Chapter 2

REVIEW OF LITERATURE

2.1 Mango in General

The mango (*Mangifera indica* Linn.), which is a dicotyledonous fruit of the family Anacardiaceae, originated in the Indo – Burmese region (Subramanyam *et al.*, 1975 ; Tjiptono *et al.*, 1984). It is produced principally in the developing countries of the tropics, with total world production estimated at 15.06 million tonnes (FAO, 1989).

Mango cultivars may be classified into two groups, Indian and Indo – Chinese, based mainly on peel pigments and sensory characteristics of the pulp. Most of the Indian varieties, which possess stronger aroma and more intense peel colouration, are monoembryonic and require asexual propagation methods for consistent reproduction of the cultivar. On the other hand, most of the cultivars grown in the South – east Asian region are largely polyembryonic (Kusumo *et al.*, 1984). Due to the contrasting flavours of these groups, populations accustomed to the taste of Indo – Chinese varieties perceive the Indian types as medicinal or possessing a 'turpentine' flavour.

Subramanyam *et al.* (1975) consider two other groups : first , mangoes of India , with character intermediate between the polyembryonic Indo – Chinese varieties and second, hybrids developed in Florida and Hawaii.

Trade in mango has been limited by the highly perishable nature of this fruit. Ripening cannot be delayed sufficiently to allow for long - distance transport. The fruit are also highly susceptible to disease, extremes of temperature and physical injury. Thus , most of the post – harvest technologies for these fruits are designed for disease control and protection against injury during packaging and transport. Technologies for longer – term storage , such as controlled or modified atmosphere ,which have been used commercially for temperate fruits have not been applied successfully to the mango. Despite the numerous studies on the physiology and biochemistry of this fruit , little is understood with repeat to the

fundamental processes relevant to the design of appropriate technologies to enhance marketable life.

India , which produces the largest volume, exports mango largely in the processed form as juice, puree, slices or pickles; in contrast, the Philippines and Mexico mainly export the fresh fruit.

2.2 Mango Anthracnose Fruit Rot in Thailand : State of the Art and Current Export Problem

The anthracnose disease of mango usually caused by the fungi *Colletotrichum gloeosporioides* (Penz.) Sacc. The fungus is classified in the class Deuteromycetes order Coelomycetes which has the telemorph stage as *Glomerella cingulata* (Stonem.) Spauld. (Sutton, 1980 ; Bailey and Jeqer, 1992). Colonies usually vary in colour from greyish white to dark grey, aerial mycelium even and felted or in turf associated with conidiomata, reverse unevenly white to gray or derker especially with age. Setate present or absent. Sclerotia absent but immature, ascomata may be mistaken for sclerotia. Appressoria clavate, ovate, obovate, sometime lobed, sepia brown, 6 – 20 x 4 – 12 µm/conidia formed in pale salmon mass, straight cylindrical, apex obtus , base truncate, 12 – 17 – 12 – 27 x 3.5 – 6 µm.

This specie is heterogenous and in culture, especially, the characteristics vary greatly. No progress in the systematic and identification of isolate belonging to this complex is likely to be made base on morphologic alone. (von Arx, 1957 , 1981 ; Mordue, 1971; Sutton, 1980 ; Baxter *et al.,* 1983 ; Baxter and van der Westhuizen, 1984 ; Holliday, 1980 , 1989) In Thailand, the average size of conidia and acervulus was 3.2 x13.4 and 19.5 – 41.2 µm. (Sukmark , 1977). Sporemasss were reproduced and outbreaked distribution by the mist or rainfall thunder. Parts of mango tree was damaged and shown symtoms at leaves,fluorescences. But immature fruit has not shown disease symptom. Because pimmature tissue of the young fruit has the fungal inhibit substance as 5 - substituted resosinol (Kobiler *et al* ., 1998) which induced the fungus formed inactive stage and called quiesent peroid that was suppressed in epidermis tissue which caused the latent infection (Bailey and Jerger, 1992 ; Danai , 1990). Disease symptoms were not appeared until the fruit grown to the ripening stage, because in this stage the inhibitant was decreased then fruit was damaged

in the next time (Prusky and Phembley, 1992) which is at the same time as harvesting peroid (Nipon, 1992).

2.3 Crude Extract from Thai Medicinal Plants to Control *Colletotrichum gloeospirioides* (Penz.) Sacc.

Studied on several ethanol extracts from Thai herbs (more than 90 varieties) to inhibit the growth of *Colletotrichum gloeosporioides* (Penz.) Sacc. by poison food technique showed that, the most effective was sweet flag (*Acorus calamus* L.) and the second was *Rhinacanthus nasutus* (Kurz) and galanga (*Alpinia galanga* Sw.) respectively. (Chareanpanit, 1990 and Korpraditsakul, 1990). These extracts were then tested for the ability to control anthracnose postharvest disease on the surface of mango fruits (Variety – NAM – DOK – MAI) and found out that the extract from *Rhinacanthus nasutus* (Kurz) could better control than the extracts from galanga and sweet flag respectively. (Korpraditsakul *et al.* 1991). Chaichantippyut (1994) reported the major active chemicals in sweet flag, which could inhibit the growth of microorganism were euginol, cineol, cadinene, pinene, methyl cinamate, sesquiterpene and dioxyflavonol. Nayudarna and Chadna (1972) and Phonglux (1987) reported that the extract form *Rhinacanthus nasutus* (Kurz) could also kill and inhibit the growth of fungi that caused of skin disease of man and house animal. *Microsporium gypseum*, *Trichophyton rubrum* , *Epidermophyton floecoseum* , *Candida albicans* , and active ingredient was oxymethyl anthrarquinone. Youngviset (1993) studied by extract stem and leaves of *R. nasutus* (Kurz). and recrystallized in ethanol found that major active ingradient was sigma sterol which has molecular weight of 402 deltons and the group of compound was naphthaquinone.

Srisornkompol (1996) and Lertvirasawat (1997) reported the test of extract from galanga by TLC - bioasssay with the fungal *Cladosporium cladosporioides* and purified the active part by preparative chromotography and indentified the structural elucidation by GC-MS spectroscopy method was confirmed as 1′ acetoxychavicol acetate. The study of Bhassabutra (1997) compared the ethanols extract from galanga, sweetflag and *Rhinacanthus nasutus* (Kurz). and found that extracts from sweet flag could be the best effectiveness to inhibit growth of *Collectotrichum gloeosporioides* (Penz.) Sacc. And the ED_{50} of the extract was 400 ppm.

She purified the extract by ethylacetate by column chromatography technique reported the efficiency to controll the anthracnose postharvest disease on the surface of mango's fruit (Variety NAM – DOK – MAI) and confirmed the same result as Benzimidazole fungicide.

2.4 Fruit Development and Havest Maturity

The final quality of the mango depends not only on the physiological processes occurring during ripening , but also on processes during fruit development and maturation.

Considerable effort has been put into identifying reliable indices of harvest maturity as this affects subsequent ripening rate , ripe fruit quality (Peacock *et al.*, 1986 ; Medlicott *et al.*, 1988), response to various postharvest treatments (Esguerra and Lizada , 1990) and processing quality (Kapur *et al.*,1985).

Mango fruit growth follows a simple sigmoid pattern (Akamine and Goo,1973 ; Lakshminarayana , 1973 ; Mendoza 1981 : Kasantikul , 1983 ; Tandon and Kalra , 1983; Lam *et al.*, 1982). The period of rapid growth is characterized by an increase in alcohol – insoluble solids, principally starch (Mendoza *et al.*, 1972 ; Tandon and Kalra , 1984) , the accumulation of which is accompanied by increases in amylase activity in 'Dashehari' (Tandon and Kalra , 1983) and 'Haden' varieties (Fuchs *et al.*, 1980). Mottoo and Kalra (1969) reported the presence of an amylase inhibitor in the unripe fruit, which might account for starch accumulation despite the increase in amylase activity during maturation. The increase in dry matter has been recommended for use as an index of maturity in 'Kensington Pride' (Baker, 1984) and might very well be the basis for the use of specific gravity as a maturity index in 'Alphonso' (Subramanyan *et al.*, 1976), 'Dashehari' (Kapur *et al.*, 1985) and 'Carabao' varieties (Cua and Lizada, 1990).

Titratable acidity correlated very well with days after flower induction in the 'Carabao' mango (Del Mundo *et al.*, 1984a), decreasing in the fully mature fruit to <44.8 meq.100 g^{-1}. In 'Alphonso' , titratable acidity increased from the sixth to the tenth week after fruit set and steadily declined thereafter as the fruit mature (Lakshminarayana, 1973).

Although endogenous ethylene and its induction of some ripening processes appear to be involved in the later stages of maturation in the mango, attempts to accelerate maturation and enhance uniformity of the process with ethephon sprays proved unsuccessful (Lertpruk,

1983; Del Mundo *et al.*,1984b). The failure to manipulate maturation with exogenous ethylene is consistent with the postulate that the outer tissues are more resistant to ethylene action (Cua and Lizada, 1990).

As the mango fruit matures, bloom develops as wax is deposited on the peel (Kosiyachinda *et al.*, 1984). Although bloom may diminish, depending on the handling the fruit undergoes, some of it persists through ripening. The cuticular layer in the fully mature 'Carabao' mango is well defined and its ultrastructure cannot be altered even by microscopy of peel sections revealed an apparent flattening of the outer layers of wax, which consequently assumed an amorphous appearance (Lizada and Kawada, unpublished). Within the smooth zones are recessed pockets revealing crystalline wax consisting of fringed platelets. As the samples were taken from fruits subjected to the usual packing operations for export, it might be assumed that the amorphous regions originated from previously crystalline platelets. The chemical nature of mango wax deposits, as well as changes during maturation, await characterization.

2.5 Ripening Processes

2.5.1 Ethylene production

A mango fruit approaching full maturity exhibits a significant decrease in starch and a distinct yellow colour in the pulp (Mendoza *et al.*, 1972 ; Medlicott *et al.*, 1988 ; Wang and Shiesh , 1990). In several varieties these changes are accompanied by a decline in pulp rupture force during the later stages of maturation (Cua and Lizada, 1990 ; Seymour *et al.*, 1990). These observations indicate that some changes associated with ripening appear to be induced prior to harvest maturity , and point to detachment.

'Carabao' mangoes were found to produce ethylene prior to full maturity (Cua and Lizada, 1990). The internal ethylene level reached a peak of 0.35 $\mu l.l^{-1}$ at 110 days after flower induction , beyond which in declined as the fruit approached full maturity ten days later. Measurement of ethylene production in the different portions of the fruit showed that the highest rate (1.25 $nlC_2H_2.g^{-1}.hr^{-1}$) occurred in the outer mesocarp , despite the observation that the highest ethylene – forming enzyme (EFE) activity could be measured in the peel (>20 nl.

$C_2H_4g^{-1}.h^{-1}$). The levels of the EFE substrate, 1 – aminocyclopropane -- 1 – carboxylic acid (ACC), were comparable in all portions of the fruit.

Despite the observation that the pre – harvest levels of ethylene in the 'Carabao' mango were higher than those in harvested fruits at colour break , no respiratory increase was induced by the former. Burg (1962) reported that mature but unripe mangoes had high ethylene levels ($1.87\mu l.l^{-1}$) , even while attached to the tree. He suggested that this ethylene is rendered ineffective by a ripening inhibitor from the parent plant. This idea of an inhibitor is consistent with the stimulation of respiration observed after detachment in fruit harvested at different stages during maturation (Lakshminarayana, 1973; Mendoza, 1981). Lakshminarayana further observed an earlier onset of the climacteric as the fruit matured beyond the ninth week after fruit set.

In the 'Carabao' mango , at least, there is apparently a differential effect of this postulated inhibitor on the various processes associated with maturation and/or ripening (e.g. climateric respiration and carotenogenesis) . There appear to be differences also in the degree of inhibition between the inner and outer portions of the mesocarp ,such that the former appears to ripen further despite comparable levels of ethylene in the entire mesocarp (Cua and Lizada, 1990).

Hardly any ethylene can be detected in the fully mature 'Carabao' mango, but ethylene production resumes as the fruit approaches colour break (Lizada and Cua, 1990). As with the ethylene production associated with maturation , postharvest ethylene production is accompanied by an increase in both ACC synthase and the ethylene – forming enzyme (EFE).

In most tissues examined, the K_m for ACC synthase is in the micromolar range (Yu *et al.*,1979 ; Hyodo *et al.*, 1985) , while that for EFE exhibits a range of values.

2.5.2 Pigments

Peel colour is an important criterion of acceptability of the mango (Satyan *et al.*, 1986). During ripening the peel colour gradually changes from green to orange / yellow. Some

cultivars develop a reddish blush which has been attributed to anthocyanins, while others , e.g. 'Harumanis' and 'Katchamita', retain most of the green colour , even at the full ripe stage.

In 'Tommy Atkins', Medlicott *et al.* (1986) observed a rapid destruction of chlorophyll , with chlorophyll a preferentially degraded relative to chlorophyll b. A more rapid loss in chlorophyll a is typically observed in senescence (Simpson *et al.*, 1976). Medlicott *et al.* (1986) also reported differing patterns of change in carotenoids and anthocyanins, with the former increasing during ripenting. In contrast, the anthocyanin levels gradually declined, indication that more unmaking account for the increased prominence of blush in some cultivars.

Peel colour development is accompanied by ultrasturctural changes associated with chloroplast – to – chromoplast transition. The thylakoid membrane systems in the peel of 'Aphonso' and 'Tommy Atkins' gradually break down, while osmiophilic globules enlarge and increase in number (Medlicott *et al.*,1986 ; Parikh *et al.*,1990). The loss of granal membrane integrity is associated with chlorophyll degradation, while the appearance of osmiophilic globules accompanies increases in carotenoid levels.

The principal carotenoids reported in ripe 'Alphonso' mango peel were β - carotene, xanthophyll esters and xanthophylls. β - carotene and auroxanthin were found to constitute 55% and 12.5% , repectively , of the peel carotenoids in 'Tommy Atkins' (Medlicott , 1985). Thus far , the only anthocyanin pigment identified in the peel is peonidin–3–galactoside, which was extracted by Proctor and Creasy (1996) from 'Haden'.

Pulp carotenoids continue to increase in the detached fruit as ripening proceeds (Chaudhary, 1950 ; John *et al.*, 1970) , with the carotenoid level in the ripe fruit varying among cultivars. (Table 2.1). In the fully ripe 'Badami' and 'Alphonso' mangoes , β - carotene constituted more than 50 % of the total carotenoids, with phytofluene the next most abundant (Jungalwala and Cama, 1963 ; John *et al.*, 1970). In 'Tommy Atkins' , β - carotene also constituted about two – thirds of the pulp carotenoids (Medlicott, 1985). The predominant xanthophyll in this variety was found to be violoxanthin.

The synthesis of carotenoids in the mango appears to proceed *via* the same biosynthetic pathway established in other species. In a series of studies Modi *et al.* (1965) and Matto *et al.* (1968) presented evidence for the role of mevalonic acid and geraniol in

mango carotenogenesis. As in the peel , the synthesis of carotenoids in the pulp is accompanied by changes in the ultrastructure of plastids. As ripening proceeds, tubularstructures visible in the plastid of unripe fruit are lost, while osmiophilic globules increase in size and number in the 'Alphonso' mango (Parikh *et al.*, 1990).

Table 2.1 Carotenoid content in the ripe pulp of some mango cultivars.

Cultivar	Carotenoids ($mg.100g^{-1}$ Fresh weight)	References
Carabao	2.75	Morga *et al.*, 1979
Nam-Dok-Mai	4.78	Kasantikul , 1983
Badami	8.92	John *et al.*, 1970
Dashehari	5.44	Mann and Singh,1976
Alphonso	4.76	Ramana *et al.*, 1984
	8.06	Subramanyam *et al.*,1976
Haden	6.82	Vazquez-Salinas and Lakshminarayana , 1986
Irwin	3.23	Vazquez-Salinas and Lakshminarayana , 1986
Kent	5.46	Vazquez-Salinas and Lakshminarayana , 1986
Keitt	3.87	Medlicott , 1985
Tommy Atkins	>5	Medlicott *et al.*, 1986
Kensington	5.06	Mitchell *et al.*, 1990

Succinic acid 2 , 2 – dimethylhydrazide , which has been used to enhance peel colour in other fruits, had no distinct effect on the pulp carotenoids of 'Alphonso' mango (Subramanyam and Sebastian,1970). However , a 5 – minute dip in water at 53°C resulted in a slight (7%) increase in total carotenoids. Increases in pulp carotenoids as a result of elevated temperatures have also been reported in 'Tommy Atkins' by Medlicott *et al.*, (1985). Subjecting 'Carabao' mangoes to vapour heat treatment or a 10 – minuted hot water dip at 52 – 55°C enhances peel colour intensity and results in a more complete disappearance of the green colour of the peel (Lizada *et al.*, 1986 ; Esguerra and Lizada , 1990).

2.5.3 Carbohydrate metabolism

The starch that has accumulated in the maturing fruit is rapidly lost during ripening (Selvaraj *et al.*, 1989 ; Morga *et al.*, 1979 ; Subramanyam *et al.*, 1976) , and this loss is evident in the chloroplast where the starch granules become progressively smaller as ripening proceeds. Starch granules completely disappear in the ripe fruit (Parikh *et al.*, 1990; Medlicott *et al.*, 1986) , which usually contains negligible levels of starch (Morga *et al.*, 1979 ; Fuchs *et al.*, 1980).

Starch hydrolysis in the ripening mango has been associated with amylase activity (Fuchs *et al.*, 1980) , which exhibits the properties of both α- and β- amylases. The complete disappearance of starch may be attributed to an upsurge of amylase as ripening is completed. Fuchs *et al.*(1980) also reported the presence of a proteinaceous inhibitor detected during the electrophoresis of amylase , which they suggested might be the inhibitor earlier observed by Mattoo and Modi (1969 , 1970; as cited by Subramanyam *et al.*, 1975).

As a consequence of starch hydroysis, total sugars increase during ripening , with glucose , fructose and sucrose constituting most of the monosaccharides (Selvaraj *et al.*, 1989). The total sugar content of the ripe 'Carabao' mango is one of the highest reported, with values exceeding 20 % (Peacock and Brown , 1984).However , the lower sugar contents reported for other varieties such as 'Golek' (Lam *et al.*, 1982) might simply reflect differences in the degree of ripeness when optimum eating quality is attained.

Sugars constitute 91% of the soluble solids from the mesocarp of the ripe 'Ngowe' mango (Brinson *et al.*, 1988). No – reducing sugars , principally sucrose, increase in the later stages of ripening (Selvaraj *et al.*, 1989; Subramanyam *et al.*,1976; Shashirekha and Patwardhan, 1976 ; Fuchs *et al.*, 1980). This is consistent with the high activity of the gluconeogenic enzyme fructose – 1 , 6 diphosphatase in the ripe fruit as reported by Rao and Modi (1976) and Kumar and Selvaraj (1990) in several mango cultivars.

In most of the varieties examined fructose was the predominant reducing sugar. Along with an observation that pentoses exhibited a five–fold increase during ripening, this was considered as suggestive of an increase in the oxidative pentose phosphate pathway , which generates the necessary reducing equivalents (NADPH) for biosynthetic processes. Increases were also reported in glucose–6–phosphate dehydrogenase and 6 – phosphogluconate dehydrogenase .

Mango ripening is accompanied by increases in gluconeogenic enzymes. In several Indian varieties , glucose – 6 – phosphatase was observed to increase up to the three – quarter – ripe stage , while fructose – 1 , 6 – diphosphatase showed increased activity as the fruits ripened from the three–quarter to the full ripe stage (Kumar and Selvaraj , 1990). In abscisic acid – treated mango , enhanced ripening as similarly accompanied by increases in these enzymes (Parikh *et al.*, 1990). These increases could be inhibited by treatment with cycloheximide , indicating *de novo* synthesis of these enzymes during ripening.

2.5.4 Structural polysaccharides and textural changes

Pronounced softening during ripening limits the marketable life of the mango. Softening is accompanied by cell wall disruption (Parikh *et al.*, 1990), with the middle lamella appearing as an electron – translucent area in electron micrographs of the ripe fruit (Medlicott, 1985).

