Flow rate : 1.5 ml/min

Sensitivity : 0.02

Chart speed : 2.5 cm/min

Detector : UV detector, at 254 nm

Sample size : 10 μl

Degas by mean of ultrasonic bath for 30 min.

2.3.3.2 The determination of the peak areas. The peak area of each compound in the HPLC chromatogram was determined by the following expression:

Peak area = Height x width at half height

The width at half height was used rather than the width of the base line of the triangular peak to avoid errors due to tailing of the base line and adsorption

To determine the width at half height, firstly the baseline of each peak was drawn from A to B, determine the half height ($\frac{1}{2}$ H). Then measure the height (H) and the width at half height (W $\frac{1}{2}$) as shown in Figure 9., page 71

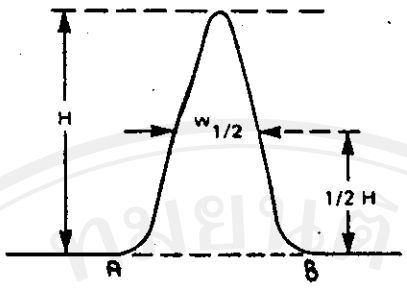


Figure 9: Determination of peak area by Height times Width at Half-Height method

2.3.3.3 Preparation of the sample for the HPLC analysis. About 3 grams of the powdered sample was weighed accurately and extracted with acetone in a soxhlet continuous extractors for 8 hours. The extract obtained was transferred quantitalively to a 100-ml volumetric flask and made to volume with acetone. Resins were removed prior to the determination by column chromatographic method. Thus, 10 ml of the extract was pipetted into a chromatographic column size 2.5 cm x 40 cm, packed with silica gel 60 for column chromatography (35-70 mesh ASTM). Elution of the pigments was made with the mixture of benzene-methanol 8:2 (500 ml). The time needed for each elution was about 30 minutes. The volume of the eluate was reduced to about 50 ml under reduced pressure. The concentrate eluate was then quantitatively transferred to a 100 ml volumetric flask and diluted to volume with methanol. The solution was filtered through a millipore filter to obtain the sample solution for HPLC analysis. Ten microliters of sample solution was injected onto the HPLC column that had been previously equilibrated

for 0.5-1 hour with the mobile solvent as mentioned above. The HPLC peaks were detected by means of an ultraviolet detector at 254 nm.

2.3.3.4 Preparation of the standard curve of the three curcuminoid compounds of turmeric pigments. Two hundred fifty milligrams of the standard curcumin (Fluka AG, containing 3 compounds) was accurately weighed into a 500-ml volumetric flask. It was dissolved and diluted to volume with methanol. This was the 500 ppm stock solution of curcumin. Solutions of curcumin of the concentrations 50,100,125,150, and 200 ppm were prepared from the above standard stock solution, using methanol as solvent. Each (10 μl) was analysed by the same HPLC apparatus and under the same conditions. The peak area of each compound in each chromatogram was measured and calculated. The data was shown in Table 17, and the standard curve of each compound was prepared.

2.3.4 Analysis of the volatile oil for the active components, turmerone and ar-turmerone. The analysis of the volatile oil of C. longa Linn was made by the gas chromatographic method under the following conditions:

Apparatus : A Perkin-Elmer Sigma 3B gas chromatograph, equipped with flame ionization detector, integrator and temperature programmer.

Column : 1/8 inch x 6 ft. glass column, packed with 5% SE-30 on 60-80 mesh chromosorb HMDS

Oven temperature : 180°C

Injection temperature : 200°C

Detector temperature : 220°C

Attenuator : 3,4

Range : x 1000

Sample size : 0.3 μl

Carrier gas : N_2

Flow rate : N_2 38 psi

: Air 30 psi

: H_2 18 psi

Ramp rate : 4 C°/min

Chart speed : 30 cm/hr

The authentic ar-turmerone, obtained from Assitant Professor Dr. Sopon Raengsamran of the Chemistry Department, Chulalongkorn University, were compared for the identification of the peaks. The percentage of each constituent in the oil was recorded automatically by means of an electronic integrator, and the data was shown in Table 13. page 76 and peaks for ar-turmerone and turmerone in Figure 11, page 77.

3. Results and discussion

Table 12 : Yield of volatile oil at different phosphorus and potassium levels of the soil

Elements added (ppm)		Yield of volatile oil* %, on dry weight basis
Phosphorus**	Potassium***	
0	0	6.19
16		7.04
46		7.94
76		8.68
106		7.55
	165	7.19
	365	7.03
	565	7.60
	765	7.09

* Average of 3 determinations of each sample.

** Phosphorus was added to the plot in form of phosphorus pentaoxide

*** Potassium was added to the plot in form of potassium oxide.

Control: containing 14 ppm of phosphorus and 435 ppm of potassium.

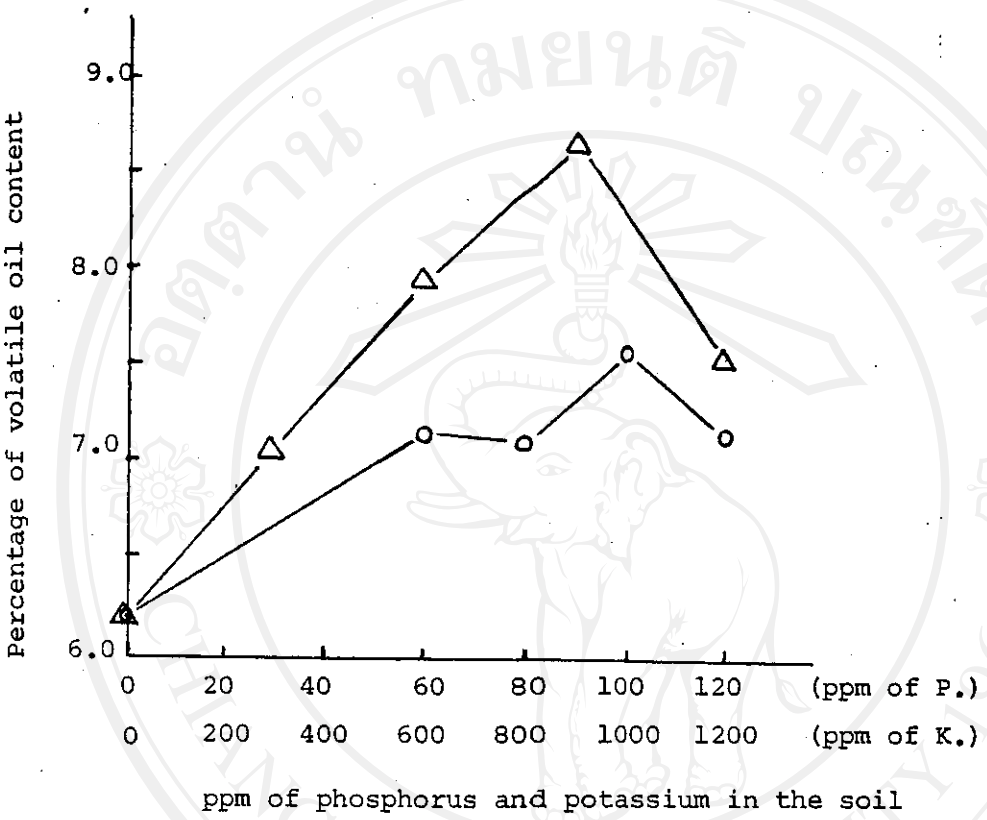


Figure 10: Effect of phosphorus and potassium on the percentage yield of the volatile oil in the rhizomes

O—O : effect of potassium

△—△ : effect of phosphorus

Table 13: Effect of phosphorus and potassium on the quality of volatile oil of C.longa Linn rhizome via the determination of ar-turmerone and turmerone contents analysed* by gas chromatographic method

Elements added (ppm)		ar-turmerone		turmerone		Total percentage of active compounds
Phosphorus	Potassium	t_R,min	Percentage	t_R,min	Percentage	
0	0	7.04	44.43	8.85	36.99	81.42
16		7.03	38.17	8.96	41.74	79.91
46		7.16	39.97	9.04	37.32	77.29
76		7.01	36.39	8.89	38.27	74.66
106		7.01	42.24	8.80	36.62	78.86
	165	7.04	38.68	8.86	38.75	77.43
	365	7.07	47.78	8.75	33.87	81.65
	565	6.94	45.41	8.60	35.02	80.43
	765	6.84	31.20	8.80	45.54	76.74

*Average of 3 determinations of each volatile oil sample.

Control: containing 14 ppm of phosphorus and 435 ppm of potassium.