In most of the varieties examined an increase in water- soluble polysaccharides has been observed during ripening (Tandon and Kalra , 1984 ; Lazan *et al.*,1986 ; Brinson *et al.*, 1988). In contrast, Roe and Bruemmer (1981) reported a decline in water soluble

polysaccharides in ripening 'Keitt' mangoes, which they attributed to the possibility of extensive polymer degradation such that the products become soluble in ethanol.

Brinson *et al.* (1988) examined cell wall constituents of 'Ngowe' mango and reported a decline in uronic acid content as a percentage weight of the whole wall, i.e. from 25 % in the unripe to 19.1 % in the ripe fruit. In contrast, the uronic acid content of the water soluble polysaccharides from these cell wall preparations increased from only 7 % in the unripe to 90 % in the ripe fruit. Moreover, galactose and arabinose each constituted about 30 % of the water – soluble polysaccharides of the cell walls of the unripe fruit. A higher uronic acid content of the water soluble polysaccharides was observed in the ripe compared to the unripe mesocarp. These observations were interpreted to indicate that during ripening.

1.) Mango cell walls are degraded, releasing the combined monosaccharides of the pectin complex;

2.) The resulting water–soluble pectic materials in the cell walls lose arabinose and galactose accounting for the galacturonan-rich polysaccharide in the mesocarp.

In a more recent study (Tucker and Seymour, 1991), using precautions to prevent cell wall degrading activity-during wall preparation and fractionation, less extensive breakdown of mango pectic polymers was reported than observed by Brinson *et al.* (1988). However, there was still a substantial in soluble pectin during ripening and a marked loss of galactose residues.

Most determinations of polygalacturonase (PG) in the ripening mango have shown low activities as measured by the reductometric method. Lazanetal (1986) reported that viscometric assays gave very low activity, although the pattern of change correlated well with that observed using the reductometric method. Brinson *et al.* (1988) failed to detect endoPG activity, while Medlicott (1985) reported very little loss in viscosity in his assays for PG. Thus, although it appears that an exoPG might be involved in cell wall degradation, the low activities measured make it difficult to account for the rather pronounced softening that the mango undergoes during ripening. It must be pointed out that all these assays use

polygalacturonic acid as substrate; use of pectic materials isolated from the mango cell wall might give different results.

In 'Carabao' mango, considerable pectinmethylesterase (PME) activity could be measured during ripening, increasing as the fruit approaches the half – ripe (50 % yellow peel colour) stage and declining thereafter. A similar pattern was observed in some of the varieties examined by Selvaraj and Kumar (1989).

The inner mesocarp of the 'Carabao' mango exhibits softening ahead of the outer mesocarp (Cua and Lizada, 1989). The difference in the degree of softening between portions of the mesocarp is evident even in the ripe fruit, and thiers difference appears to be variety – dependent (Chaplin *et al.*, 1990). The jelly seed disorder (Van Lelyveld and Smith, 1979) and premature ripening around the seed (Winston, 1984) might be extreme examples of such differences.

2.5.5 Organic acids

Titratable acidity declines as the mango ripens , dropping from 46 mwq. 100 g^{-1} in the preclimacteric to 5.6 meq. 100g^{-1} in the postclimacteric 'Badami' mango (Shashirekha and Patwardhan, 1976). Similar patterns have been reported for other varieties (Morga *et al.*, 1979 ; Medlicott and Thompson, 1985 ; Selvaraj *et al.*,1989). The predominant acid is citrate, with malate and succinate also found in significant quantities (Shashiekha and Patwadhan, 1976). Although both citrate and succinate consistently decline in all varieties examined, malate exhibits different patterns of changes in different cultivars. In a study of mitochondrial enzymes of the tricarboxylic acid cycle. Baqui *et al.*(1974) reported that citrate synthase decreases during ripening , while isocitrate dehydrogenase and succinate dehydrogenase each increase. These changes are consistent with the observed decline in citrate and succinate.

Isocitrate lyase has been reported to decline rapidly at the early stages of ripening, with a concomitant decrease in glyoxylate , which was found to inhibit this activity (Baqui *et al.*, 1977).

Malic enzyme has been purified from mango pulp (Krishnamurthy and Patwardhan, 1971). The changes in its activity during ripening paralleled those of respiration during the climacteric. This has been reported in 'Haden' (Dubery *et al.*, 1984), 'Dadomia' (Baqui *et al.*, 1977) and 'Pairi' (Krishnamurthy *et al.*, 1971). In 'Alphonso', cytosolic malate dehydrogenase increased during ripening (Parikh *et al.*, 1990).

An increase in citrate cleavage enzyme during ripening has also been reported (Mattoo and Modi , 1970). This activity could be stimulated by a crude fatty acid extract from mango. Mattoo and Modi further suggested that, along with the malic enzyme and malate dehydrogenase , the citrate cleavage enzyme provides the reducing equivalents for synthesis. Its products , acetyl CoA and oxaloacetate , might also be utilized for synthetic processes during ripening.

In 'Pairi' mango , oxaloacetate and α - ketoglutarate reached a maximum prior to the climacteric rise, progressively declining to very low levels (Krishnamurthy *et al.*, 1971). In contrast , aspartate and glutamate exhibited a minumum immediately prior to the climacteric peak, and , although the levels increased slightly at the postclimacteric stage , they were only about 50 % and 39 % , respectively, of the maximum preclimacteric aspartate and glutamate levels. In 'Badami', aspartate and glutamate, in addition to arginine and lysine levels, are higher at the preclimacteric stage than at either the climacteric or postclimacteric stage (Shashirekha and Patwardhan, 1976). These patterns might be related to the requirement for protein synthesis during ripening (Parikh *et al.*,1990).

2.4.6 Lipid metabolism

The total lipid increased in the ripening 'Alphonso' mango ,as evidenced by the increase in ether – extractable components of the pulp (Bandyopadhyay and Gholap, 1973 a). Similar patterns were also obtained from the other Indian cultivars that Selvaraj *et al.* (1989) examined. The glyceride content in 'Alphonso' also increased , while the fatty acid profile

changed through ripening. The more unsaturated fatty acids, i.e. palmitoleate and linolenate were found in higher levels in the ripe relative to unripe fruits. These changes were found to correlate with aroma and flavour. In a later study, Bandyopadhyay and Gholap (1973b) reported that the cultivars with stronger aroma and flavour had lower palmitate / palmitoleate ratios.

Applying [12-^{14}C]acetate to 'Alphonso' mangoes, Gholap and Bandyopadhayay (1980) found that acetate was maximally incorporated into saturated fatty acids , while the radioactivity in [1-^{14}C] palmitic acid was incorporated largely in hydroxy fatty acids. Hydroxy fatty acids are precursors of lactones , which are major aroma constituents in 'Alphonso' mangoes (Engel and Tressl , 1983; Idstein and Schries, 1985).

Mitochondria from unripe 'Dadomia' fruits oxidized fatty acids and this was stimulated by glyoxylate (Baqui *et al.*, 1977). The capacity of the mitochondria to oxidize fatty acids increased in the preclimacteric and climacteric fruits. When used as substrates stearic and oleic acids elicited the highest increases. The products of the β - oxidation of fatty acids are utilized in the synthesis of both carotenoids and terpenoid volatiles.

Selvaraj (1989) examined some enzymes involve in the production of carbonyl volatiles and reported the presence of lipase, lipoxygenase and alcohol dehydrogenase (aldehyde forming) activities in several Indian cultivars. All of these activities were high at the unripe stage , and declined when the fruits were beyond the half – ripe stage.

2.5.7 Volatile constituents

One can distinguish between cultivars of mango on the basis of flavour and aroma. The volatile constituents of the 'Alphonso', which is considered to have a very strong aroma among the Indian cultivars (Selvaraj, 1989), have been examined by two laboratories (Engel and Tressl, 1983 ; Idstein and Schrier, 1985) and both indentified (Z) – ocimene as a major component. However, the two analyses differed in the approximate concentrations of β - myrcene and (E) – ocimene.

According to Engel and Tressl (1983) characteristic mango flavour could not be attributed to any single component; however, the typical green aroma of the unripe mango

might be attributed to cis – ocimene and β - myrcene (Gholap and Bandyopadhyay, 1975), while dimethylstyrene has been described as having mango character (MacLeod and Pieris, 1984).

Distinct varietal differences can be attributed to volatile components unique to each variety. In a comparison of 'Alphonso' with 'Baladi' Macleod and Pieris identified (Z) – 3 – hexenyl esters as being responsible for the fresh , green fruity note of the former but these cannot be detected in the latter. The C_9-lipid oxidation product, (E) – 2 – nonenal , could only be detected in 'Baladi' and might be responsible for the melon – like flavour of this cultivar. Other notable differences include :

1) the level of limonene, which was 40μ $l.l^{-1}$ Baladi , in contrast to 300 $nl.l^{-1}$ in 'Alphonso' ;
2) the presence of the hydrocarbons α - guriunene, germacrene D, bicyclogermacrene, τ-and δ - cadinene and α-selinene and the corresponding oxygenated sesquiterpenoids in 'Baladi', but not in 'Alphonso';
3) the relatively high levels of the ethyl esters of even – numbered fatty acids from C_2 to C_{16} in 'Baladi' ;
4) the relatively high levels of C_6 aldehydes and alcohols in 'Alphonso'.

2.5.8 Phenolics

Mango latex exudes from the freshly harvested fruit , but this exudate decreases during ripening. The major component of latex in freshly harvested 'Alphonso' mango was found to be 5 – [2(Z) – heptadecenyl] resorcinol ,which is structurally similar to known phenolic allergens found in species of the Anacardiaceae family (Bandyopadhyay *et al.*, 1985). Alkenylresorcinols have also been detected in the peel and flesh of freshly harvested mangoes and appear to be the basis for resitance to fungal pathogens (Prusky, 1990).

Astringency remains perceptible in the 'Carabao' mango until the table ripe stage is attained, and the progressive loss of astringency is associated with a loss in total phenolic content (Tirtosoekotjo , 1984). On the other hand, the 'Pico', which, like the 'Carabao' mango, contains > 0.1 % total phenolics and is non – astringent at the ripe stage, shows no significant decrease in phenolic content during ripening. Selvaraj and Kumar (1989) reported differences in tannin content among Indian cultivars, although all showed declining levels in the course of ripening. Gallotannins, which have been detected in the peel and pulp of the mango fruit, were also observed to decrease to negligible levels in the ripe fruit.

Polyphenoloxidase (PPO) showed an increase in the course of ripening in 'Malgoa' and 'Harumanis' (Lazan *et al.*,1986), and the increase occurred concomitantly with the decline in ascorbic acid levels. In the freshly harvested fruit , the activity measured was $< 0.1\ A_{420nm}$ $min^{-1}. g^{-1}$ fresh weight , using catechol as substrate. In contrast , the PPO activity, measured by using pyrogallol as substrate , generally declined in the course of ripening in several Indian varieties including the 'Alphonso' (Selvaraj and Kumar , 1989).

The properties of PPO isolated from ripe 'Haden' mangoes have been characterized (Park *et al.*, 1981). The enzyme was found to possess no monophenol oxidase activity and was specific for o – diphenolic substrates, showing the highest activity in the presence of catechol as substrate. It exhibited a pH optimum range of 5.6 – 6.0 , and was most effectively inhibited by sulphite. As with PPO isolated from other fruits, some inhibition could be effected by ascorbic acid. Two isoenzymes acting on catechol were detected.

Earlier, Joel *et al.* (1978) reported the presence of two PPOs in the green mango fruit, one possessing typical o – diphenol oxidase activity, and the other, m – phenol oxidase (laccase) activity. The latter was found only in the secretory duct cavities, and could, therefore, be detected also in the exudates from the cut pedicel of the freshly harvested fruit. Which pointed out that the presence of laccase appears to be characteristic of species belonging to the Anacardiaceae family.

2.6 Antifungal Compounds in Plants

Two major questions that can be asked about the establishment of fungal growth of the common nonpathogenic fungi in plants tissue. What prevents the growth of pathogenic fungi in resistant cultivars of host plants. These are clearly complex problems, and no single answer will be obtained to either of them, but antifungal secondary metabolites of plants are hypothesized to play a role. In this section which consider some of the diverse secondary metabolic of plants that have antifungal activity. No commercial development of plant products as fungicides has been made; interest in these compounds stems primarily from studies on plant resistance to fungal attack. There is great interest in the possibility that biotechnology may greatly expand the usefulness of this approach to plant disease control (Griffin, 1994).

2.6.1 Inhibitins and lectins

These fungal toxins, discussed by Griffin (1993), antifungal plant metabolites are products of secondary metabolism and are mostly related to three pathways of biosynthesis : the mevalonic acid pathway for terpenoid biosynthesis and the two pathways involved in the biosynthesis of phenolics, the shikimic acid and the acetate malonate pathways. Some representative compounds are shown in Figure 2.1 Pinosylvin , thugapliclin , and mansonone have been isolated from decay – resistant heartwood trees. Protocatechuic acid and cinnamic acid (also implicated as endogenous spore germination inhibitors) and the chlorogenic acids derived from cinnamic acid) are found in many plant species. Derivertives of benzoxazolinone are found in several agriculturally important grasses. They usually exist as nontoxicglycosides that are converted to their active forms by the action of fungal glucosidase. All of the above compound are constitutive, do not increase during infection, and can be classed as inhibitins.

Plant lectins are another group of interesting plant materials that have potential significance to host - parasite interaction (Callow, 1977 ; Brambl and Gade, 1985). Lectin are proteins or glycoproteins that bind carbohydrates with great specificity. They may include enzymes involved in carbohydrate metabolism (for example lysozyme) but others, such as concanavalin A, have no measurable enzyme activity. Lectins are often detected by their ability to agglutinate red blood cells, and they have several interesting effects on animal cells. They are refered to as phytohemaglutinins because of their red cell agglutinative activity ; some authors would like to limit the term lectin to this usage. However , as Callow (1977) point out, this is clearly not the normal function of lectins and an understanding of their normal function is necessary.

Fungi also contain lectins – the yeast agglutination factors, for example. This illustrate the possible roles of these materials in specific cell recognition processes; and it is possible that lectins are involved in host – parasite interactions by inhibiting the growth of incompatible fungi. Lectins from several different plant seeds were found to affect germ – tube growth and development of several fungi (Brambl and Gade, 1985; Mirelmon *et al.*, 1975). Inhibition , stimulation , and no effect were observed as responses that were highly depend on the fungus – lectin combination. Of 11 different lectin sources , only one (pock weed) inhibited Neurospora, five inhibited *Botryodiplodia theobromae* , and only are inhibited more than one of the three fungi tested (Brambl and Gade , 1985). Interestingly , growth stimulation was observed in several instances , but this effect did not necessarily accompany growth inhibition of surviving hyphae. The specificity of lectin binding to fungal spores was demonstrated with differential agglutination reactions of *Fusarium roseum* and *F. solani* spores by concanavalin A, wheat germ agglutinin, and ricin under various conditions (Kleinschuster and Baker „ 1974). These experiments suggest that lectins may play some role in plant resistance to fungal attack.

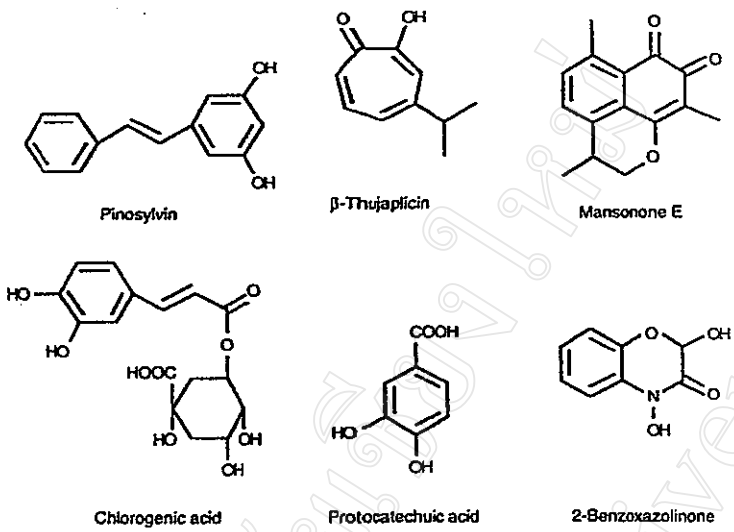


Figure 2.1 Some inhibitins from plant.

2.6.2 Phytoalexins

Phytoalexins are antimicrobial plant metabolite that appear or increase in plants in response to microbial infection. They were first postulated by Muller in 1940 from studies on the infection of potato tuber by *Phytophthora infestans*. Rishitin (Figure 2.2) and several other terpenoids have been obtained from potato and other members of the solanaceae after infection with *Phytophthora infestans*. Inoculation with an incompatible strain of the fungus conferred resistance to the later introduction of a compatible strain. An incompatible strain is from a race of the fungus that will not parasitize the particular host cultivator used. Phytoalexins are usually distinguished from inhibitions by their induction upon infection of the host. Inhibitions are constitutive compounds present in uninfected tissues at constitutives effective in the inhibition of spore germination or growth. While this terms have been defined in other, more restrictive fashions, and several other terms have most commonly accepted aspects. The induction of phytoalexin by plants, including application of heavy metal ions, antimetabolites, and other inhibitors. Bruising and freezing may induce them and

presumable so will fungal toxins involved in necrosis. However, fungal metabolites, called elicitors, that induce the formation of the phytoalexins have been discovered (Deverall, 1976).

The roles that these various materials play in host resistance to fungal attack is still a matter of speculation. Work on the identification of antifungal metabolites and their roles in host parasite relations has been reviewed (Callow, 1977 ; Deverall, 1976 ; van Etten and Puepke, 1976)

One of the largest families of antifungal compounds are the pterocarpan isoflavonoids, of which phaseollin is an example. Found only in the Leguminosae , they are derived partly from the acetate malonate pathway and partly from the shikimic acid pathway. Pisatin, kievetone, and coumesterol are other isoflavonoids isolated from legumes. These are only a few of many antifungal compounds of plant origin that has been identified ; numerous phenolic and quinone compounds have been described that have antifungal properties , but few have been studied in detail and little is known about their role in plant disease resistance (Fawcett and Spencer , 1969).

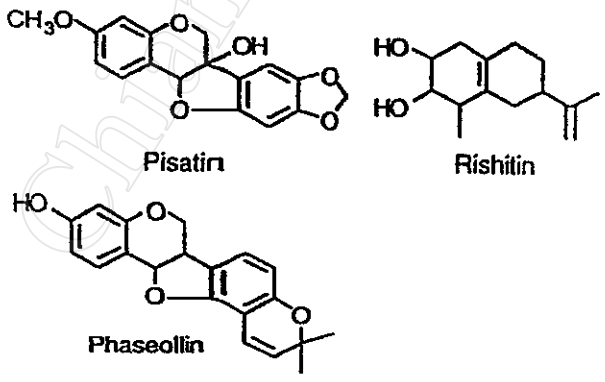


Figure 2.2 Representative phytoallexins.

2.7 Method of Plant Analysis

2.7.1 Method of extraction, isolation and identification

1) The plant material

Ideally, fresh plant tissues should be used for phytochemical analysis and the material should be plunged into boiling alcohol within minutes of its collection. Sometimes, the plant under study is not at hand and material may have to be supplied by a collector living in another commitment. In such cases, freshly picked tissue, stored dry in a plastic bag, will usually remain in good condition for analysis during the several days required for transport by airmail.

Alternatively, plants may be dried before extraction. If this is done, it is essential that the drying operation is carried out under controlled condition to avoid too many chemical changes occurring. It should be dried as quickly as possible, without using high temperatures, preferably in a good air draft. Once thoroughly dried, plants can be stored before analysis for long periods of time. Indeed, analyses for flavonoids, alkaloids, quinones and terpenoids have been successfully carried out on herbarium plant tissue dating back many years.

One example of the use of herbarium material is the essential oil analysis that was carried out on type specimens of *Mentha* leaf, the material being obtained from the original collection of Linneaus made before 1800 (Harlay and Bell, 1967). Quantitative chances in essential oil content may occur in both leaf and fruit tissue with time and this possibility must be taken into account. For example, Sanford and Heinz (1971) found that the myristicin content of nutmeg, *Myristica fragrans* fruits increased slowly on storage, while the more volatile β-pinene content decreased with time. On the other hand, a leaf sample of *Strychnos nuxvomica* originally collected in 1675 still contained 1 – 2% by weight of alkaloid (Phillipson, 1982).

Some subclasses of polyphenol are best extracted under more controlled conditions than those indicated above. For example, the yield of condensed tannin from willow leaves is greatest when measurements are made on fresh leaves that have been vacuum-dried rather than air-dried.

By contrast, the simple phenolic glycosides in the same plants are best extracted after simple air-drying (Orians, 1995).

The freeing of the plant tissue under study from contamination with other plants is on obvious point to watch for at this stage. It is essential, for example, to employ plants, which are free from disease, i.e. which are not affected by viral, bacterial or fungal infection. Not only may products of microbial synthesis be detected in such plants, but also infection may seriously alter plant metabolism and unexpected products could be formed, possibly in large amounts.

Contamination may also occur when collecting lower plant material for analysis. When fungi growing parasitically on trees are collected, it is important to remove all tree tissue from the samples. Earlier reports (Paris *et.al.*,1960) of chlorogenic acid, a typical higher plant product, in two fungi are almost carefully cleaned material incorrect because of contamination; repeat analyses on carefully cleaned material showed no evidence of this compound being present (Harborne and Hora, unpublished results). Again, mosses often grow in close association with higher plants and it is sometimes difficult to obtain them free from such litter. Finally, in error. Two closely similar grass species growing side by side in the field may be incorrectly assumed to be the same, or a plant may be collected without the realization that it has a parasite (such as the dodder, *Cuscuta epithymum*) intertwined with it.

In phytochemical analysis, an acknowledged authority must authenticate the botanical identity of the plants studied at some stage in the investigation. So many mistakes over plant identity have occurred in the past that it is essential to authenticate the material whenever reporting new substances from pants or even knows substances from new plant sources. The identity of the material should either be beyond question (e.g. a common species collected in the expected habitat by a field botanist) or it should be possible for the identity to be established by a taxonomic expert. For these reasons, it is now common practice in phytochemical research to deposit a voucher specimen of a plant examined in a recognized herbarium, so that future reference can be made to the plant studied if this becomes necessary.

2) Extraction

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated. In general,

it is desirable to 'kill' the plant tissue, i.e. prevent enzymic oxidation or hydrolysis occurring, and plunging fresh leaf or flower tissue, suitably cut up where necessary, into boiling ethanol is a good way of achieving this end. Alcohol, in any case, is a good all-purpose solvent for preliminary extraction. Subsequently, the material can be macerated in a blender filtered but this is only really necessary if exhaustive extraction is being attempted. When isolating substances from green tissue, the success of the extraction with alcohol is directly related to the extent that chlorophyll is removed into the solvent and when the tissue debris, on repeated extraction, is completely free of green colour, it can be assumed that all the low molecular weight compounds have been extracted.

The classical chemical procedure for obtaining organic constituents from dried plant tissue (heartwood, dried seeds, root, leaf) is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents, starting in turn with ether, petroleum, and chlorofrom (to separate lipids and terpenoids) and then using alcohol, and ethyl acetate (for more polar compounds). This method is useful when working on the gram scale. However, one rarely achieves complete separation of constituents and the same compounds may be recovered (in varying proportions) in several fractions.

The extract obtained is clarifide by filtration through celite on a water pump and is then concentrated *in vacuo*. This is now usually carried out in a rotary evaporator, which will concentrate bulky solutions down to small volumes, without bumping, at temperatures between 30 and 40°c. Extraction of volatile components from plants needs special precautions.

There are short cuts in extraction procedures, which one learns with pratice. For example, when isolating water-soluble components from leaf tissue, the lipids should strictly speaking be removed at an early stage, before concentration, by washing the exract is concentrated on a rotatory evaporator, almost all the chlorophyll and lipid is deposited on the side of the flask, and, with skill, the concentration can be taken just to the right point when the aqueous concentrate can be pipetted off, almost completely free of lipid impurities.

The concentrated extract may deposit crystals on standing. If so, these should be collected by filtration and their homogeneity tested for by chromatography in several solvents.

If a single substance is present, the crystals can be purified by recrystallization and then the material is available for further analysis. In most case, mixtures of substances will be present in the crystals and it will then be necessary to redissolve them in a suitable solvent and separate the constituents by chromatography. Many compounds also remain in the mother liquor and these will also be subjected to chromatographic fractionation. As a standard precaution against loss of material, concentrated extracts should be stored in the refrigerator and a trace of toluene added to prevent fungal growth.

When investigating the complete phytochemical profile of a given plant species, fractionation of a crude extract is desirable in order to separate the main classe of constituent from each other, prior to chromatographic analysis. One procedure based on varying polarity that might be employed on an alkaloid-containing plant is indicated in Figure 2.3. The amounts and type of compounds separated into different fractions will, of course, vary from plant to plant. Also, such a procedure may have to be modified when labile substances are under investigation.

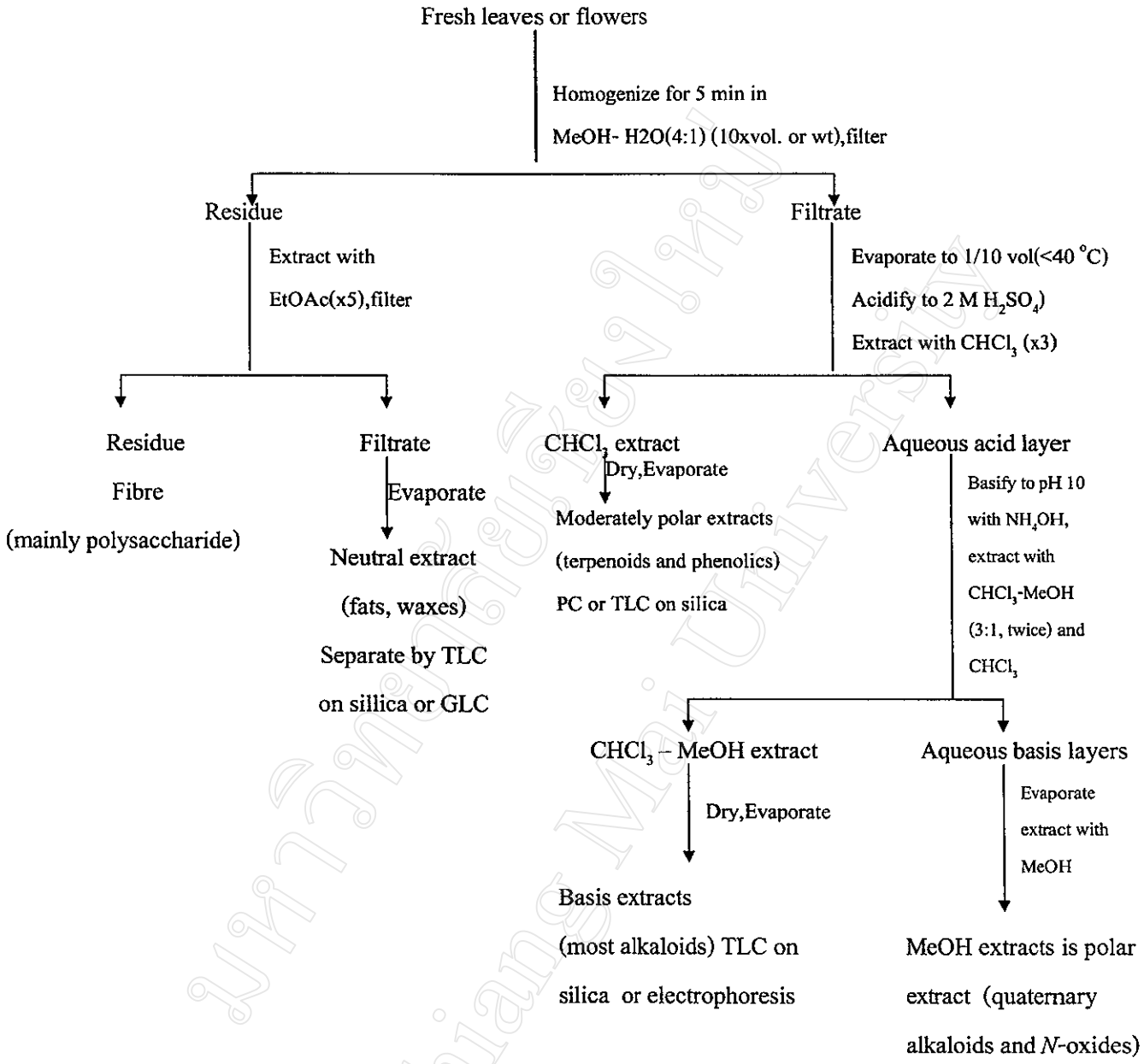


Figure 2.3 A general procedure for extracting fresh plant tissues and fractionating into different classes according to polarity.

2.7.2 Method of separation

1) General

The separation and purification of plant constituents is mainly carried out using one or other, or a combination, of four chromatographic techniques: paper chromatography (PC), thin layer chromatography (TLC), gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC). The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. PC is particularly applicable to water-soluble plant constituents, namely the carbohydrates, amino acids, nucleic acid bases, organic acids and phenolic compounds. TLC is the method of choice for separating all lipid-soluble components, i.e. the lipids, steroids, carotenoids, simple quinones and chlorophylls. By contrast, the third technique GLC finds its main application with volatile compounds, fatty acids, mono-and sesquiterpenes, hydrocarbons and sulphur compounds. However, the volatility of higher boiling plant constituents can be enhanced by converting them to esters and/or trimethylsilylethers so that there are few classes which are completely unsuitable for GLC separation. Alternatively, the less volatile constituents can be separated by HPLC, a method which combines column efficiency with speed of analysis. Additionally, it may be pointed out that there is considerable overlap in the use of the above techniques and often a combination of PC and TLC, TLC and HPLC or TLC and GLC may be the best approach for separating a particular class of plant compound.

All the above techniques can be used both on a micro- and a macro-scale. For preparative work, TLC is carried out on thick layers of adsorbesnt and PC on thick sheets of filter paper. For isolation on an even larger scale than this, it is usual to use column chromatography coupled with automatic fraction collecting. This procedure will yield purified components in gram amounts.

One further technique which has fairly wide application in phytochemistry is electrophoresis. In the first instance, this technique is only applicable to compounds which carry a charge, i.e. amino acids, some alkaloids, amines, organic acids and proteins. However, in addition, certain classes of neutral compounds (sugars, phenols) can be made to move in an electric field by

converting them into metal complexes (e.g. by use of sodium borate). Surgent (1969) has provided a simple introduction to eletrophoretic techniques.

Capillary electrophoresis, which is currently in vogue, is carried out in very small bore fused silica tubes about one metre long. High voltage (30 kV) is applied and UV detection is practised at the cathode end. The sample, applied at the anode end, can be of the order of one nanogram, but the concentration should be relatively high. This technique has proved to be a valuable analytical tool for most classes of secondary metabolite, but especially for the plant polyphenols (Tomas-Barberan, 1995).

Besides the techniques so far mentioned, a few others are used occasionally in phytochemical research. Separation by simple liquid-liquid extraction is still of some value in the carotenoid field. The means for automatic liquid-liquid extraction, as embodied in the Craig counter current distribution apparatus, has been available for some time but it tends only to be used as a last resort when other techniques fail. A more convenient apparatus for liquid-liquid extraction has been developed, called droplet counter-current chromatography (DCCC), which works on a preparative scale for separating water-soluble constituents (Hostettmann *et al.*, 1986). Separation of plant proteins and nucleic acids often requires special techniques not yet mentioned, such as feltration through Sephadex gels, affinity chromatography and differential ultracentrifugation.

Since so much has been written elsewhere on chromatography (e.g. Heftmann, 1992), it is only necessary here to discuss the main separation techniques as they are applied in phytochemical research and to give a few leading references to other available texts.

2) Paper chromatography

One of the main advantages of PC is the great convenience of carrying out separations simply on sheets of filter paper, which serve both as the medium for separation and as the support. Another advantage is the considerable reproducibility of R_f values determined on paper, so that such measurements are valuable parameters for use in describing new plant compounds. Indeed, for substances such as the anthocyanins, which do not have other clearly

defined physical properties, the R_f is the most important means of describing and distinguishing the different pigments (Harborne, 1967).

Chromatography on paper usually involves either partition or adsorption chromatography. In partition, the compounds are partitioned between a largely water-immiscible alcoholic solvent (e.g. n-butanol) and water. The classic solvent mixture, n-butanol-acetic acid-water (4 : 1 : 5, top layer) (abbreviated as BAW) was indeed devised as a means of increasing the water content of the n-butanol layer and thus improving the utility of the solvent mixture. Indeed, BAW is still widely applicable as a general solvent for many classes of plant constituent. By contrast, adsorption forces are one of the main features of PC in aqueous solvent. Pure water is a remarkably versatile chromatographic solvent and it can be used to separate the common purines and pyrimidines and is also applicable to phenolic compounds and to plant glycosides in general. (Harborne, 1998)

The choice of apparatus for PC depends to some extent on the amount of laboratory space available. Horizontal or circular PC, for example, takes up little more space than a standard TLC tank. It has remarkably good resolution and is used, for example, for separating carotenoids. In most laboratories, PC is carried out by descent, in tanks which will accommodate Whatman papers of the size 46 x 57 cm. Descending PC is most useful since the solvent can be more easily over-run if this is desired; it is also slighty more convenient for two-dimensional separations.

Considerable ranges of 'modified' filter papers are avaiable commercially for achieving particular chromatographic separations. For example, incorporating silicic acid ro alumina into the papers, making them more suitable for separating lipids can reduce the polar properties of cellulose. Papers can likewise be modified in the laboratory, for example, by soaking them in paraffin or silicone oil in order to carry out 'reversed phase' chromatography, again for lipids. For large-scale separations, thick sheets if chromatography filter paper are available (Whatman no.3 or 3MM) and these will cope with several miligrams of material per sheet.

In PC, compounds are usually detected as coloured or UV-fluorescent spot, after reaction with a chromogenic reagent, used either as a spray or as a dip. For large sheets, dipping is usually easier but the solvent content of the spray should be modified in order to facilitate quick drying and thus avoid diffusion during the dipping. The paper may then be heated in order to develop the colours.

The R_f value is the distance a compound moved in chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the centre of the spot produced by the substance, and this is divided by the distance between the origin and the solvent front (i.e. the distance the solvent travels). This always appears as a fraction and lies between 0.01 and 0.99. It is convenient to multiply this by 100 and R_fs are quoteding this book as R_fs (x 100). Elsewhere, R_f (x 100) is sometimes refered to as the R_fs value.

When comparing R_f values of a series of structurally related compounds, it is useful to refer to another chromatographic constant, the R_m value. This is related to R_f by the expression:

$$R_m = \log \left[1/R_f - 1\right]$$

Table 2.2 R_f data of flavonol 5-methyl ethers: comparison of actual R_f and R_f calculated from ΔR_m

	R_f (x 100)			
Flavonol	Forestal	50%HOAc	PhOH	BAW
Kaempferol	62	44	58	91
Quercetin	45	31	28	79
Myricetin	29	21	10	41
Kaempferol 5-methyl ether				
Actual value	70	43	78	82
Value predicted from ΔR_m	70	41	76	89
Quercetin 5-methyl ether (azaleatin)	53	29	42	55
Myricetin 5-methyl ether	37	21	23	27

*Measured on Whatman no. 1 paper.

It is valuable for relating chromatographic mobilily to chemical structure, since ΔR_m values in a homologous series are usually constants. Thus, with the flavonoid compounds, ΔR_ms are constant for the number of hydroxyl and glycosyl substitutions present in the molecule (Bate-Smith and Westall, 1956). The procedure can be used to calculate the R_f value of an unknown member of a series of compound in plant extracts. Such a procedure was employed, for example, in characterizing a new methyl ether of kaempferol, where the predicated and actual R_f values were in very good agreement (Table 2.2) (Harborne, 1969).

Within the vast literature on PC, a useful introductory account written for the novice is that of Peereboom (1971). Books on PC which are particulartly valuable as sources of R_f data are Lederer and Lederer (1957), Linskens (1959) and Sherma and Zweig (1971).

3) Thin layer chromatography

The special advantages of TLC compared to PC include versatility, speed and sensitivity. Versatility is due to the fact that a number of different adsorbents besides cellulose may be spread on to a glass plate or thei support and employed for chromatography. Although silica gel is most widely used, layers may be made up from aluminium oxide, celite, calcium hydroxide, ion exchange resin, magnesium phosphate, polyamide, Sephadex, polyvinylpyrrolidone, cellulose and from mixtures of two or more of the above materials. The greater spread on a plate and is an adventage when working with labile compounds. Finally, the sensitivity of TLC is such that separations on less than μg amounts of material can be achieved if necessary (Harborne, 1998).

One of the original disadvantages of TLC was the labour of spreading glass plates with adsorbent, a labour somewhat eased by the later introduction of automatic spreading devices. Nevertheless, even somewhat these, certain precautions are necessary. The glass plates have to be carefully cleanded with acetone to remove grease. Then the slurry of silica gel (or other adsorbent) in water has to be vigorously shaken for a set time interval (e.g. 90s) before spreading. Depending on the particle size of the adsorbent, calcium sulphate hamihydrate (15%) may have to be added to help bind the adsorbent on to the glass. Finally, plates after spreading have to be air dried and then activated by heating in an oven at 100 – 110°c for 30 min. In some separations, it is advantageous to modify the properties to the adsorbent by adding an inorganic salt (e.g. silver nitrate for argentation TLC) and this is best done when the plate is being spread. Another reason for still

using plates coated in the laboratory is that the moisture content of the silica gel can be controlled, a factor which is critical for some separations.

Nowadays, however, it is usual to employ precoated of commercial manufacture in most work, since these are more uniform and provide more reproducible results. There is a range of such plates available with different adsorbents, coated on glass, aluminium sheets or plastic. These may be with or without a fluouescent indicator, the addition of which allows the detection of all compounds which quench the fluourescence, when the plate is observed in UV light of 254 nm wavelength. The most recent type of TLC plate is that coated with the same fine microparticles of silica that are used in the columns for HPLC. Such chromatography is termed HPTLC and it usually gives more efficient and rapid separations than on conventional silica layers.

A wider range of solvents have been applied to TLC than to PC and in general, there is more latitude in the exact proportions of different solvents used in a solvent system. R_f values are considerable less reproducible than on paper and it is therefore essential to include one or more reference compounds as markers. It is possible to standardize conditions for accurate measurement of R_f in TLC, but this is a very tedious process. TLC is usually carried out by ascent, in a tank which is paper-lined so that the atmosphere inside is saturated with the solvent phase. Horizontal TLC is employed, either when plates need to be over-run with solvent or when TLC is used in combination with electrophoresis.

Spraying normally carries out detection of compounds on TLC plates, the smaller area of the plate (20 x 20 cm) making this a relatively simple procedure. One advantage over PC is that glass plates may be sprayed with conc. H_2SO_4 a useful detection reagent for steroids and lipids.

Preparative TLC is carried out using thick (up to I mm) instead of thin (0.10 – 0.25 mm) layers of adsorbent. Manufactured plates are available for this. Separated constituents are recoverde by scraping off the adsorbent at the appropriate places on the developed plate, eluting the powder with a solvent such as ether and finally centrifuging to remove the adsorbent.

Such is the strength of the adsorbent layers on glass that it is possible to repeatedly develop a plate with one or several different solvent systems, drying the plate in between development. Alternatively, a multiple elimination TLC system, devised by van Sumere (1969), can be used. This involves cutting a long rectangular glass plate spread with adsorbent with a glass cutter at

appropriate steps in a complex separation and even spraying fresh adsorbent on to the plate in between separations.

Some of the literatures on TLC are Stahl (1969), Truter (1963), Bobbitt (1963), Kirchner (1978) and Touchstone and Dobbins (1978) and Wagner and Bladt (1996), respectively.