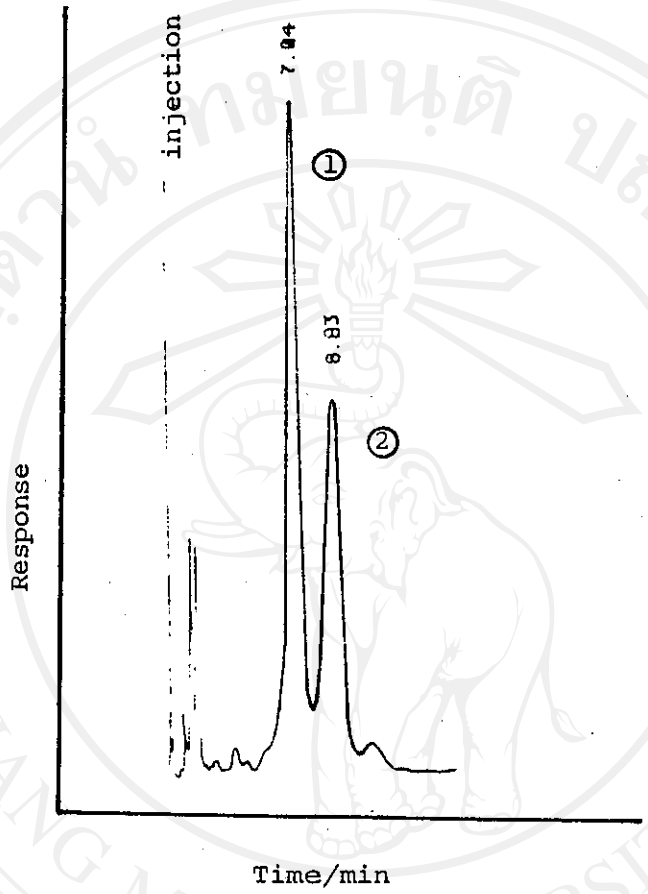


Figure 11: Gas chromatographic separation of turmerone and ar-turmerone in volatile oil

Peak ① : ar-turmerone

Peak ② : turmerone

Table 14: *Percentage constituents of C. longa Linn rhizomes cultivated in the soil treated with fertilizers containing phosphorus and potassium.

Elements added (ppm)		Total ether extractive %	Non-volatile ether extractive %	Total ash %	Acid-insoluble %	Loss on drying %
Phosphorus	Potassium					
0	0	11.94	9.22	7.17	1.45	14.01
16		10.98	8.92	7.54	1.44	14.21
46		10.39	9.39	7.60	1.75	13.18
76		12.24	8.40	7.41	1.38	14.53
106		9.74	7.62	7.19	1.82	13.47
	165	11.65	9.89	8.16	1.58	14.36
	365	10.76	8.28	7.59	1.52	12.91
	565	11.92	8.10	7.38	1.41	14.93
	765	11.33	9.07	7.72	1.49	14.61

* Average of 3 determinations of each sample

Control: containing 14 ppm of phosphorus and 435 ppm of potassium.

Table 15: Effect of phosphorus and potassium in the fertilizer on the percentage of curcuminoid pigments in C.longa Linn rhizomes.

Elements added (ppm)		*Percentage of pigments produced			
phospho-rus	Potas-sium	p-hydroxycinnamoyl feruloyl methane (peak 1)	bis-(p-hydroxycin-namoyl)-methane (peak 2)	curcumin (peak 3)	Total curcuminoid compounds
0	0	0.70	1.04	3.43	5.17
16		0.79	1.07	3.56	5.42
46		0.81	1.14	3.42	5.37
76		0.71	1.16	3.61	5.48
106		0.79	1.15	3.47	5.41
	165	0.84	1.50	3.59	5.93
	365	1.16	1.63	3.99	6.78
	565	0.86	1.52	3.56	5.94
	765	0.84	1.50	3.59	5.93

* Average of 3 determinations of each sample
Determination of curcuminoid compounds by means of HPLC
Control: containing 14 ppm of phosphorus and 435 ppm potassium

Table 16: Effect of potassium on the yield of individual curcuminoid compounds

Amount of potassium added (ppm)	% increase in pigment		
	p-hydroxycinamoyl-feruloyl methane (peak 1)	bis-(p-hydroxy-cinnamoyl)-methane (peak 2)	curcumin (peak 3)
165	20.00	44.23	4.66
365	65.71	56.73	16.32
565	22.85	46.15	3.79
765	20.00	44.23	4.66

Control: containing 14 ppm of phosphorus and 435 ppm of potassium.

Table 17: Peak areas of curcuminoid compounds from various concentrations of standard curcumin (Fluka) determined by HPLC

Concentration of standard curcumin, (ppm)	Peak areas (cm^2)*		
	p-hydroxy-cinnamoyl-feruloyl methane (peak 1)	bis-(p-hydroxy-cinnamoyl)-methane (peak 2)	Curcumin (peak 3)
50	0.24	0.67	2.15
100	0.49	1.34	4.58
125	0.59	1.74	5.46
150	0.75	2.01	6.46
200	0.98	2.66	8.73

*calculated by Height times Width at Half-Height method, mobile phase:acetonitrile:water:formic acid 45:75:2

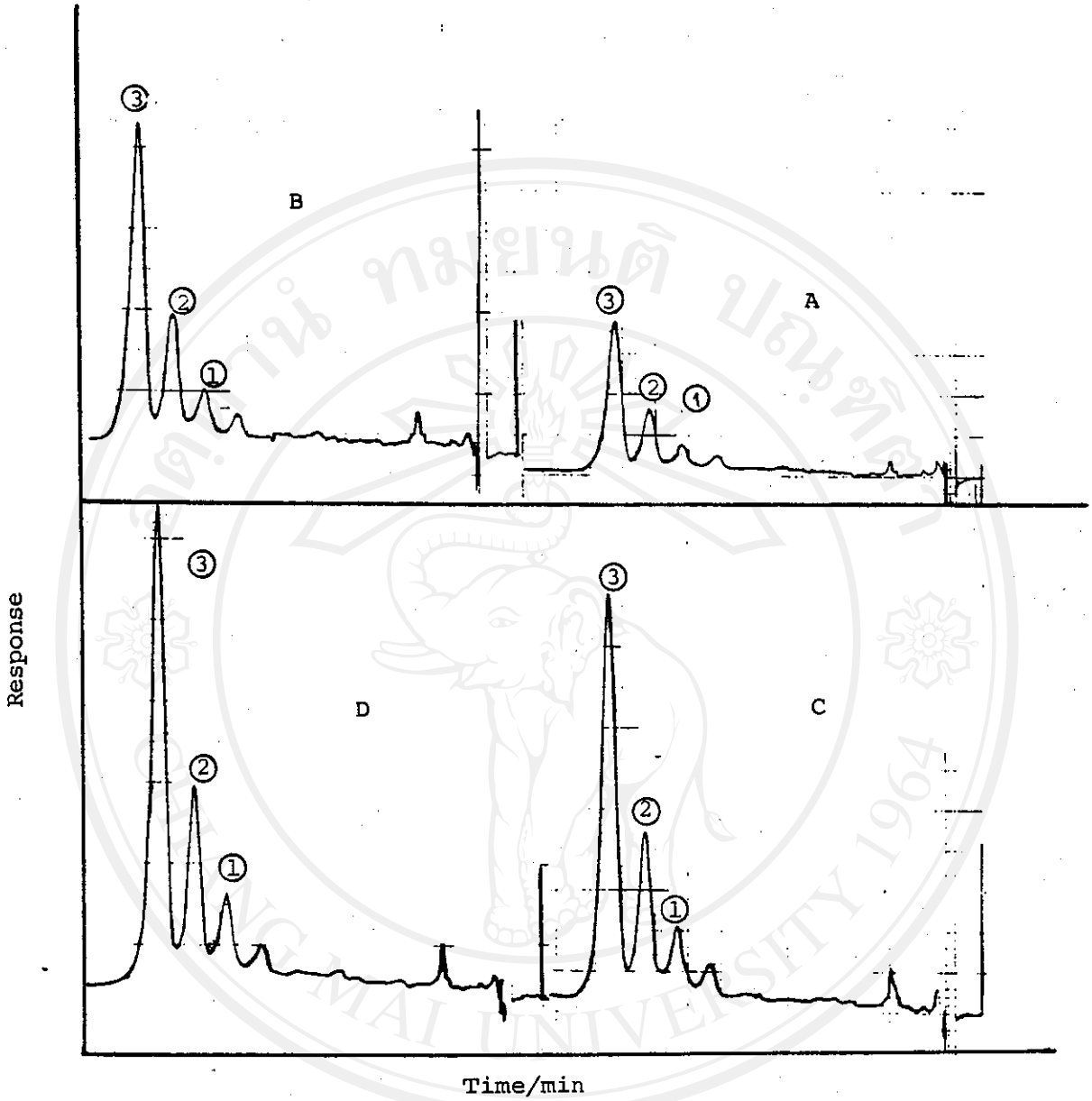


Figure 12: HPLC separation of curcumin and minor curcuminoids using acetonitrile:water:formic acid 45:75:2 as eluent A,B,C,D: concentration of standard curcumin (Fluka) 50,100,125,150 ppm respectively

Detection by UV detector at 254 nm.

①: demethoxycurcumin

②: bisdemethoxycurcumin

③: curcumin

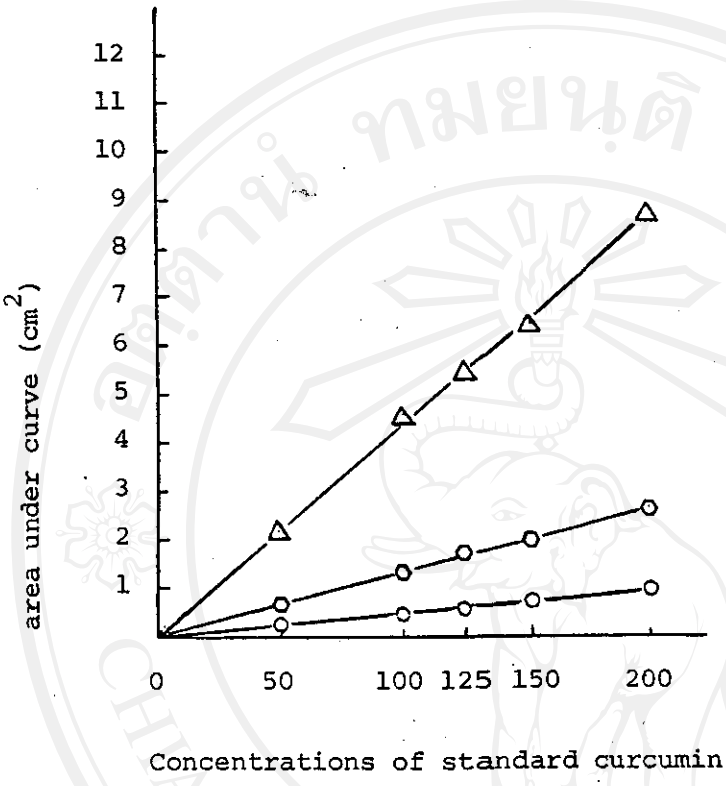


Figure 13: The standard curve of curcuminoid compounds determined by the HPLC method