4) Gas liquid chromatography (Harborne, 1998)

The apparatus required for GLC is sophisticated and expensive, relative to that required for PC or TLC. In principle, however, GLC is no more complicated than other chromatographic procedures.

Apparatus for GLC has four main components as follows:

(1) *The column* is a long narrow tube (e.g. 3m x 1 mm) usually of metal made in the form of a coil to conserve space. It is packed with a stationary phase (e.g. 5 – 15% silicone oil) on an inert powder (Chromosorb W, celite, etc.). The packing is not essential and alternatively an open silica column is used in which the stationary phase is spread as a film on the inside surface (capillary GLC).

(2) *The heater* is provided to heat the column progressively from 50 to 350°C at a standard rate and to hold the temperature at the higher limit if necessary. The temperature of the column inlet is separately controlled so that the sample can be rapidly vaporized as it is passed on to the column. The sample dissolved in ether or hexane is injected by hypodermic syringe into the inlet port through a rubber septum.

(3) *Gas flow* consists of an inert carrier gas such as nitrogen or argon. Separation of the compounds on the column depends on passing this gas through at a controlled rate.

(4) *A detection device* is needed to measure the compounds as they are swept through the column. Detection is freqently based on either flame ionization or electron capture. The former method requires hydrogen gas to be added to the gas mixture and to be burnt off in the actual detector. The detection device is linked to a potentiometric recorder, which produce the results of the separation as a series of peaks of varying intensity (Figure 2.4).

The results of GLC can be expressed in terms of retention volume R_v which is the volumn of carrier gas required to elute a component from the column, or in terms of retention time R_t which is the time required for elution of the sample. These parameters are nearly always

expressed in terms relative to a standard compound (as RR_s or RR_f) which may be added to the sample extract or which could take the form of the solvent used for dissolving that sample.

The main variable in GLC are the nature of the stationary phase of the column and the temperature of operation. These are varied according to the polarity and volatility of the compounds being separated. Many classes of substance are routinely converted to devatives (especially to trimethylsilyl ethers) before being subjected to GLC, since this allows their separation at a lower temperature.

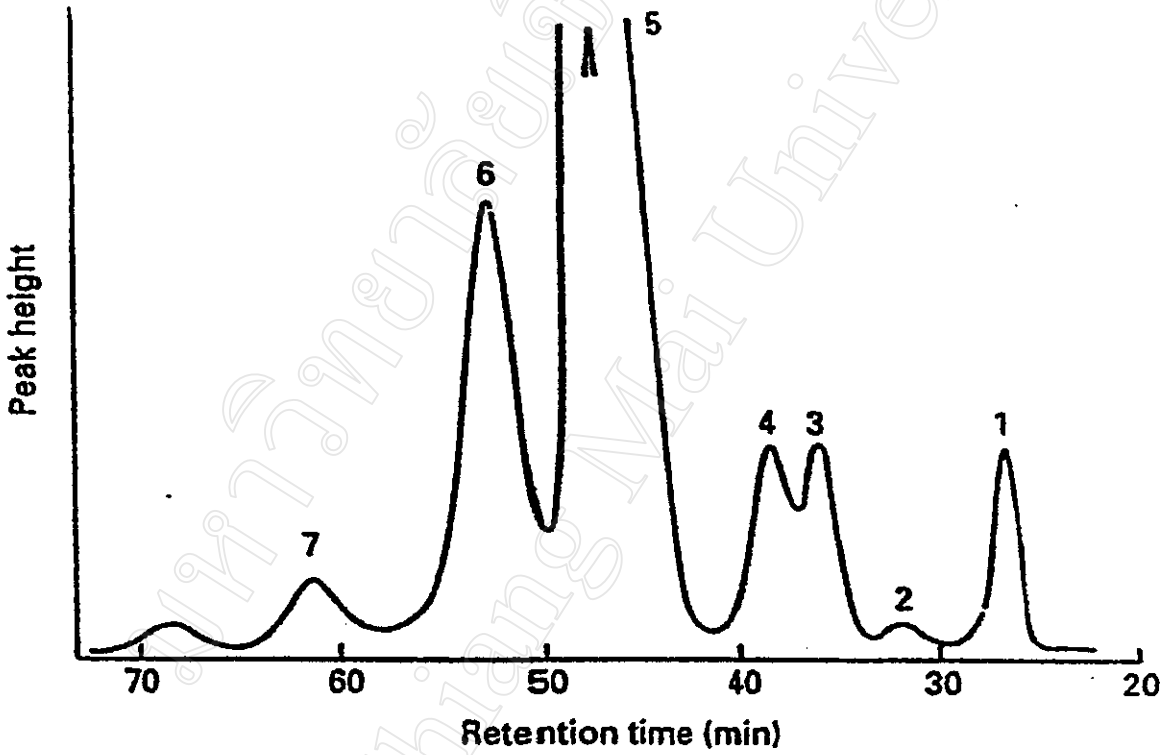


Figure 2.4 GLC trace of the separation of the sterol acetates present in oat seed.

Key: 1 cholesterol; 2 brassicasterol; 3, compesterol; 4 stigmasterol; 5, sitosterol; 6, Δ^5- avenasterol; and 7, Δ^7- avenasterol. Stationary phase is 1% cyclohexanedimethanol succinate + 2% polyvinylpyrrolidone on acid – washed Gas chrome P 225 (from Knights, 1965).

GLC provides both quantitative data on plant substances, since measurements of the area under the peaks shown on the GLC trace (Figure 2.4) are directly related to the concentrations of the defferent components in the original mixture. There are two general formulae for measuring these areas: (a) peak height X peak width at half the height = 94% of the park area (this only applies to symmetrical peaks), and (b) peak area is equivalent to that of a triangle produced by drawing tangents through the points of inflection. Peak areas can be determined automatically, e.g. by use of an electronic integrator.

The GLC apparatus can be set up in such a way that the separated components are further subjected to spectral or other analysis. Most frequently, GLC is automatically linked to mass spectrometry (MS) and the combined GC-MS apparatus has emerged in recent years as one of the most important of all techniques for phytochemical analysis,which a useful introductory reference that review on GLC, the practical point of view are Simpson (1970) and Burchfield and Storrs (1962).

5) High performance liquid chromatography (HPLC)

HPLC is analogous to GLC in its sensitivity and ability to provide both quantitative and qualitative data in the single operation. It differs in that the stationary phase bonded to a porous polymer is help in a narrow-bore stainless steel column and the liquid mobile phase is forced through under considerable pressure. The apparatus for HPLC is more expensive than GLC, mainly because a suitable pumping system is required and all connections have to be screw-jointed to withstand the pressures involved. The mobile phase is a miscible solvent mixture, which either remains constant (isocratic separation) or, may be changed continuously in its proportions, by including a mixing chamber into the set-up (gradient elution). The compounds are monitored as they elute off the column by means of a detector, usually measuring in the ultraviolet. A computing integrator may be added to handle the data as they emerge and the whole operation can be controlled through a microprocessor.

A major difference between HPLC and GLC is that former procedure normally operates at ambient temperature, so that the compounds are not subjected to the possibility of thermal re-

arrangement during separation. Temperature control of the HPLC column may, however, be advantageous for critical separations so that a thermostatically controlled small spherical particles of silica coated of bonded with stationary phase, is particularly sensitive to poisoning by impurities, so that it is essential to purify and filter plant extracts before infecting them at the head of the column.

HPLC is mainly used for those classes of compound, which are nonvolatile, e.g.higher terpenoids, phenolics of all types, alkoloids, lipids and sugars. It works best for compounds, which can be detected in the ultraviolet or visible regions of the spectrum. An example of the separation of flavonoids by HPLC is shown in Figure 2.5. For sugars, which do not show any UV absorption, it is possible to use a refractive index detector, but this is a less sensitive procedure. Proteins have been separarted by HPLC on columns of modified Sephadex, silica gels or ion exchangers.

In most modern HPLC separations, prepacked columns are employed and many types are available from the manufacturers. However, it is possible to carry out most separations using either a silica microporous particle column (for non-polar compounds) or a reverse-phase C_{18} bonded phase column (for polar compounds) (Hamilton and Sewell, 1982). One final practical detail needs mentioning; the solvents have to be ultrapure and need to be degassed before use.

HPLC is the latest chromatographic technique to be added to the phytochemist's armoury. Apart from the expense of the apparatus and the solvents, it is a most important and versatile method of quantitative plant analysis but it has yet to prove itself for separations on a preparative scale.

2.7.3 Methods of Identification

1) General

In identifying a plant constituent, once it has been isolated and purified, it is necessary first to determine the class of compound and then to find out which particular substance it is within that class. Its homogeneity must be checked carefully beforehand, i.e. it should travel as a single spot in several TLC and/or PC systems. The class of compound is usually clear from its response to colour tests, its solubility and R_f properties and its UV spectral characteristics. Biochemical test may also

be in valuable:presence of a glucoside can be confirmed by hydrolysis with β- glucosidase, of a mustard oil glycoside by hydrolysis with myrosinase and so on. For growth regulators, a bioassay is an essential part of identification.

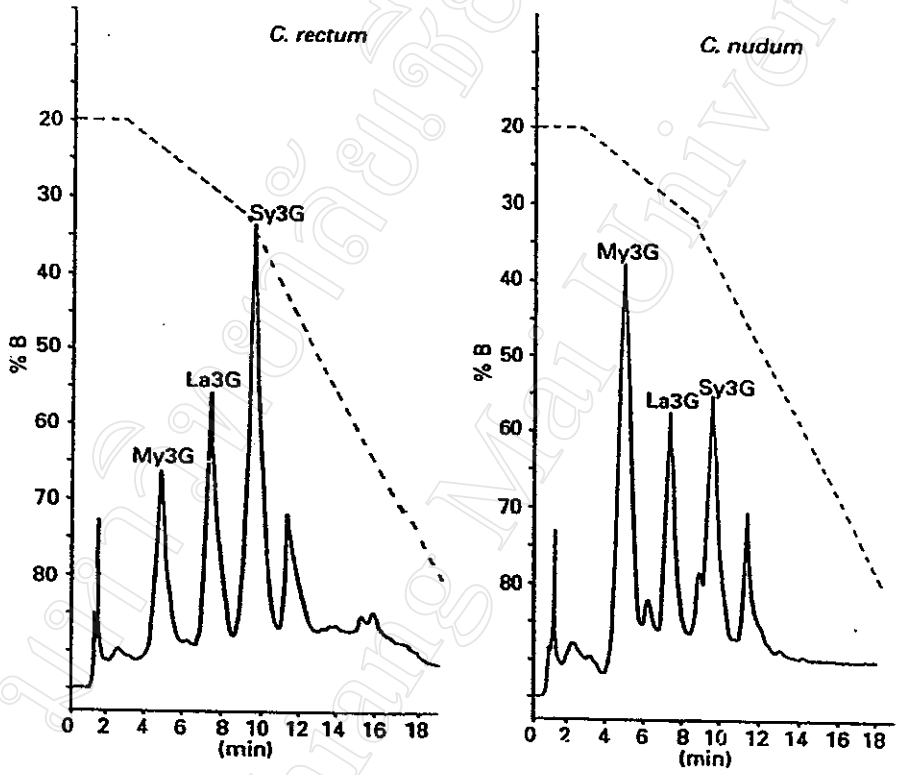


Figure 2.5 HPLC traces of the flavonoids of two species of *Chondropetatum,* where the same compounds are present but in different amount.

Key: Species *A, C. rectum;* species *B,C. nudum;* My3G = myricetin 3-galactoside; La3G – laricytrin 3-galactoside; Sy3G = syringetin 3-galactoside; 4th peak = syringetin 3-diglycoside. Separation on Spherisorb 5μm C_8 column (25 cm x 4.6 mm), gradient elution with MeOH-H_2O-HOAc (1 : 18 : 1) and MeOH-H_2O-HOAc (18:1:1) and detection at 365nm. (from Haborne,1998).

Complete identification within the class depends on measuring other properties and then comparing these data with those in the literature. These properties include melting point (for solids), boiling point (for liquids), optical rotation (for optically active compounds) and R_f or RP_t (under standard conditions). However, equally informative data on a plant substance are its spectral characteristics: these include ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectral (MS) measurements. A known plant compound can usually be identified on the above basis. Direct comparison with authentic material (if available sholud not be carried out as final confirmation. If authentic material is not available, careful comparison with literature data may suffice for its identification. If a new compound is present, all the above data should be sufficient to characterize it. With new compounds, however, it is preferable to confirm the identification through chemical degradation or by preparing the compound by laboratory synthesis.

Identification of new plant compounds by X-ray crystallography is now routine, and can be applied whenever the substance is obtained in sufficient amount and in crystalline form. It is particularly valuable in the case of complex terpenoids, since it provides both structure and stereochemistry in the same operation.

Brief comments will now be given on the different spectral techniques and on their comparative importance for phytochemical identification. For a more detailed treatment of spectral methods, the reader is referred to one of the many books available on the application of spectroscopic methods to organic chemistry (Williams and Fleming, 1966; Sheinmann, 1970, 1974; Harwood and Claridge, 1996).

2) Ultraviolet and visible spectroscopy

The absorption spectra of plant constituents can be measured in very dilute solution against a solvent blank using an automatic recording spectrophotometer. For colourless compounds, measurements are made in the range 200 to 400 nanometres (nm); for coloured compounds, the range is 200 to 700 nm. The wavelengths of the maxima and minima of the absorption spectrum so obtained are recorded (in nm) and also the intensity of the absorbance (or optical density) at the particular maxima and minima (Figure 2.6). Only traces of material are required, since the standard spectrophotometric cell (1 x 1 cm) only holds 3 ml of solution and, using special cells, only one tenth of this volume need be available for spectrophotometry. Such

spectral measurements are important in the identification of many plant constituents, for monitoring the eluates of chromatographic columns during purification of plant products and for screening crude plant extracts for the presence of particular classes of compound such as polyacetylenes.

A solvent widely used for UV spectroscopy is 95% ethanol since most classes of compound show some solubility in it. Commercial absolute alcohol should be avoided, since it contains residual benzene which absorbs in the short UV. Other solvents frequently employed are water, methanol, hexane, petroleum and ether. Solvents such as chloroform and pyridine are generally to be avoided since they absorb strongly in the 200 – 260 nm region; they are, however, quits suitable for making measurements in the visible region of the spectrum with plant pigments such as the carotenoids.

When substances are isolated as crystalline compounds and their molecular weights are known or can be determined, then the intensities of the wavelength maxima are normally recorded in terms of log 8, where 8 = A/C1 (A = absorbance, C = concentration in g moles/litre, 1 = cell path length in cm, usually 1). With compounds where neither the concertration nor the molecular weight are known, absorbance values have to be used. In these cases, the heights of the various maxima may be compared by considering absorbances as a percentage of that at the most intense peak.

Purification is an essential preliminary to spectral studies and plant constituents which exhibit characteristic absorption properties should be repeatedly purified till these properties are constant. In chromatographic purifications, allowances for UV-absorbing impurities present in the filter paper can be made by using an eluate of a paper blank, prepared at the same time as the sample, for balancing in the spectrophotometer against the eluate containing that sample. A similar procedure should be adopted when purification is being done on TLC plates.

The utility of spectral measuraments for identification purposes can be greatly enhanced by repeating measurements made in neutral solution, either at a range of different pH values or in the presence of particular inorganic salts. For example, when alkali is added to alcoholic solutions of phenolic compounds, the spectra are characteristically shifted towards longer wavelengths (they undergo bathochromic shifts) with increases in absorbance (Figure 2.6). By contrast, when alkali is added to natural solution of aromatic carboxylic acid the shift is in the opposite direction towards shorter wavelengths (hypsochromic shifts). Reactions such as chemical reduction (with sodium

borohydride) or enzymatic hydrolysis can equally will be followed in the cell cuvette of a recording spectrophotometer and absorption measurements made at regular time intervals will indicate whether reduction or hydrolysis has taken place.

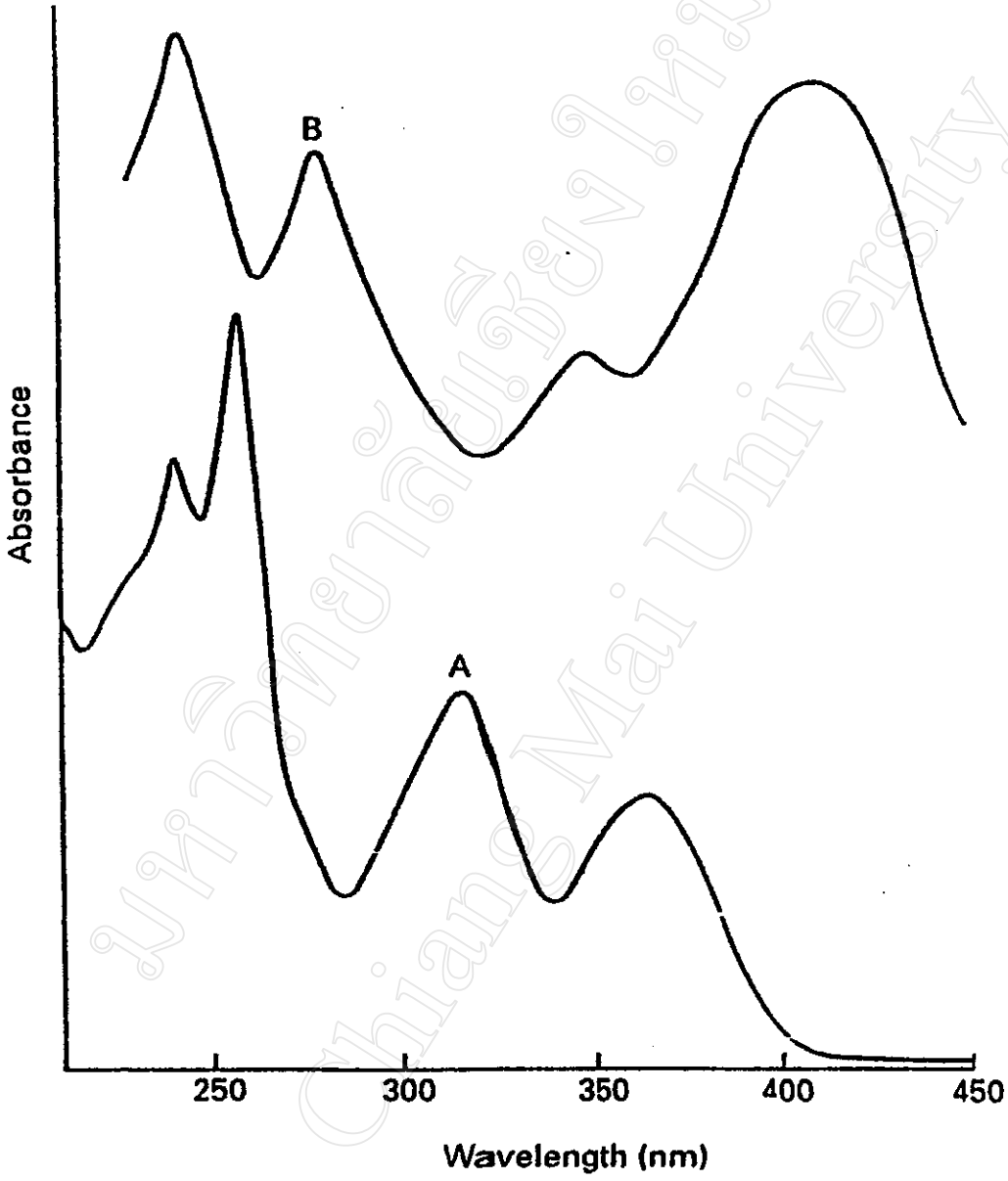


Figure 2.6 Ultraviolet adsorption spectrum of the *Xanthone mangiferin*. Curve A, solvent is 95% EtOH. Wavelength maxima are at 240, 258,316 and 364 nm; minima are at 215, 248, 285 and 338 nm. Relative intensities of absorbance at the maxima are 82, 100, 50 and 37% respectively. Curve B, 95% EtOH + 2 drops 2N NaOH.