△—△ : curcumin (peak 3)

⬡—⬡ : bis-(p-hydroxycinnamoyl)-methane (peak 2)

○—○ : p-hydroxycinnamoyl-feruloyl methane (peak 1)

Table 18: Peak areas of curcuminoid compounds from the extracts of the cultivated C. longa Linn rhizomes samples determined by HPLC

Concentration of phosphorus or potassium added, (ppm)	Peak areas*, cm^2			
	p-hydroxycinnamoyl feruloyl methane (peak 1)	bis-(p-hydroxycinnamoyl)-methane (peak 2)	curcumin (peak 3)	Total area
0 (control)	0.70	1.04	3.43	5.17
16 (p)	0.79	1.07	3.56	5.42
46 (p)	0.81	1.14	3.42	5.37
76 (p)	0.70	1.16	3.61	5.47
106 (p)	0.79	1.15	3.47	5.41
165 (K)	0.84	1.23	3.86	5.93
365 (K)	1.161	1.63	3.99	6.78
565 (K)	0.86	1.52	3.56	5.94
765 (K)	0.84	1.50	3.59	5.93

* Average of 3 determinations of each sample

(P) and (K) repressents phosphorus and potassium respectively

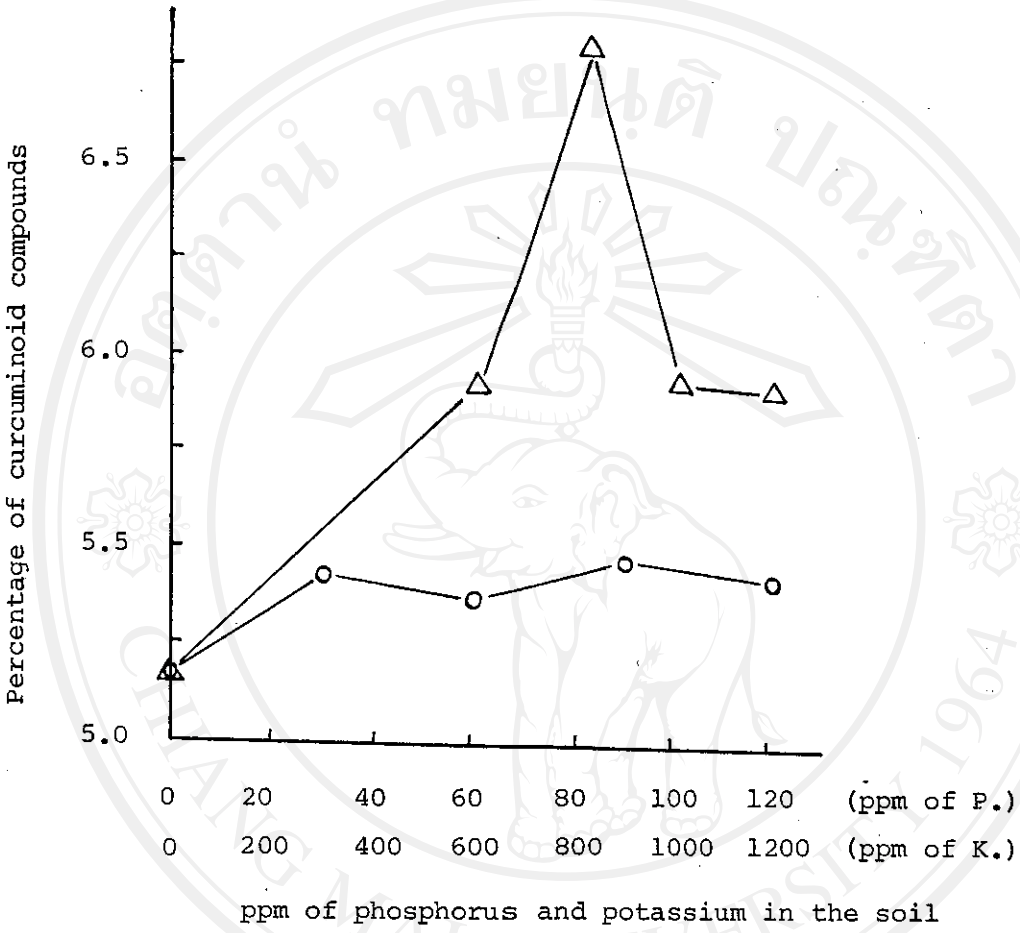


Figure 14: Effect of Potassium and phosphorus on the percentage of total curcuminoids produced.