Table 2.3 Spectral properties of the different classes of plant pigment.

Pigment class	Visible spectral range (nm)*	Ultraviolet range (nm)
Chlorophylls (green)	640 – 660 and 430 – 470	Intense short UV Absorption due to Protein attachment
Phycobilins (red and blue)	615 – 650 or 540 – 570	
Cytochromes (yellow)	545 – 605 (minor band sometimes at 415 – 440)	
Anthocyanins (mauve or red)	475 – 550	Ca. 275
Betacyanins (mauve)	530 – 554	250 – 270
Carotenoids (yellow to orange)	400 – 500 (a major peak with two minor peaks or inflections)	-
Anthraquinones (yellow)	420 – 460	3 – 4 intense peaks between 220 and 290
Chalcones and Aurones (yellow)	365 – 430	240 – 260
Yellow flavonols (yellow)	365 – 390	250 - 270

*All values are approximate; actual values vary according to the solvent used, the pH and the physical state of the pigment.

The value of UV and visible spectra in identifying unknown constituents is obviously related to the relative complexity of the spectrum and to the general position of the wavelength maxima. If a substance shows a single absorption band between 250 and 260 nm, it could be any one of a considerable number of compounds (e.g, a simple phenol, a purine or pyrimidine, an aromatic amino acid and so on). If, however, it shows three destinct peaks in the 400-500 nm region, with little absorption elsewhere, it is almost certainly a carotenoid. Furthermore, spectral measurements in two or three other solvents and comparison with literature data might even indicate which particular carotenoid it is.

The above statements suggest that absorption spectra are of especial value in plant pigment studies and this is certainly true for both water- and lipid–soluble plant colouring matters (Table 2.3). Other classes which show characteristic absorption properties include unsaturated compounds (particularly the polyacetylenes), aromatic compounds in general (e.g. hydroxycinnamic acids) and ketones. The complete absence of UV absorption also provides some useful structural information. It is indicative of the presence of saturated lipids of alkanes in lipid fractions of plant extracts, or organic acids, aliphatic amino acids or sugars in the water – soluble fractions.

3) Infrared (IR) spectroscopy

IR spectra may be measured on plant substances in an automatic recording IR spectrophotometer either in solution (in chloroform or carbon tetrachloride (1 – 5%)), as a mull with nujol oil or in the solid state, mixed with potassium bromide. In the latter case, a thin disc is prepared under anhydrous conditions from a powder containing about 1 mg of material and 10 to 100 mg KBr, using a mould and press. The range of measurement is from 4000 to 667 cm^{-1} (or 2.5 to 15 μm) and the spectrum takes about three minutes to be recorded. Typical IR spectra obtained in this way are shown in Figure 2.7.

The region in the IR spectrum above 1200 cm^{-1} shows spectral bands or peaks due to the vibrations of individual bonds or functional group in the molecule under examination (Table 2.3). The region below 1200 cm^{-1} shows bands due to the vibrations of the 'fingerprint' region. Intensities of the various bands are recorded subjectively on a sample scale as being either strong (S), medium (M) or weak (W).

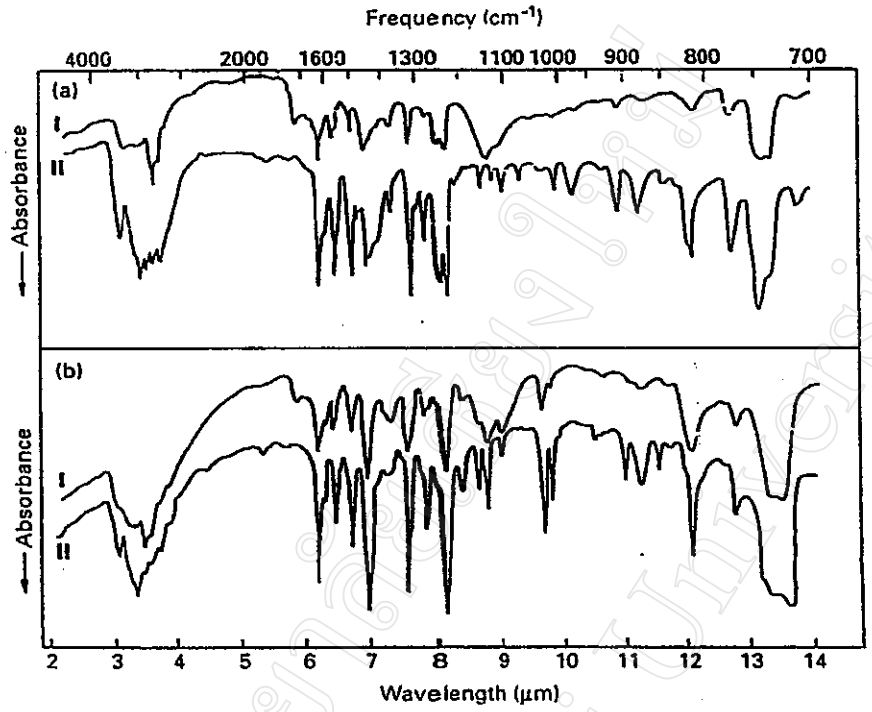


Figure 2.7 Infrared spectra of two alkaloids from tobacco smoke.

Key (a) harmane,natural (I) and synthetic (II); (b) norharmane, natural (I) and synthetic (II). Note that IR spectra are traditional recorded upside down compared to the UV and visible spectra . Thus, the absorbance bands here point downwards.

The fact that many functional groups can be identified by their characteristic vibration frequencies (Table 2.3) makes the IR spectrum the simplest and often the most reliable method of assigning a compound to its class. In spite of this, IR spectroscopy is most frequently used in phytochemical studies as a 'fingerprinting' device, for comparing a natural with a synthetic sample (Figure 2.8). The complexity of the IR spectrum lends itself particularly well to this purpose and such comparisons are very important in the complete identification of many types of plant constituent. IR spectra, for example, have been used extensively for identifying known essential oil components as they are separated by GLC on a preparative scale.

Table 2.4 Characteristic infrared frequencies of some classes of natural products

Class of compound	Approximate positions of characteristic bands* above 1200 cm^{-1}
Alkanes	2940 (S), 2860 (M), 1455 (S), 1380 (M)
Alkenes	3050 (W-M), 1850 (W), 1650 (W-M), 1441 (W)
Aromatics	3050 (W-M), 2100 – 1700 (W), 1600, 1580, 1500 (W-M)
Acetylenes	3310 (M), 2225 (W), 2150 (W-M), 1300 (W)
Alcohols and Phenols	3610 (W-M), 3600 – 2400 (broad), 1410 (M)
Aldehydes and Ketones	2750 (W), 2680 (W), 1820 – 1650 (S), 1420 (W-M)
Esters and Acids	1820 – 1680 (S)
Carboxylic acids	3520 (W), 3400 2500 (broad,M), 1760 (S), 1710 (S).
Amines	3500 (M), 3400 (M), 3400 – 3100 (variable), 1610 (M)
Cyanides	2225 (W – S).
Isocyanates	2270 (VS)

* Bands in 'fingerprint' region are omitted for simplicity. Data adapted from Brand and Eglinton (1965).

An illustration of the use of IR spectra for 'finger – printing' alkaloids is given in Figure 2.8. Two trace components of tobacco smoke are identified as the bases harmane and norharmane, using the KBr disc procedure (Poindexter and Carpenter, 1962). It may be noted that some of the detail in the fingerprint region of both alkaloids is absent from the natural samples, probably due to the presence of trance of impurity. It may also be observed that although the alkaloids are closely similar in structure (they differ only in that harmane is the C – methyl derivative of norharmane), they can be readily distinguished by their IR spectra.

IR spectroscopy can also usefully contribute to structural elucidation, When new compounds are encountered in plant. Although there are many listed correlations between chemical structure and IR absorption peaks, the actual interpretation of a complex spectrum can be difficult and the operation requires much experience. With some classes of compounds,

however,interpretation can be relative simple matter. Measurement of the carbonyl frequencies between 1800 and 1650 cm^{-1} in an adjacent hydroxyl or not. With the anthraquinones, for example, nonchelated quinones have a band between 1678 and 1653 cm^{-1} ; a quinone with one α-OH shows two bands, at 1675 – 1647 cm^{-1} and at 1637 - 1621 cm^{-1} ; and a quinone with two α-OH groups has bands at 1675 – 1661 cm^{-1} and at 1645 – 1608 cm^{-1} .

For source of IR data, various catalogues can be consulted, e.g. Hershenson (1959, 1964). For a good introductory reciew on IR spectroscopy, see Eglinton (1970). A popular introductory practical textbook, now, in its third edition, is that of Cross an Jones (1969).

4) Mass spectroscopy (MS)

MS, since its relatively recent introduction (about 1960), has revolutionized biochemical research on natural products and has eased the task of the phytochemist in many ways. The value of the technique is that it requires only microgram amounts of material, that it can provide an accurate molecular weight and that it may yield a complex fragmentation pattern which is often characteristic of (and may identify) that particular compound.

MS, in essence, consists of degrading trace amounts of an organic compound and recording the fragmentation pattern according to mass. The sample vapour diffuses into the low pressure system of the mass spectrometer where it is ionized with sufficient energy to cause fragmentation of the chemical bonds. The resulting positively charged ions are accelerated in a magnetic field which disperses and permits relative abundance versus mass constitutes the mass spectral graph, which thus consists of a series of lines of varying intensity at different mass units (Figure 2.8).

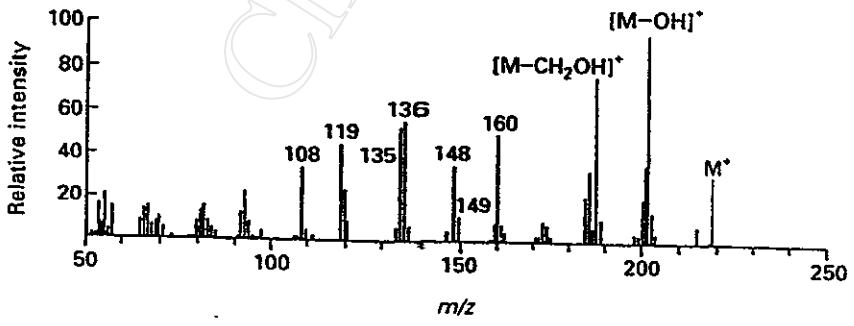


Figure 2.8 Mass spectrum of the growth regulator zeatin.

In many cases, some of the parent compound will survive the vaporization process and will be recorded as a molecular ion peak. Very accurate mass measurements (to 0.0001 mass units) can then be performed on this (and any other) particular ion. The accuracy is such as to indicate the exact molecular formula of the substance, so that conventional elemental analysis (which normally requires several milligrams of substance) is no longer necessary.

Unlike the UV and IR spectrophotometers which are operated by the phytochemist himself, instruments for mass spectral and NMR determinations are more expensive and much more sophisticates, so that they are normally operated by trained personnel. The phytochermist therefore, hands his sample over for analysis and receives back the results in the form of the graph shown in Figure 2.8. The technique works successfully with almost every type of low-molecular weight plant constituent and it has even been uses for peptide analysis. Those compounds which are too involatile to vaporize in the MS instrument are converted to trimethylsilyl ethers, methyl esters or similar derivatives. This is true of the gibberellins. MS is frequently used in conjunction with GLC, and the combined operation provides at one go a qualitative and quantitative identification of the many structurally complex components that may be present together in a paritcular plant extract.

One example must suffice to illustrate the value of MS measurements in phytochemical research. This is the case of zeatin, the first naturally occurring cytokinin growth regulator to be isolated from higher plants. Its structure as 6 – (4-hydroxymethyl-trans-2-butenylamino) purine was determined by Shannon and Letham (1966); the results of the MS (Figure 2.8) considerably helped in this identification. Thus, there was a prominent molecular ion at 219, confirming the melecular formula $C_{10}H_{13}ON_5$. The presence of a primary alcohol was revealed by fragments at m/z 202 (M-OH) and m/z 188 (M-CH_2OH). The location of the alkyl group attachment at N-was indicated by the fragment at m/z 148. Finally, confirmation of the adenine nucleus was obtained form the characteristic fragments (shown by most adenine derivatives) at m/z 136, 135 and 108.

New technical developments continue to emerge in mass spectroscopy and modern spectrometers may be provided with a Fast Atom Bombardment (FAB) source and are than capable of analysing fragile or involatile organic compounds, including salts and higher-molecular weight materials. Previously, when using MS in the analysis of plant glycosides, the O-glycosidic sugars

were lost in the process and escaped detection but it is now possible with FAB-MS to obtain molecular ions for the original glycoside.

Some of the many applications of MS data to plant biochemical research are covered in two treatises (Waller, 1972; Waller and Dermer, 1980).

5) Nuclear magnetic resonance spectroscopy (NMR)

Proton NMR spectroscopy essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as-CH_2,- CH_3,- NH_2,-CHOH-, etc.)and the proton NMR spectrum provides a record of the number of hydrogen atoms in these different situatoins of the carbon skeleton of the molecule; this can only be obtained by carbon-13 NMR spectroscopy.

In practice, the sample of the substance is placed in solution, in an inert solvent, between the poes of a powerful magnet and the protons undergo different chemical shifts according to their molecular environments within the molecule. These are measured in the NMR apparatus relative to a standard, usually tetramethylsilan (TMS), which is an inert compound, which can be added to the sample solution without the likelihood of chemical reaction, occurring.

Chemical shifts are measured in either δ (delta) or τ (tau) units; where $\tau = 10\delta$ and $\delta = \Delta v \times 10^6$ / radio frequency, Δv being the difference in absorption frequency of the sample and the reference compound TMS in Hertz units. Since total radio frequency in usually 60 MegaHertz (60 million Hertz) and shifts are measured in Hertz units, they are often referred to as ppm. Also, the intensity of the signals may be integrated to show the number of protons resonating at any one frequency.

The solvent for NMR measurements has to be inert and without protons. One is, therefore, limited to using carbon terterachloride, deuterochloroform ($CDCI_3$), deuterium oxide (D_2O), deuteroacetone (CD_3COCD_3) or deuterated dimethylsulphoxide. Polar compounds are often sparingly soluble or insoluble in the available solvents and they have to be converted to the trimethylsilyl ethers for measurement (Figure 2.9). At least 5-10 mg of sample is needed and this limits the use of NMR spectroscopy in many phytochemical experiments. However, instruments

which only require mg samples for analysis are now available. One advantage of NMR spectroscopy over MS is that the sample can at least be recovered unchanged after the operation and used for other determinations.

Table 2.4 Some proton nuclear magnetic resonance chemical shifts characteristic of different classes of plant products

Class	Type of proton	Range of shift, δ (ppm.)
Alkanes and fatty acids	$CH_3 - R$	0.85 – 0.95
	$R - CH_2 - R$	1.20 – 1.35
Alkenes	$CH_3 - C \equiv C$	1.60 – 1.69
	$- CH = C$	5.20 – 5.70
Acetylenes	$HC \equiv C$	1.45 – 2.65
Aromatic compounds	$Ar - H$	6.60 – 8.00
	$Ar - CH_3$	2.25 – 2.50
	$Ar - CHO$	9.70 – 10.00
Nitrogen compounds	$N - CH_3$	2.10 – 3.00
	$N - CHO$	7.90 – 8.10
	$N - H$	variable

As with other spectral techniques, proton NMR spectroscopy can be used by the phytochemist as a fingerprinting technique. It has to be remembered, however, that the complexity of the spectrum is directly related to the number of different types of proton present, so that a highly substituted complex alkaloid may in fact give fewer signals that a simple aliphatic

hydrocarbon. The major use of proton NMR is for structural determination, in combination with other spectral techniques. Its use for determining the class of compound is quite considerable; some examples of chemical shifts which are typical of certain classes of natureal products are listed in Table 2.4. Aromatic protons (either in benzene derivatives or in heterocyclic compounds) are clearly distinct from aliphatic protons. Within a class of compound, too, NMR measurements may often provide the means of identifying individual structures.

Proton NMR spectra can be quite complex. For example, due to the interaction between protons attached to adjacent cabon atoms, the spectral signals may appear as 'doublets' or 'triplets' instead of as single peaks. Interpretation, therefore, requires much skill. In spite of this, useful phytochemical information can be obtained without necessarily analyzing the spectrum in close detail. Two examples will show this. First, while determining the structure of sterculic acid, chemists could not at first accept that this unique fatty acid had an apparently strained cyclopropane ring in its structure and alternative formulae in which the double bond was placed in positions adjacent to a cyclopropane ring were prefered. The proton NMR spectrum, however, showed clearly that it must have a cyclopropene ring, since there was no signal for protons in the olefinic region (5.2 – 5.7δ) and the alternative formulae were thus impossible.

A second example is from a structural study of a yellow flavonoid pigment, tambuletin. These was described, by the original Indian workers who isolated it, as a flavonol aglycone but the proton NMR spectrum at once revealed the presence of an unsuspected sugar in it, since there were signs at 5.15 and 5.26 δ due to sugar protons well separated from signals

shown by protons attacted to the flavone nucleus (between 6 and 7.5 δ). Thus, the pigment was clearly a glycoside, not an aglycone (Harrbone *et al.,* 1971).

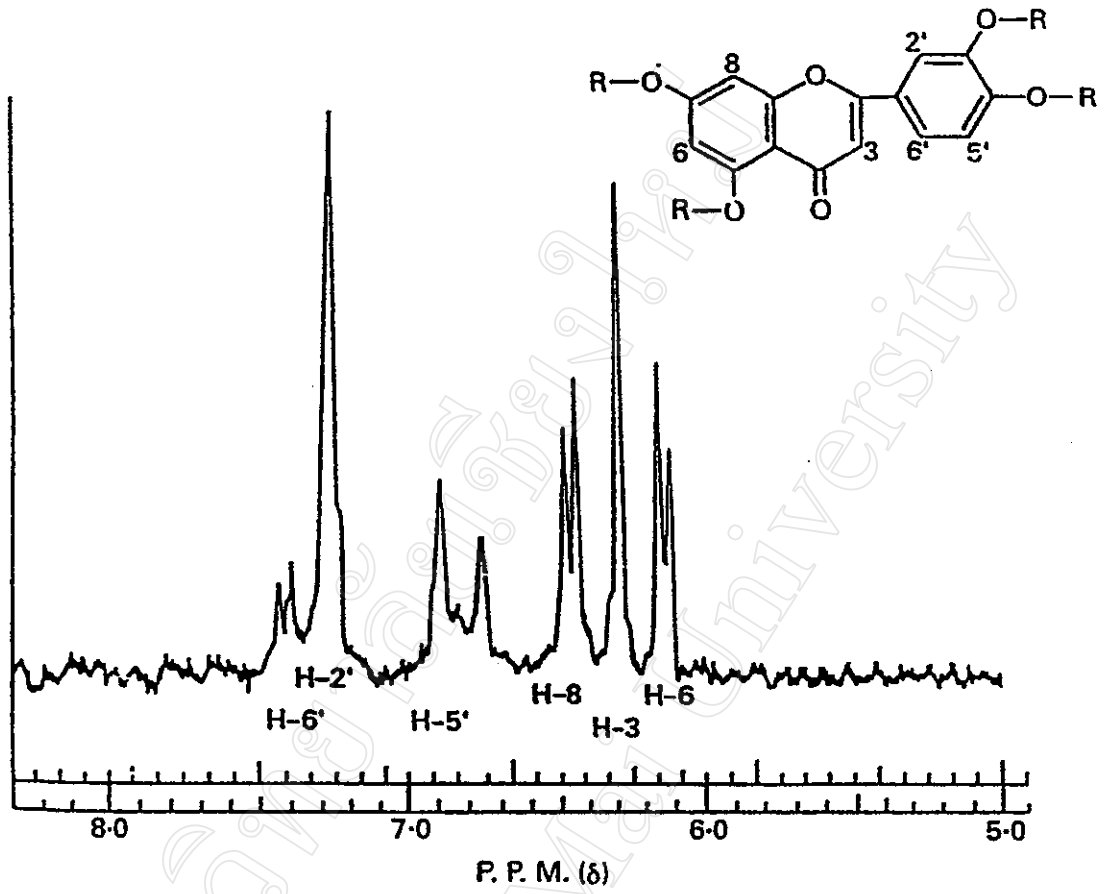


Figure 2.9 Proton NMR spectrum of the flavone luteolin (as the trimethysilyl ether).Chemical shifts are relative to TMS (Mabry, 1969).