O—O : effect of phosphorus

△—△ : effect of potassium

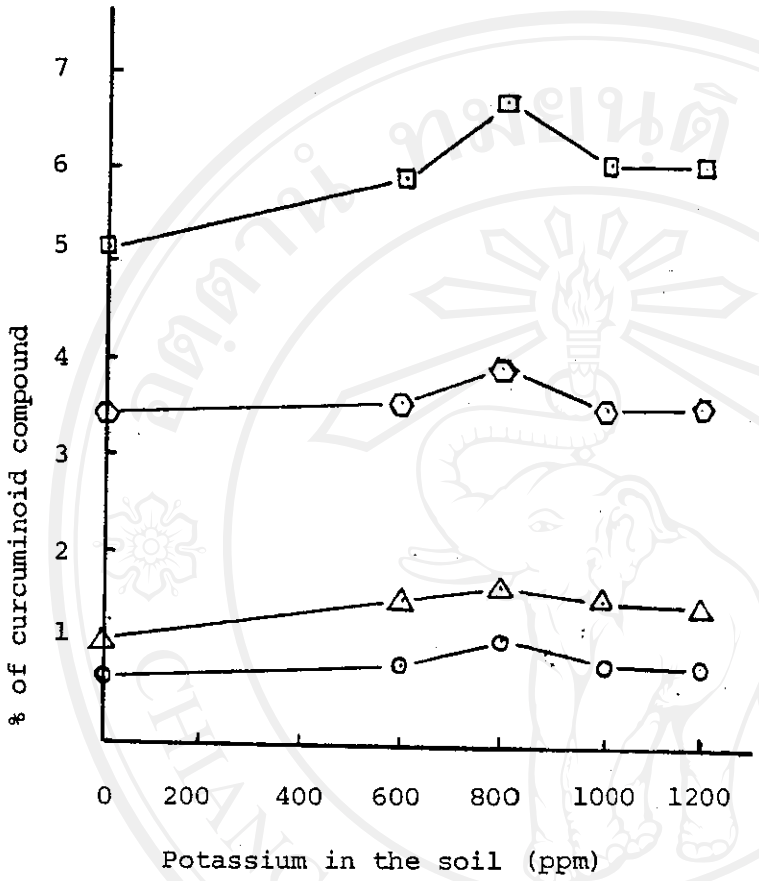


Figure 15: Effect of potassium on the percentage of curcuminoid compounds

o—o : p-hydroxycinnamoyl-feruloyl methane

△—△ : bis-(p-hydroxycinnamoyl)-methane

⬡—⬡ : curcumin

□—□ : total curcuminoid compounds

The method employed in the determination of the curcuminoid pigment content in C. longa Linn rhizomes was the High Performance Liquid Chromatographic method developed in the pharmacognosy research laboratory at ChiangMai University, based on the original work of Asakawa et al, 1981 in which the following conditions were described:

Column : 4.6 mm x 15 cm, packed with 5 μ Nucleosil C_{18}

Elution solvent: mixture of acetonitrile-water-acetic acid 51:49:5

Detector : UV-detector

Preliminary experiment for the separation of the three curcuminoid compounds in standard curcumin (Fluka) by reverse phase HPLC was carried out under the same conditions as those described by Asakawa et al, but the 10 μ Nucleosil C_{18} column was used instead of the 5 μ Nucleosil C_{18} column. Under such conditions poor resolution was obtained probably owing to the unsuitability of the mobile phase, flow rate and sample size used. Attempt at optimizing the experimental conditions namely the mobile phase, flow rate, the sensitivity and chart speed was made. Various proportions of the mobile phase, acetonitrile:water:acetic acid, were tried. Results were shown in Table 19 . It was seen that again poor resolution was obtained. In order to acheive the suitable mobile phase for the separation and/or determination of curcuminoid compounds a mixture containing acetonitrile, water and formic acid at various proportions were investigated. Results were shown in Table 19 . The

optimal proportion for acetonitrile:water:formic acid was found to be 45:75:2 V/V at the flow rate of 1.5 ml/min. Since it provided high resolution. The chromatogram was shown in Figure 16. , page 90

Spectrophotometric method was used extensively in the analysis of pigment in turmeric. The method was suitable for the determination of total pigment since every curcuminoid compound showed maximum absorption at the same wavelength, 425 nm. Spectrophotometric method was employed in the evaluation of turmeric in the markets, as shown in section 1.5, chapter II. The excellent method for the determination and/or separation of the three curcuminoid compounds was found to be HPLC under optimun conditions. Since it exhibited three well-resolved peaks in the chromatogram.