Note that the six protons in luteolin give well separated shifts. If luteolin is further substituted, the position of substitution is clear from the disappearance of one or other of the six signals. Tambuletin is 8 – substituted and its NMR spectrum is similar in this region to luteolin except that the doublet signal at 6.5 δ has disappeared.

The detection of signals from carbon atoms in the NMR apparantus is possible, due to the presence of small amounts (ca. 1.1%) of carbon-13 along with carbon-12 in matural plant substances. The smaller magnetic moment genarated by ^{13}C compared to that of a proton means that the signal is much weaker. Only since the technical advances of pulsed NMR and Fourier Transform analysis has ^{13}C – NMR spectroscopy become generally available and, even with these

advances, more instrument time may be required than with proton NMR. This procedure is widlly used in structural analysis, although the need to have a sample weighing about 10 mg is still a limitation.

Similar solvents are used, as in proton NMR, but the range of ^{13}C resonances is much greater, namely 0-200 ppm. downfield from TMS compared with a range form 0-10 ppm. for proton resonances. Thus, ^{13}C –NMR spectra are more highly resolved and, in most cases, each carbon within the molecule (Figure 2.10) can be assigned to a separate signal. As with proton NMR (Table 2.4), differently substituted carbon atoms give shifts within specific ranges; for example, aliphatic carbon atom give shifts between 0 and 40 ppm., aromatic carbon between 110 and 150 ppm. and ketonic carbons between 160 and 230 ppm.

^{13}C-NMR spectroscopy is essentially complementary to proton NMR and the combination of the two techniques provides a very powerful means of structural elucidation for new terpenoids, alkaloids or flavonoids. It is useful in the analysis of glycosides, in indicating the linkage between sugar moieties and their configurations. Both proton and ^{13}C-NMR measurements have been successfully applied to structural and other analyses of proteins and other macromolecules (Jones, 1980). There is no simple guide to NMR spectroscopy written for the plant scientist. There are, though, many works dealing with its application to organic chemistry (Jackman, 1959; Scheinmann, 1970) or to biochemistry (James, 1975).

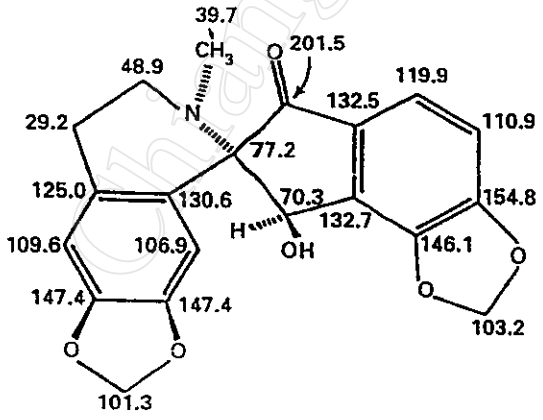


Figure 2.10 Carbon – 13 shifts relative to TMS (in ppm.) for the different carbon atoms in the molecule of the spirobenzylisquinoline alkaloid sibiricine from *Corydalis sibirica* (Fumariaceae).

In the last decade, the application of NMR spectroscopy to the structural elucidation of plant products has advanced considerably. This has been through the production of high resolution spectra using more powerful instruments and through the development of additional procedures designated as NOE (nuclear overhauser effect). COSY, NOESY, INEPT, DEPT and so on (Derome, 1986). Many complex structures have been solved using these techniques. In addition, X – ray crystallography has become more readily available for the complete structural analysis of such molecules.

6) Criteria for phytochemical identifications

As already mentioned above, a known compound discovered in a new plant source can be identified on the basis of chromatographic and spectral comparison with authentic material. Authentic samples may be obtained commercially from a firm of suppliers, by resolution from a known source or, a last resort, by request from the scientist who originally isolated and described it. The extent of the comparison to be made varies according to the class of compound being studied but as a general principle, it is desirable to use as many criteria as possible to be certain of the correctness of the identification (Table 2.5).

Chromatographic comparison should be based on co – chromatography of the compound with authentic material, without separation, in at least four systems. If TLC is the main basis of comparison, then there are obvious advantages in employing different adsorbents (e.g. cellulose and silica gel) as well as different solvents on the same adsorbent (Table 2.5). Whenever possible, it is desirable to compare unknown and standard by three distinct chromatographic criteria, e.g. by retention time by GLC and HPLC and R_f on TLC or by R_f or more procedures should be adopted wherever possible. Ideally, UV, IR, MS and H – NMR spectra should all be compared.

With new plant substances, it is usually possible to establish the structure on the basis of spectral and chromatographic measurements, especially in relationship to those made on known compounds in the same series. Confirmation of structure may be possible by chemical conversion

to a known compound. At one time, an essential step in structural identification was the determination of molecular formula by micro – analysis, with at least a carbon, hydrogen determination. Such micro – analyses are still very desirable, but when only micrograms of material are available, it is possible to use a precise mass measurement on the molecular ion, determined by mass spectrometry (Table 2.5). Derivatization is also valuable with new compounds, e.g. the preparation of acetates, methyl ethers, etc., since their analysis provides useful confirmation of the molecular formula of the original substance.

Table 2.5 The type of criteria needed for the identification of known plant constituents.

The identification of 6 – hydroxyluteolin 7 – methyl ether in leaves of *Crocus nimus.*

Criterion	Property recorded
1.) Physical properties	Yellow powder, m.p. 245 – 6°C
2.) Molecular formula by MS	Molecular ion found 316.0574 $C_{16}H_{12}O_7$ requires 316.0582
3.) MS fragmentation pattern	Fragment ion by demethylation at 301.344 ($C_{15}H_9O_7$ requires 301.0345), etc.
4.) UV spectral properties (and shifts with alkali, etx.)	Maxima at 254, 273, 346 nm, etc.
5.) Colour on TLC plate	Yellow in daylight Dark brown in UV $\pm NH_3$
6.) TLC on cellulose	R_f 0.73 in n-BoOH–HOAc – H_2O (4:1:5) R_f 0.59 in 50% HOAc R_f 0.67 in $CHCI_3$- HOAc – H_2O (90:45:6)
7.) TLC on polyamide	R_f 0.36 in C_6H_6 – MeCOEt – MeOH (4:3:3)
8.) Chemical conversion	Demethylation with pyridinium chloride to 6 – hydroxyluteolin

2.7.4 Analysis of results

1) Qualitative analysis

Much plant analysis is devoted to the isolation and identification of secondary constituents in a particular species or group of species with the expectation that some of the constituents may be novel or of an unusual structure. In such cases, it is important of recognize that many of the more readily isolated components are either commonly present or universal in occurrence. Sucrose may crystallize out from an aqueous plant concentrate and sitosterol from a phytosterol fraction. The more interesting components are often those present in lower amounts.

When an apparently new structure has been obtained, it is necessary to check carefully that it has not been reported before. Reference may be made to the various literature compilations available (e.g. *Dictionary of Alkaloids*, Southon and Buckingham, 1989) but a computer search in *Chemical Abstracts* is also desirable. The best source of information on known natural products is *Dictionary of Natural Products* (Buckingham, 1994). This runs to seven volumes, with three later supplements, and is available as a CD – ROM.

Another motive for phytochemical analysis is the characterization of an active principle responsible for some toxic or beneficial effect shown by a crude plant extract when tested against a living system. In such cases, it is essential to monitor the extraction and separation procedures at each stage in order to follow the active material as it is purified. The activity can sometimes vanish during fractionation, due to its liability and a pure crystallite compound may be eventually obtained which lacks the activity of the original extract. The possibility of damage to the active principle during isolation and characterization must always be borne in mind.

Similarly, it is essential to realize that the production of artifacts is a common feature of plant analysis. Many of the compounds occur in plant tissues are quite labile and almost inveritably may undergo change during extraction. The plastid pigments, the chlorophylls and carotenoids, are susceptible to modification during chromatography. All plant glycosides are liable to some hydrolysis, either enzymic or non – enzymic, during istation, while esters may undergo transesterification by the presence of alcoholic solvents. The volatile terpenes are susceptible to molecular rearrangements during steam distillation and the racemization of optically active

constituents may occur, unless special precautions are taken. Again, proteins may be subject to protease attack during isolation procedures.

Additionally, artifacts may be introduced unwillingly form laboratory equipment during purification. The most common additive is butyl isophthalate, which is a plasticizer that almost always contaminate plant extracts. It has actually been reported as a plant constituent, in spite of its obvious origin from a plastic wash bottle used by the operator during the isolation. In avoiding artifacts, it is necessary to check the original crude plant extract to see if a compound isolated only after extensive purification is actually present there.

2) Quantitative analysis

Equally as important as qualitative measurements on a plant extract are determinations of the amounts of the components present. In the simplest approach, quantitative data can be obtained by weighing the amount of plant material initially used (assuming dried tissue) and the amount of the product obtained. Such a yield as a percentage of the whole will be a minimal figure, since inevitably some material will be lost during purification. Losses can be estimated by adding a known weight of pure substance to the crude extract, repeating the purification and determining the amount recovered. If fresh tissue is extracted, a conversion factor (most plant leaves are 90% water) will be needed to express the result as percentage dry weight.

Quantitative measurements can also be conducted on dried, powdered plant material to determine the total content of sugar, nitrogen, protein, phenol, tannin and so on. Some of the procedures that can be used will be mentioned in later chapters. Such procedures are liable to error due to interference from other components present. Whether such determinations have much value in terms of, for example, the amount of herbivory a particular plant organ suffers needs to be assessed.

Ideally, in quantitative measurements, the amounts of the individual components within a particular class of constituent need to be determined and this is now readily achieved by either GLC or HPLC. The amounts of fatty acids bound in neutral plant lipids can, for example, be determined in a thoroughly reproducible way after saponification, methyl ester formation and quantification of

the methyl esters by GLC, Similarly, HPLC measurements can be used to determine the amounts of flavonoid pigments in different varieties and genotypes of garden flowers.

The importance of repeating measurements so that they can be seen to be statistically significant is obvious but not always appreciated. Variation in amounts due to environmental parameters needs to be eliminated and sampling has to be considered in relation to plant age and provenance. Guidance on these matters are available in most modern plant texts.

2.7.5 Applications

1) General

While phytochemical procedures have today an established role in practically all branches of plant science, this has not always been so. Although these methods are obviously essential in all chemical and biochemical studies, their application in more strictly biological spheres has only come within the last two decades. Even in disciplines so remote from the chemical laboratory as systematics, phytogeography, ecology and palaeobotany, phytochemical methods have become important for solving certain types of problems and will undoubtedly be used here with increasing frequency in the future.

There is only room in this book to mention a few of the many applications of phytochemical methods. Some of the obvious applications in agriculture, in nutrition and the food industry and in pharmaceutical research must be taken for granted. The following applications are simply given to illustrate the value of phytochemical techniques in some the major branches of plant science.

2) Plant physiology

The major contributions of phytochemical studies to plant physiology are undoubtedly in determining the chemical structures, biosynthetic origins and modes of action of nature growth hormones. As a result of continuing collaboration over the years between physiologists and phytochemists, five classes of growth regulators are now recognized : the auxins, cytokinins, abscisins, gibberellins and ethylene. Methods of detection, which vary form GLC through TLC to PC. One of the more remarkable aspects of the gibberellin group of hormones is

the large number of know structures (over a hundred), all apparently with a similar range of growth properties. The need for very precise methods of detection and distinction between gibberellins led to the development of combined GC – MS for their analysis. A general book on methods for the isolation of plant growth substances, edited by Hillman (1978), can be consulted for more details. The necessary requirements for accurate hormone analysis are critically considered by Reeve and Crozier (1980). An excellent review of techniques, including the use of radio – immunoassay, is that of Horgan (1981). A sixth class of growth regulator described recently are the brassinosteroids.

3) Plant pathology

Phytochemical techniques are primarily important to the pathologist for the chemical characterization of phytotoxins (products of microbial synthesis produced in higher plants when invaded by bacteria or fungi) and of phytoalexins (products of higher plant metabolism formed in response to microbial attack). A range of different chemical structures are involved in both cases. The most familiar phytotoxins are lycomarasmin and fusaric acid, amino acid derivatives, which are wilting agents in the tomato. Other toxins that have been isolated are glycopeptides, naphthaquinones or sesquiterpenoids (Durbin, 1981). Some phytotoxins are chemically labile so that special precautions have to be taken during their isolation and identification.

Phytoalexins also have different structures, according to the plant source (Bailey and Mansfield, 1982). They may be sesquiterpenoid (rishitin from *Solanum tuberosum),* isoflavanoid (pisatin from *Pisum sativum),* acetylenic (wyeronc acid from *Vicia faba)* or 'phenolic' (orchinol from *Orchis militaris).*

'Pre – infective' substances (naturally occurring secondary constituents) are considered by some plant pathologists to be important in imparting disease resitance to plants. Phenolic compounds, such as phloridzin in apple, tannin in raspberry.

4) Plant ecology

Two research areas where secondary plant consitituents are significant in plant ecology are in plant – animal and plant – plant interactions. The analyltical problems in both cases

are difficult because of the very limited amounts of biological material at the disposal of the phytochemist. For example, in following the fate of secondary compounds in insects feeding on plants, it is necessary to analysis different organs of the insect to see where the compounds are stored ; such analyses are often complicated and time – consuming.

Compounds so far known to be involved in plant –animal interactions are primarily alkaloids and cardiac glycosides, mustard oil glycosides, cyanogens, steroids or volatile terpenes. The plant compounds may variously act as feeding attractants or repellents, have hormonal effects on the insects or provide the insects with a useful defence mechanism against predation (Harborne, 1993).

Plant – plant interactions involve so – called allelopathic substance which one plant exudes from its roots or leaves in order to prevent the growth of ther plant species in its vicinity. The compounds are either volatile tepenes (e.g. cineole) or simple phenolic acids, depending on whether the plant is growing in a semi – tropical or a temperate climate. The phytochemical study of allelopathy can be difficult since it requires determinations on whole leaf extracts, natural leaf leachates and on soil samples too. The possible rapid turnover of active substances in the soil also provides another analytical hazard in this field (Putnam and Tang, 1986).

5) Plant tissue culture

Phytochemistry has an important role in plant tissue culture in the detection and analysis of the products of secondary metabolism that may be formed in such tissues. During culture, the concentrations of secondary metabolites may vary depending on the plant hormones added, the presence of light and other factors. In some cases, metabolites are produced which are not present in the plant from which the tissue is derived. For example, anthocyanins may be produced in culture of plants which lack cyanic colour. New pharmaceutically active constituents may be produced in cell or callus culture and good phytochemical techniques are necessary for their detection. Production of such structures may be elicited in cell culture by adding bacterial or fungal cell extracts (Staford and Pazoles, 1997).

Some secondary metabolites such as alkaloids may be produced in cell culture in vanishingly small amounts and radioimmunoassay techniques may be required for their analysis (Zenk *et al.*, 1997). And excellent series of nine volumes has been published, describing

experimental conditions for secondary product synthesis in cell culture and methods of phytochemical analysis for most plants that have taken into cell culture (Bajaj, 1996).

6) Plant genetics

In the past, phytochemistry has contributed to higher plant genetics in providing the means of identifying anthocyanin, flavone and carotenoid pigments occurring in different colour genotypes of garden plants. The results have shown that the biochemical effects of these genes have a simple basis and have indicated the probable pathway of pigment synthesis in these organisms (Alsoton, 1964). The inheritance in plants of other chemical attributes (alkaloids, terpenes, etc.) has also been successfully mapped out by phytochemical analysis.

A more recent contribution of phytochemistry to genetics is in the identification of hybrid plants and in the elucidation by chemical means of their parental origin. Phytochemistry has been won increasing recognition as a useful tool, together with cytology, to be used in the analysis of genetic variation within plant populations (Harborne and Turner, 1984).

7) Plant systematics

One of the most rapidly developing fields in phytochemistry at the present time is the hybrid discipline between chemistry and taxonomy, known as biochemical systematics or chemotaxonomy. Basically, it is concerned with the chemical survey of restricted groups of plant, mianly for secondary constituents but also for macromolecules and the application of the data so obtained to plant classification (Harborne and Turner, 1984).

Perhaps the most useful class of compounds for such study are the flavonoides. Surveys of many other classes of compounds (notably of alkaloids, non – protein amino acids, terpenes and sulphur compounds) have also yielded potentially useful new information for taxonomic purposes. Accurate methods are essential, both in preliminary screening of plants and in the more detailed analysis of individual components.

Chemical analyses of the amino acid sequences of plant proteins have also been brought to bear on systematic problems, at the higher levels of classification. Results have been obtained with cytochrome c, plastocyanin and ferredoxin ; the sequencing of plant nucleic acids has also yielded data of taxonomic interest (Soltis *et al.*, 1992).

8) Medicinal plant research

The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a small fraction of plants with medicinal activity have been assayed. Nearly 50% of drugs used inmedicine are of plant origin. There is therefore much current research devoted to the phytochemical investigation of higher plants which have ethnobotanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity. Cytotoxicity *via* the brine shrimp test is studied in order to reveal now anticancer compounds. Toxol. The new hospital drug from the bark of *Taxus brevifolia*, was discovered in this way. Alternatively, crude plant extracts can be first assayed for particular activities and the active fractions then analysed phytochemically. A variety of bioassays are now available for the phytochemist to use in such work (Hostettmann, 1991).

2.8 Bioassay

The effectiveness of fungicides is determined initially by bioassay *in vitro* and subsequently by practical tests with the host or material to be protected. Several tests for evaluating the practical usefulness of fungicides for controlling plant diseases have been developed (Zehr , 1978). *In vitro* bioassays are most commonly based on measurements of spore germination or growth. The American Phytopathological test in which a spore suspension is mixed with appropriate nutrients for germination and several dilutions of the fungicide to be tested (American Phytopathological Society Committee on Standardization of Fungicadal Test, 1943). Drops of this mixture are placed on microscope slides and incubated , and spore germination is scored after and appropriate time interval. The time interval chosen depends on the time required for spore germination. The time of examination will affect the results obtained and it has been recommended that germination be scored after 6 hr and 24 hr.

Colony diameter measurement is the most popular growth test of fungicidal effectiveness for mycelial fungi. This measures a different process in the fungus and may yield quite different results. For example , dicarboximides inhibited conidial germination of *Botrytis cinerea* less than hyphal growth (Pommer and Lorenz , 1987). Both kind of testing have validity and the preferred test will depend on the expected application. For compounds used in protective measures to prevent initial infection by spores the spore germination assay is most relevant , but for compounds used systemically or therapeutically the growth test may be more meaningful.

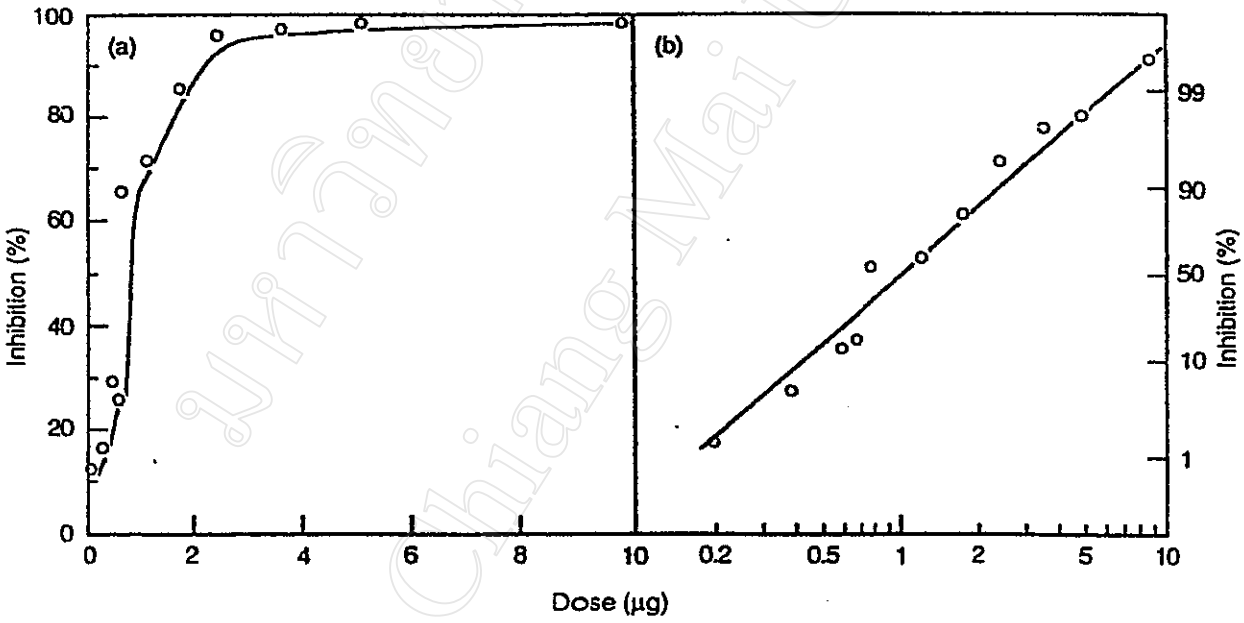


Figure 2.11 The effect of cuprousoxide on germination of condia of *Macrosporium sarcinaeforme* (a) Probability plot of inhibition against dose. (b) linear plot of inhibition against dose. Redrawn from the data of Dimond *et al.* 1941.

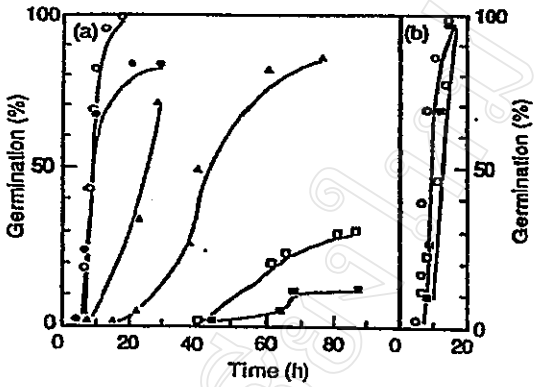


Figure 2.12 Effect of cycloheximide on the germination of *Cladoporium* sp. Condia. (a) germination of spore from culture grown on medium lacking cyclohexane.Cycloheximide concentrations in germination medium were 0 (O), 0.0018 (●), 0.018 (Δ),0.18 (▲),1.8(□) and 18(■) mM. (b) Germination of spores from cultures grown on medium contain 0.18 mM cycloheximide. Cycloheximide concentrations in germination medium were 0 (O),1.8(□) and 18 (■) mM. Redrawn from the data of Griffin *et al.* (1978)

Bioassays usually result in sigmoid curve of mortality or inhibition plotted against dosage or concentration (Dimond *et al.*, 1941) (Figure 2.11 a) . These data can often be transformed to a linear plot by using a probability - log plot (Figure 2.11 b). Such a plot is useful because it is easier to compare straight lines and determine critical points. The critical point most commonly used in comparing inhibitory chemicals are the following :

ED_{50} effective dosage for 50 % inhibition (also called EC_{50} for effective concentration)

ED_{95} effective dosage for 95 % inhibition

ED_{100} mininum effective dose for 100 % inhibition

The latter is also referred to as the threshold response or the minimum inhibitory concentration (MIC). Some materials may not cause 100% inhibition , but even with highly effective inhibitors , the shallow slope of the upper and of the curve with a linear scale plot may make it difficult to accurately determine ED_{95} and ED_{100}. The linear transformation afforded by the propability log plot is useful in estimating these values.

The straight line of a probability plot suggests that individual spores have different resistances to the toxicant, and the variation in resistance among spores follows a gaussian (normal) distribution. The spores of the probability plot measures the dispersion of this distribution of response with a broad gaussian distribution.

The effect of the timing of measurement using the spore germination assay can be seen from tests of the effect of cycloheximide on the germination of *Cladosporium conidia* (Figure 2.12 a). Scoring germination at 24 hr gave and ED_{50} of 11 μM and an ED_{95} of 200 μM. However , continued incubation to 90 hr gave nearly complete germination at these concentrations , and ED_{50} ad ED_{95} values are estimated αs 120 μM and 20 mM , repectively. The sensitivity of this fungus would be misjudged by an assay that terminated after 24 hr.

Another difference among antifungal compounds is that some kill outright and others are merely inhibitory. This can be tested most conveniently after spore germination tests in broth by transfering spores from those test concentration of test compound in the initial incubation that results in no growth after transfer is the minimal fungicidal concentration (MFC). Many compounds do not kill spores at all, their effects being completely reversible in the time frame used. In strict usage these should be termed fungistats rather than fungicides , but this distinction is rarely made and the term fungicides is used generally for antifungal compounds.

2.9 Mode of Action

2.9.1 Introduction

The first class of organic fungicides with captan or the ethylene (bisdithio) - carbamates as typical representatives is classified as "multisite inhibitors". This term described their nonspecific desactivation of several and thus rarely specified enzymes , most often *via* the chemical modification of essential SH groups. (Oven, 1963 ; Sijpestein , 1984). The Adventage of this particular mode of action is the strong inhibition of spore germination mediated by the inhibition of enzymes involved in the mobilization of storage products and respiration. The disadvantage of the multisite mode of action are toxic side effects if antifungal compounds would enter the tissue of the respective host. Systemic movement within plant tissue and , thus , a curative activity is not feasible. The treatment of deep – seated and systemic mycotic diseases with multisite inhibitor would lead to similar toxic side effects.

Several classes of antifungal fungicides and drugs with specific mode of action have been developed over the last two decades. The benzimidazoles , the phenylamides and the large group of sterol biosynthesis inhibitor have reached a high level of acceptance due to their broad spectrum of important diseases (Koller, 1992). In particular , the latter group has become an example for the feasibility to develop a very similar class of inhibitors for both the control of plant diseases and for the treatment of human mycoses. The first representatives of most of our current antifungal groups with site specific properties were introduced between 1965 and 1975. since then , many derivatives with identical modes of action have been developed, but progress with regard to inhibitors with novel target sites has been slow.

For the farmer the lack of "new chemistries" has led to a sometimes alarming situation with respect to the development of fungicide resistance. Regrardless of the availability of excellent fungicides for most of resistance has led to a revival of traditional contact fungicides , used either alone as alternatives or in mixtures with the systemic compound (Koller , 1991). However , some of the older and nonspecific fungicides are, in

many countries under toxicological scrutiny (Nownan, 1988), and the development of new fungicides with different mode of action will become important in the future, either as alternatives to fungicides with resistance problems, or as components of antiresistance strategies. The demand for novel site – specific antifungal inhibitors has, at the same time, dramatically increased in the field of medicine (Tling, 1987). Immunocompromised patients (AIDS, cancer therapy, organ transplants) are prone to deep – seated and systemic mycoses, which are sometimes hard to treat with the currently available arsenal of antimycotic drugs (Spencer and Jackson, 1989; Trelkeld and Dismukes, 1989). Future antimycotic drugs must be fungal specific to warrant a long – term treatment of internal mycoses with out side effects on the patient. Scientists engaged in the discovery of new antifungal agents might be advised to cross discipline more frequently in the future.

Most of our modern fungicides and antifungal drugs have been found *via* random synthesis, biological screening, and empirical optimization of lead structures. Some have been derived from natural product screening and approach not fundamentally different from random synthesis. The mode of action of particular antifungal groups were only identified in the large stages of development. Most often, this initial biochemical work was done by scientists at academic institutions and, thus, outside corporate research, undoubtedly, the "screening appoach" has been remarkably successful in the past, and the role and importance of molecular tools in the discovery of new antifungal inhibitors is not inmediately apparent. It is certainly justified that the "traditional" way of fungicide discovery and the eminent importance of appropiate and reliable screening systems and methods have been emphasized in recent reviews (Ryley and Rathmell, 1983; Ehephard, 1987). Regardless of the undisputed merits of random synthesis and screening, however continuous biochemical studies have yielded valuable contributions to fungicide discovery programs , in particular in the group of sterol demethylation inhibitors with a mode of action identified in 1974 and under continuous development for more than two decades (Berh *et al.*, 1988 ; Janssen, 1957 ; Vanden Bossche *et al.*, 1988, and Vanden Bossche *et al.*, 1989b).

Although a large number of antifungal agents have been introduced in the 1980s, most of these new compounds were derivatives of the groups discovered and

biochemically characterized in the 1970s, and progress with regard to new major antifungal groups with novel modes of action has slowed down. This lack of success is surprising, because effects in fungicide discovery programs did not diminish. Considering an average number of 500 to 10,000 compounds screened for fungicidal activity per year at a major company, and 10 to 15 companies engaged in the development of fungicides and antifungal agents , approximately 1 million compounds have been synthesized during the last decade. Triggered by the slower pace of success, the value of molecular information received increasing recognition, and the integration of biochemistry into fungicide discovery programs was frequently proposed as a more rational tool in the search for novel inhibitor lead structures (Brent, 1983 ; Balduin, 1984 ; Balduin and Rathmell, 1988 and Kuhn, 1989). The advent of fungal molecular biology has added, without doubt, a new dimension to these molecular programs.

Unfortunately , the term "rational" approach is frequently used to distinguish between a biochemically guided discovery program and the traditional screening approach. This appears to imply that the screening approach lacked rationale, which is not the case. The organic chemist engaged in the synthesis of new inhibitor structures applies and contributes to the progress made in the field of (economical) chemical synthesis, and the screening biologist is continuously challenged by the improvement and adjustment of screening systems. Furthermore , the application of quantitative analysis of structure activity relationships (QSAR) (Richardson *et al.*, 1988) of analog series of the basic chemical structure of fungicides has combated both to the design of new fungicides and to the understanding of their mechamism action, studies has a long history not only in drug but also in fungicide research (Rwamura and Fujita, 1982 ; Fujita and Iwamura, 1983). In broad terms, QSAR analysis is the correlation of biological activity with those physicochemical parameters of the inhibitors that are easy to determine. Although QSAR studies can be applied to the analysis of particular enzymes (Deardeu, 1989), Knowledge of the mode of action of an antifungal compound is not essential for these studies. Rather, QSAR studies reflect the rationale of the "random" discovery approach.

Unlike the clear goals of biological screening of compounds derived from random synthesis, the role of biochemistry remains less focused and had been defined in

various ways. In principle, two distinctly different strategies are feasible. One approach would call for the identification of the mode of action soon after biological activities of a randomly synthesized inhibitor have been identified. Within the frame work of this approach, molecular studies would be initiated as soon as a "new chemistry" has emerged from random synthesis and screening. The clear goal would be the contribution of biochemical information to inhibitor optimization. A second strategy would call for the rational design of antifungal inhibitors. This strategy would involve, as a prerequisite, the definition and characterization of a suitable target first, followed by the design of respective inhibitors. Goals such as high inhibitor specificity could guide the initial search for appropriate target sites prior to the initiation of synthesis programs (Koller, 1992)

2.9.2 Inhibitor lead structures derived from random screening

The overall antifungal activity evaluated in biological screening tests in the must integral and almost perfect biological parameter of a newly synthesized compound. The antifungal activity describes not only the intrinsic inhibitory potency, but also additional and essential parameters such as chemical stability, penetration into the fungal cell, and the (systemic) distribution within the host. Considering this wealth of information gained from the overall parameter "Biological performance", it is not immediately apparent why the mode of action and the availability of *in vitro* enzyme tests should contribute to success. This concern is justified and the role of biochemistry within frame work of random synthesis can neither be a substitution of biological screening, nor the mere identification of a fungicidal mode of action. It is the application and integration of biochemical information as tool for optimization of lead structures, once fungicidal activity of "new chemistries" has been discovered.

2.9.3 Biochemistry and ongoing programs

Molecular studies integrated into the random screening of synthetic chemicals or natural products are, by definition, not directly involved in the discovery of novel inhibitor. It would be of restricted value to establish *in vitro* tests for several selected enzymes, and then to search for particular enzyme inhibitors among compounds derived from random synthesis. The response of a whole fungal organism to a putative inhibitor constitutes and elegant, simple, and simultaneous "assay" of hundreds of potential targets, and the statistical changes to find an inhibitor of one specified enzyme by chance are magnitudes lower than those experienced with *in vitro* tests. The goal of biochemical work at this early stage should be the identification of respective target site (s) in order to provide biochemical information for the optimization of lead structures. The advantage of the approach is that the antifungal potency of a lead structure has already been validated. The disadvantage of the approach is that the required for the precise identification of inhibitor target site is unpredictable. It has to be precise. Any synthetic work guide by the catalytic mechanism of a particular enzyme idenitified as the target site would become counteractive if the proposed mode of action is incorrect.

In order to avoid misperceptions, the inhibitory site of "new chemistries" should be narrowed down in a systematic step – by – step procedure. A scheme for the early steps in mode of action evalulation is shown in diagram (Figure 2.13) : Appropriate experimental procedures have been described innumerous publication, e.g. Davidse *et al.* (1983), Ziogas and Davidse (1987) Clemon and Sisler (1971) and Nakanishi and sister (1983). The first step is the careful choice of the fungal test organism according to the antifungal spectrum of the compound. Mode of action studies done with relatively insensitive fungi might lead to results reflecting secondary rather than primary target sites. Once a suitable organism has been chosen, it will be required to determine a suitable time period of inhibitor pretreatment prior to biochemical and anabolic pathways with *in vivo* activities on growth parameters, the inhibitor should be fully penetrated into the cell, but inhibitor action should not be allowed to proceed for long periods. Prolonged inhibitor treatment might lead to metabolic effects reflecting the consequence rather than the cause of inhibitor action.

The first test should be the inhibitor action on respiration. Any target site in catabolic routes but also the inhibition of nutrient uptake will be reflected in a decreased oxygen consumption of whole cells. Pronounced inhibition of respiration implies that AIP synthesis is decreased or abolished , and all catabolic sequences would be inhibited as a consquence of inhibitor action. Inhibitor, that test positive should be tested with host cell tissue , for agricultural fungicides most appropiately with plant cell cultures. This would provide first indications for the suitability of catabolic inhibitors to be optimized toward systemic movement with in the host. The lack of effects on respiration are a first indication for specific inhibitor site in anabolic pathways. The identity of this site can then be narrowed down by incorporation of early radioactive precusors of different classes of and products (Figure 2.13). The incorporation of radioactivity into end products can be easily measured ; it requires an additional analytical step for acetate incorporated into lipids. Here, a particular precursor can accumulate to high levels with out effect of the incorporation of acetate into the total lipid fraction.

The initial steps of evaluation described above are relatively easy to perform, not overly time – consuming , and well suited for a routine setting. It has to be pointed out that the set of data derived from this preliminary evaluation only provides guidance for subsequent studies. However, the informative value should not be underestimated. The data will be too preliminary for the guidance of structural optimization efforts, but they are a valuable contribution to research decisions. For example, extensive efforts to modify a promising antifungal group toward systemic movement in plants would have little changes for success if the first representatives of the group were identified as equally inhibitors of respiration in both the fungal and the plant system. In this case , efforts should concentrate on the question whether the inhibitors represent a multisite or site – specific mode of action (The Carboximides are an example for site – specific respiration inhibitors) (White and Georgopoulos, 1992) and whether selectivity could be improved toward fungal respiration once a specific mode of action has been identified. To the contrary , a clear inhibitory action in a biosynthetic pathway not yet reported for one of

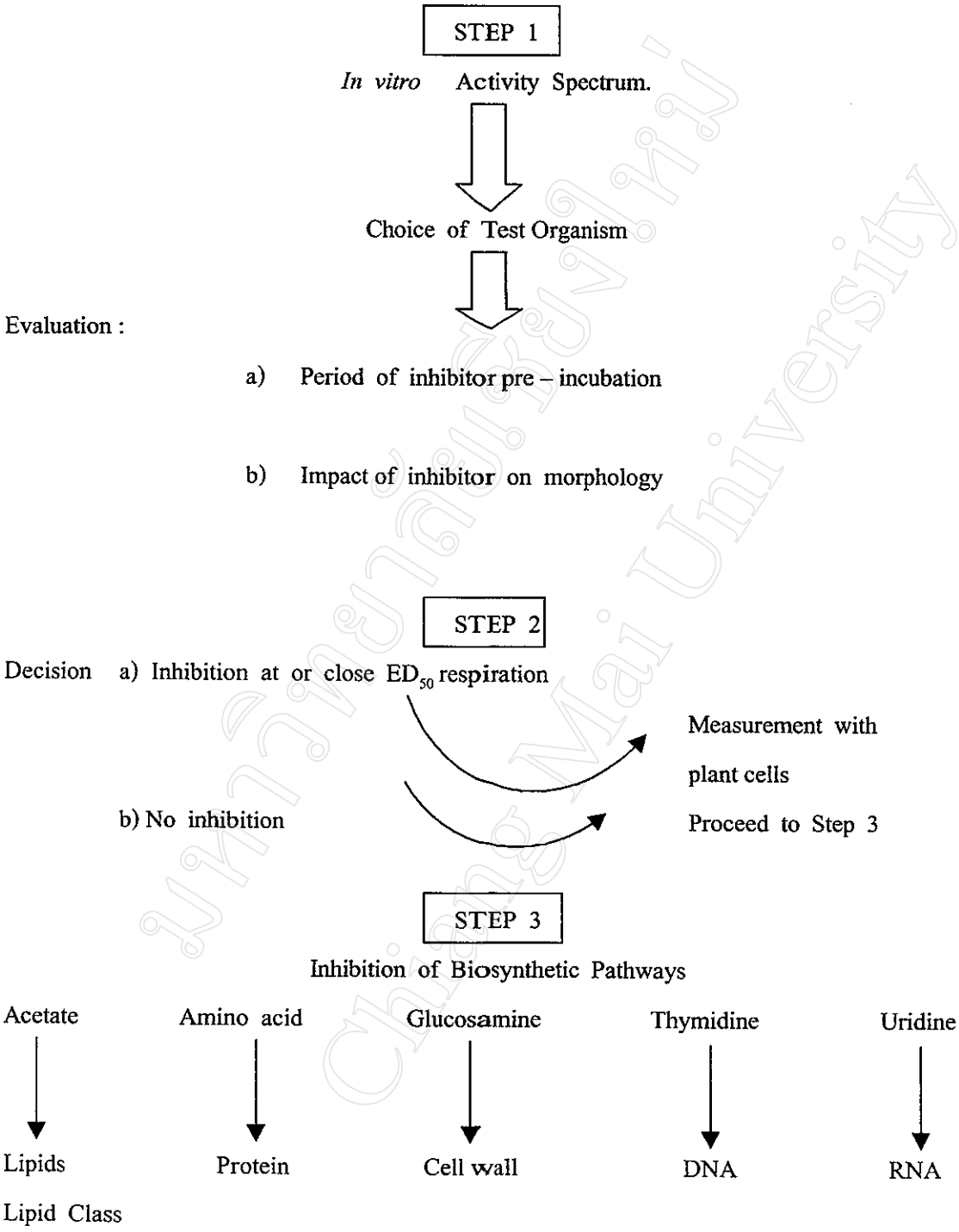


Figure 2.13 Investigative steps of initial mode of action studies. (Koller, 1992)

the established antifungal agents is good indication for a novel and site – specific mode of action and might make a new class of compounds considerably more attractive.

It might be argued that a new chemistry combined with antifungal and probably even systemic activity in screening system is sufficient indication for a novel mode of action. Many examples from the past have shown that this is not the case. For instance, the common mode of action of the first representatives of sterol demethylation inhibitors (DMIs; Koller, 1992) triarimol, clotrimazole and miconazole, is not immediately apparent from their structures (Figure 2.14). In addition, even trained eye of an experienced biochemist would not have recognized the potential of any of the three inhibitors to compete with 24 – methylenedihydro lanosterol for binding to the respective enzyme , the 14 α - sterol demethylase (Koller, 1992) the common mode of action of the three structurally different DMIs, however, would have been recognized in an initial mode of action evaluation according to the scheme outlined above. This would have opened the opportunity to broaden the chemistry of DMIs much earlier than it eventually happened.