Turmeric has been used in the traditional medicine for quite a long time as an antiseptic. The volatile oil content in turmeric was responsible for the action. Not less than 20 components were reveled in the gas chromatogram of turmeric volatile oil. Among these turmerone and ar-turmerone were the major constituents, the latter occured from turmerone during extraction process. The work of Assistant Professor Dr.Sopon Raengsamran* of the Chemistry Department, Chulalongkorn University has been reported about the inhibition of curcuma oil against bacteria and fungus as follows:

Type of bacteria and fungus	Zone of inhibition of curcuma oil (cm)
E-Coli	0.9
Streptomyces aureus	1.3

* Personal communication

Bacillus subtilis	1.4
Shigella flexneri	1.4
Salmonella typhi	1.3
Candida albicans	1.2

He concluded that if the inhibition zone was broad, the curcuma oil would be very effective.

The site of the cultivation experiments was on the slope (20-25%) of a high hill (about 1500 meters above the average sea level) in Amphur Mae-Chaem. Although the land was previously a rice field and corn field, the composition of the soil was rich. Analysis of the soil before the experiments showed adequate organic matter content (2.76%) and high potassium content (435 ppm). Therefore, the turmeric rhizomes obtained from the control plot showed rather high quality:volatile oil content 6.19%, total active components in the oil 81.42%, total ether extractive 11.94% and total pigment content 5.17%.

However, the effects of potassium and phosphorus added to the soil in the experiments could be clearly seen (Figure 10). Addition of phosphorus to the soil increased the yield of the volatile oil content in the turmeric rhizomes up to a certain point (90 ppm, yield 8.68% compared to 6.19% of the control), then more phosphorus did not have an advantage. Potassium also had the same effect, although the increase was less than the effect of phosphorus (maximum effect on addition of 565 ppm potassium, yield 7.60% compared to 6.19% of the control).

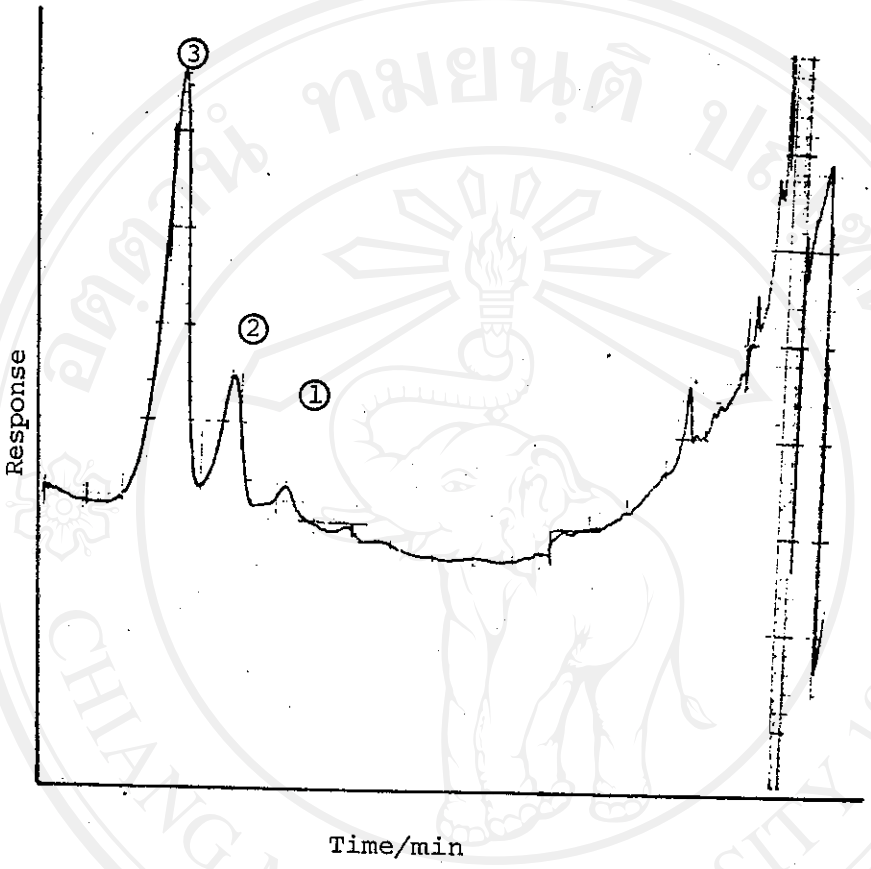


Figure 16: Investigation of HPLC separation of curcumin and minor curcuminoids

using acetonitrile:water:formic acid (45:72:2) as eluent

Concentration 125 ppm

Flow rate 1.5 ml/min

Detection by UV detector at 425 nm

① : demethoxycurcumin

② : bis-demethoxycurcumin

③ : curcumin

Table 19 Investigation of the optimal experimental conditions for determining curcuminoid compounds by HPLC

Mobile phase	Flow rate (ml/min)	t_x (min)			Peak height (cm)		
		Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
Acetonitril:water:acetic acid 40:60:5	2.0	1.22	1.38	1.50	0.70	2.50	9.50
45:70:7	1.6	1.50	1.62	1.82	0.50	2.90	10.80
45:70:7	2.0	1.28	1.40	1.52	0.70	3.10	11.70
35:70:7	1.6	3.24	3.64	4.06	0.20	0.96	3.40
35:70:7	2.0	2.76	3.06	3.42	0.25	1.10	4.00
35:65:5	1.6	2.26	2.58	2.82	0.25	1.30	5.00
35:65:5	2.0	2.96	3.22	3.60	0.20	1.10	4.10
40:72:5	2.0	2.50	3.96	4.44	0.30	0.70	4.80
Acetonitrile:water:formic acid 45:55:2	2.0	1.56	1.72	1.88	1.29	5.65	18.70
45:55:2	1.5	1.66	1.82	2.06	1.40	6.60	21.80
45:65:2	2.0	2.40	2.64	2.88	0.50	3.90	12.60
45:60:2	1.5	2.48	2.80	3.06	1.30	5.00	16.20
45:70:2	2.0	1.60	1.80	2.00	0.70	2.90	10.00
45:70:2	1.5	2.10	2.30	2.60	0.68	1.65	8.95
45:75:2	1.5	2.78	3.04	3.36	0.40	1.60	5.60
45:75:2	2.0	2.04	2.26	2.48	0.40	1.90	6.90
45:75:2.5	1.5	2.48	2.76	3.00	0.25	1.20	4.30
45:75:3	1.5	2.34	2.62	2.86	0.40	1.48	5.00
45:75:1.5	1.5	2.48	2.72	3.04	0.80	3.20	11.10

Peak 1 : p-hydroxycinnamoyl-feruloyl methane

Peak 2 : bis-(p-hydroxycinnamoyl)-methane

Peak 3 : Curcumin

Sample size used : 10 μl of standard curcumin solution, detection: UV at 254 nm

Although phosphorus at 90 ppm increased the yield of the volatile oil content 40.22%, the content of the active components (turmerone and ar-turmerone) did not accordingly increased. Phosphorus, therefore, was advantageous to the cultivation of turmeric only in the aspect of the yield of the volatile oil, but not the therapeutic quality of the oil.

Potassium, on the other hand, although causing increase of the yield of the volatile oil only 22.77% at 1,000 ppm, it did not increased the percentage of total active components.

Phosphorus and potassium also increased the pigment content of turmeric rhizomes. Potassium showed significant effect. Addition of 365 ppm of potassium caused 31.14% increase of total pigment from the control. (from 5.17% content in the control to 6.78%). Phosphorus had very slight effect, maximun increase was only 5.80% from the control. Figure 14 showed the sharp contrast of the effect of potassium and phosphorus to the total yield of the pigments.

Potassium caused the increase of bis-(p-hydroxycinnamoyl)-methane (peak 2) more than p-hydroxycinnamoyl-feruloyl methane (peak 1) and curcumin (peak 3) as shown in Table 16., page 80.

Therefore, potassium content in the soil was an important factor to produce the high-coloured, high quality turmeric for commerce.

The total ether extractive was an indicator of quality for the production of oleoresin from turmeric rhizomes. Oleoresin was

another form of products from turmeric in commerce. The results obtained in Table 14 (P. 79) showed that increase of potassium in the soil did not affect the total ether extractive yield, and phosphorus had insignificant effect. Addition of phosphorus 76 ppm to the soil, while it had important effect on the yield of the volatile oil and slight effect on the production of total pigments, it affected the total ether-extractive only insignificantly (increase of 2.51% from control)

The rhizomes from the control plot where the phosphorus content was 14 ppm showed the total ether-extractive of 11.94%, just about the same level of content as in the fertilizer experimenting plots. Therefore, addition of phosphorus and potassium caused a better quality rhizome, both the oil yield and the pigment yield, but not the quality of the volatile oil and the resins of the turmeric.

Turmeric from many provinces of Thailand showed better quality than the turmeric produced in the experiments on the high hill (Table 8 , page 43)

Turmeric rhizomes, as well as other plant rhizomes, need the soil rich in nitrogen. On the hills, the new clearings of the rather horizontal land should be the better sites for cultivation since the soil would be rich in organic matters and heavy rain would not wash out the nutrients from the soil surface as with the growing sites on the slopes of the mountain. Other elements in the soil, besides phosphorus and potassium, would have significant

effects on the quality of turmeric since samples of the rhizomes from various places showed the color ranging from yellow to deep orange brown (very high content of pigments) and from certain places the volatile oil content rised up to more than 15%. Research on the effects of minerals should be recommended to be conducted furthur.