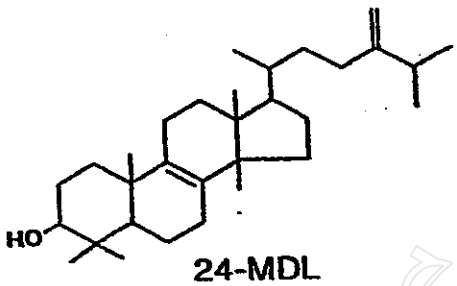
Even if the data derived from a systematic and early biochemical evaluation comprise a contribution to research and development decisions, the question remains whether an in depth study aimed at the precise characterization of the respective target site would add more information and opportunities to the development of a particular group. In contrast to the initial evaluation , these studies may consume considerable time. Furthermore, the dicarboximides and benzimidazoles are good examples for inhibitors, where the schematic initial evaluation would have provided very little information with regard to the mode of action. Even if these concerns are justified at times, the identification of the target site has its clear intrinsic values and might open group of already developed opportunities for efforts to reinitiate research on a group of already developed inhibitors. The melanin biosynthesis inhibitors described by Yamaguchi and Kubo (1992) are an excellent example to demonstrate this opportunity. The chemical
structures of melanin biosynthesis inhibitors (MBIs) are diverse and are not indicative for a common mode of action. Once the mode of action had been clarified for tricylazone

(Figure 2.14), it became apparent the compounds such as pyoquilon and others were identical with regard to their mode of action. Employing the antimelanization activity as criterion , two approaches for the synthesis of new MBIs. Inoue *et al.* (1985) identified a benzo – bicyclic ring system containing a substituted nitrogen in the ring in α - position to the benzene ring, and two additional substitutions at specific sites (Figure 2.14) was common structural features; new MBIs previously not reported were synthesized. Although this structure – activity study was guided by the known mode of action of structurally different compounds, the approach to utillize this information was basically chemical nature. A more biochemically approach was reported by Omata *et al.* (1989). According to the published mode of MBI action (Yamaguchi and Kubo , 1992) the authors assumed a competitive inhibition of the 1 , 3 , 8 – trihydroxy – naphthalene (Figure 2.14) and compared the known MBIs with the structure of the respective enzyme substrate. Based on this structural information, a series of phthalazine derivertives was synthesized, and several representatives of these designed inhibitors were indeed active as MBIs.

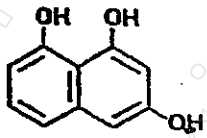
Lead structures of novel antifungal agents are not only derived from organic synthesis programs, but also from natural products. The screening of microbial products or plant extracts is, more often, random and not principally different from synthetic chemicals. However, the selection of microorganisms or plants can be based on existing information. For example , an antifungal component present in garlic extracts, a component of "folk love" medicine , has been found to inhibit lipid biosynthesis in *Candida albicans* (Adetumbi *et al.*,1986). Although the structures of numerous antifungal inhibitors produced by microorganisms or extracted from plants have been described in the past (Marston and Hostettman, 1987 ; Fawcett and Spencer, 1969 and Harborne , 1987) , respective modes of action are rarely known. This lack of information, however , does not exclude that interesting lead structures can be derived form natural sources. An example of biochemical considerations in the characterization of a plant product, guided by a comparison to known fungicides with similar biological activities, is a study on the rice blast activity of the diterpene pisiferic acid (Figure 2.15), a performed antifungal plant product isolated from Cupressaceal (Kobayashi *et al.*, 1978). Computer graphic analysis of pisferic acid analysis revealed similarities with the rice blast fungicide probendazole, a saccharin derivative

(Figure 2.15). Indeed, the two compounds shared similarities when the effects in the hostpathogen interaction were compared , indicating a similar mode of action. Although not all details have been clarified yet, the mode of action of probendazole, as discussed in greater detail below, has been shown to be indirect *via* the activation of defense reactions within the host plant rice (Watanabe *et al.*, 1985 ; Shekizawa and Mase , 1981 and Shekizawa *et al.*, 1985)

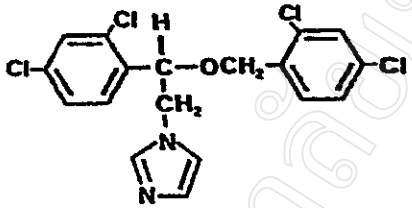
A second class of preformed antifungal plant metabolites are two groups of plant steroids, the saponines and glycoalkaloids of the *Solanum* type (Roddick, 1988 ; Schlosser, 1988) These compounds are characterized by a steroidal aglycone attacheded to a carbohydrate moiety; the structure of α - tomarine as a typical representative of this class of compound with a relatively broad spectrum of antifungal activities is shown in Figure 2.15. Although the question exists of whether the glycosides or the aglycones formed by glycosides action are the more active compound, little doubt exists that the saponines act by forming 1 : 1 complexes with sterols in fungal membranes (Roddick,1987). The mechanism of membrane disturbance is very similar to the action of the polyene macrolide antibiotic which reported by Koller (1992). The expression of fungal resistance to saponines has been related to a decreased ergosterol content in fungal membranes, again very similar to the macrolider (Defago and Kern, 1983 ; Defago *et al.*, 1983). The more indirect and not highly selective action on biological membranes implies that saponines have a restricted value in providing lead structures for novel modes of antifungal action. A non specific mode of action might also apply to laetisaric acid (Figure 2.15) , a midchain hydroxyllated linoleic acid produced as a allelopathic agent by the soil fungus *Laetisaria arvalis* a potential biocontrol agent (Bower *et al.*, 1986). Leatisaric and caused rapid lysis of *Phytium* membranes (Bower *et al.*, 1986). Similar fatty and derivertives isolated from plants have been reported to act as antifungal agents (Harborne, 1987)



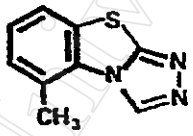
24-MDL



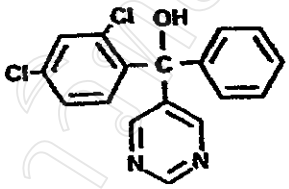
1,3,8-THN



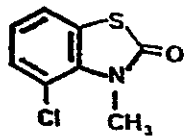
Miconazole



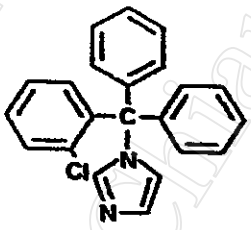
Tricyclazole



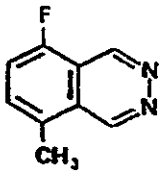
Triarimol



Chlobenthiazone



Clotrimazole



Phthalazine
Derivative

Figure 2.14 Comparison of enyzme substrates with inhibitor stuctures.

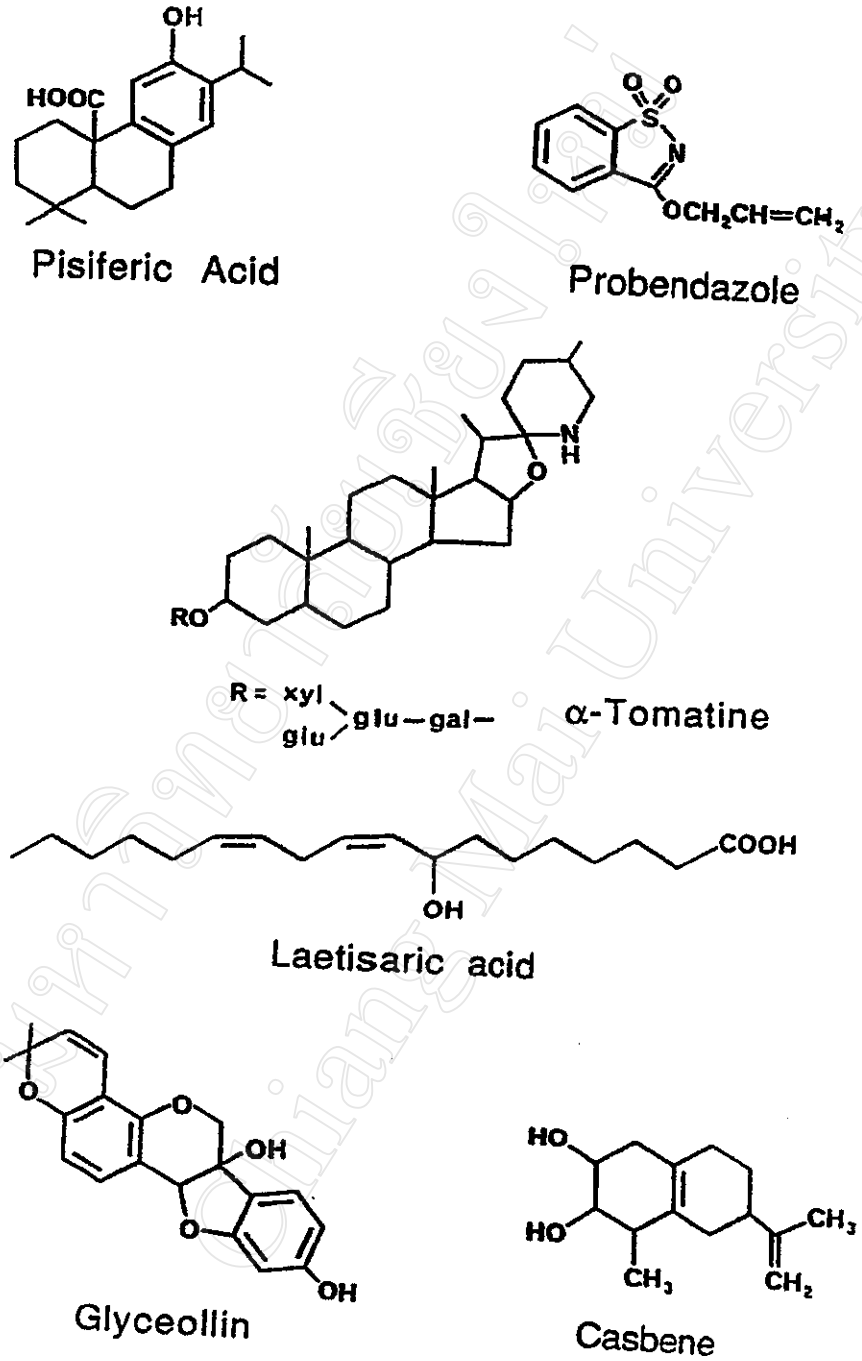


Figure 2.15 Natural product with antifungal activities.

A second class of antifungal plant metabolites are phytoalexins. Which are synthesized *de novo* as a defensive response to fungal attack , but also under abiotic stress condition (Baily and Mansfield,1982 ; Harborne, 1986). Since the development of the phytoalexin concept in 1940 and the first structural identification of the first phytoalexin pisati in 1960, research in this area has been a major subject of molecular plant pathoglogy. The most extensively studied phytoalexins belong to the class of isoflavonoid – derivertive compound , such as glyceollin , and to terpenoids such as rishitin (Baily and Mansfield, 1982 ; Harborne, 1986). Considering the wealth of information , it is surprising that studies on the mode of action are relatively rare. van Etten and Bateman (1987) first demonstrated phaseollin, and isoflavonoid – derived phytoalexin, drastically affected the functions and integrity of plasmamembranes of *Rhizoctonia solani*. A nonspecific membrone effect was indicated by plant cells as well as erythrocytes. These results were confirmed and substantiated by Yoshikawa *et al.*, working with *Phytophthera capsii* (Yoshikawa *et al.*, 1987). The fact that artificial liposomes became permeable after treatmant with phaseollin , and that the presence of sterols had no effect on the membrane – disturbing action indicated that the phytoalexin acts as a general membrane antagonist. Fluorescent depolarization studies supported the view that fungal plasmamembranes are the most sensitive site of action (Yoshikawa *et al.*, 1987). A non specific membrane action of glyceollin is supported by the inhibition of electron transport in isolated soybean mitochondria (Boyston *et al.*, 1983), and the plasma membrane ATPase isolated from red beet (Giannini *et al.*, 1988). A similar membrane – disturbing effect has been reported for other isoflavonoid- derived phytoalexins , but also for the structurally different terpenoid phytoalexins such as rishitin (Figure 2.14) (Yoshikawa *et al.*, 1987; Smith, 1982). Overall, the phytoalexins appear to act , similar to most of the investigated preformed antifungal plant metabolites, as multisite toxicants with biocidal effects on membranes rather than as site – specific compounds with distinct target site.

Considering the widespread occurrence of secondary plant metabolites with antifungal activities, be it preformed or induced under stress conditions, it is astounding that no site specific inhibitor has been identified thus for. Consequently, the chemical structures of

know antifungal plant products are not ideal chemical lead for the synthesis of specific inhibitors. As discussed by Mc Laren (1986), phytoalexins as such have little chances to be considered as chemical control agents. The vast majority is less active than chemical fungicides. ED_{50} values are typically in the range of 20 to 50 µg/ml, and chemical derivatives are normally not dramatically more active (Schullz *et al.*, 1990; Lake and Pruner, 1990). The current lack of specific antifungal inhibitors of plant origin, however does not necessarily indicate that plant metabolites of this quality do not exist, and screening programs combined with mode of action studies might be successful. The question of whether these programs would have advantages over conventional synthetic programs is not easy to asseses. It should be mentioned, however, that both screening strategies are mainly based on chance, and that the toxicological risk of man – made vs. natural chemicals appears to be more or less equal (Ames *et al.*, 1990a ; Ames and Gold, 1990 ; Ames *et al.*, 1990b).

The future potentials of most antifungal natural products such as phytoalexins might be in the genetic engineering of disease – resistance plants rather than in their usefulness as leads for novel synthetic fungicides. Although phytoalexins have been the subject of extensive studies over the last three decades, the question of to what degree these antifungal metabolites are involved in the expression of resistance is still under debate (Baily and Mansfield, 1982 ; Harborne,1986). Perhaps role of phytoalexins has emerged from work with *Fusarium solanum* f.sp. *pisi* and its host pea, a plant that responds to fungal attack and other stress by the synthesis of pisatin. Van Etten and co – workers have shown that pathogenicity of *F. solani* was dependent on the detoxification of pisatin by an inducible cytochrome P – 450 mono oxygenate (pisatin demethylase) (Van Etten *et al.*, 1989). Furthermore, the transformation of the corn pathogen on the non host pea (Schafer *et al.*, 1989). As critically discussed by van Etten (van Etten *et al.*, 1989) the alternation of phytoalexin synthesis in a given plant would implicate that a given pathogen would not be able to tolerate this "foreign" antifungal metabolite. The success of this and similar strategies would rely on several premises. For example, the new phytoalexin would have to be under the control of an inducible promoter (constitutively expressed phytoalexins would be phytotoxic), and even the tissue – specific expression might be required potential

mammalian toxicity of phytoalexin – containing plant tissues). However, rapid process triggered by the novel techniques of molecular genetices is being made in this field, and it can be expected that the merits of these strategies will be tested in the nearer future.

A disadvantage inherent to many natural products is their complex chemical structures. For example, diterpenes such as sclareol (Figure 2.16) isolated from *Nicotiana* spp. Have been shown to control rust and powdery mildews (Severson *et al.*, 1985; Banthorpe *et al.*, 1990). The stereochemical complexity of these secondary plant products would prohibit the chemical modification of these compounds, a strategy frequently applied to natural products produced by microorganisms, would not be applicable. The yields obtained from plant extracts are too low to be of economical interest. The only approach could be the identification of the mode of action, followed by attempts to simplify the inhibitors toward chemically accessible structures. In some cases, however, the structures of antifungal natural products are simple enough to be utilized in the synthetic programs. Methoxyacrylates such as strobiturin A (Figure 2.16) are an example of recent interest (Beautement *et al.*, 1991). These structures, which have been identified as respiration inhibitors with a complex III target site in beef heart mitochondria (Becker *et al.*, 1981) are of simple nature and have served as chemical leads in synthetic programs (Anke et. *al.*, 1983 ; Beautement *et al.*, 1991) a relatively simple structure has also been identified for harzianopytridone, an antifungal metabolite produced by *Trichoderma harzianum* (Rodgers , 1983). Antifungal activities of a small number of chemical derivertives were not largely improved (Rodgers, 1983).

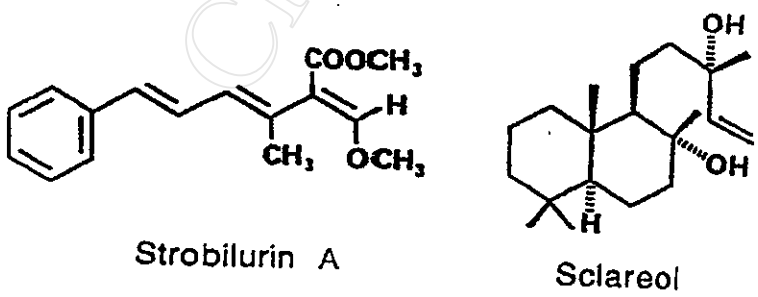


Figure 2.16 Differences in chemical complexity of natural inhibition.

2.10 Influence of Fungicides on Fungal Fine Structure

Different strategies of antimicrobial therapy in plant, human, and veterinary medicine have been developed to protect biological tissue from fungal infection and damage. The discovery of systemic fungicides has been important advance in the development of new chemical therapies. Unlike protective, nonsystemic, and in most case nonspecific compounds, systemic fungicides are characterized by mobility in plant tissue and selective activity against the pathogen at the infection site. The selectivity is based on structural and physiological differences between host and fungal parasite. For a comprehensive understanding of the mode of action of antifungal agents, biochemical and cellular aspects have to be taken into account. Electron microscopy can provide unique information concerning the influence of fungicides on the fine structure of the pathogen and host. The present review gives a current view of the ultrastructure research in the pharmacology of some important selectively acting agents. More details has been reviewed by Hippe (1998).

2.10.1 Sterol - biosynthesis – inhibiting fungicides

This class, sterol – biosynthesis – inhibiting (SBI) fungicides, contains functionally related compounds belonging to different chemical (e.g. azole, pyrimidines, morpholines, and allylamines). Their selective fungi toxic action occurs at various steps in the sterol pathway (Figure 2.17). Fungi that synthesize their own sterols are generally considered to be sensitive to this class of fungicides, although some of the SBI's exhibit a high degree of selectivity. On the basis of their generally broad antifungal spectrum, SBI fungicides have become the most important group of chemical agents used at present in agricultural and medicine against filamentous and dimorphic fungi, as well as pathogenic yeast (Trinci and Ryley, 1984). Morphological and ultrastructural modifications induced by various SBI compounds have been analyzed in both qualitative and quantitative electron microscopic investigation. Striking morphological abnormalities were observed in cell culture of *Ustilago avenal* and other phyto – and human, pathogenic fungi treated with various SBI's by scanning electron

microcopy (Barug *et al.*, 1983 ; Hippe′, 1983 ; Meingassner *et al.*, 1981). These data suggest that SBI's dramatically affect the regulation of morphologenesis and fungal development. Rapid modifications of the fine structure of hyphal tips are especially relevant in this regard. Following short – term treatment with propiconazole , disruption of SpitzenkÖrper and cytoskeletal integrity in hyphal tips of germ tubes of *Puccinia graminis* f.sp. *tritici* has been documented (Dahmen *et al.*, 1988). Varying the SBI – fungicides concentration and the time of application, both *in vitro* and *in vivo* experiment resulted in a common sequence of ultrastructural changes leading to cellular degeneration and finally cell death. The sequence included undulations of the plasmalemma, increases and destabilization of the ER, disturbances of the mitochondria , accummulation of lipid – bodies , vacuolization and exocytosis , considerable thickening of cell walls, and incomplete septa formation (Hippe′ *et al.*, 1980 ; Hippe′, 1983 ; Meingassner *et al.*, 1981 ; Sancholl *et al.*, 1988). Concerning the increased occurrence of lipid containing vacuoles in triazole resistant strain of *U. avenae* , an involvement in the resistance mechanism against SBI compounds can not be excluded since an accumulation of fungicide has been found in these area (Hippe′ , 1987).

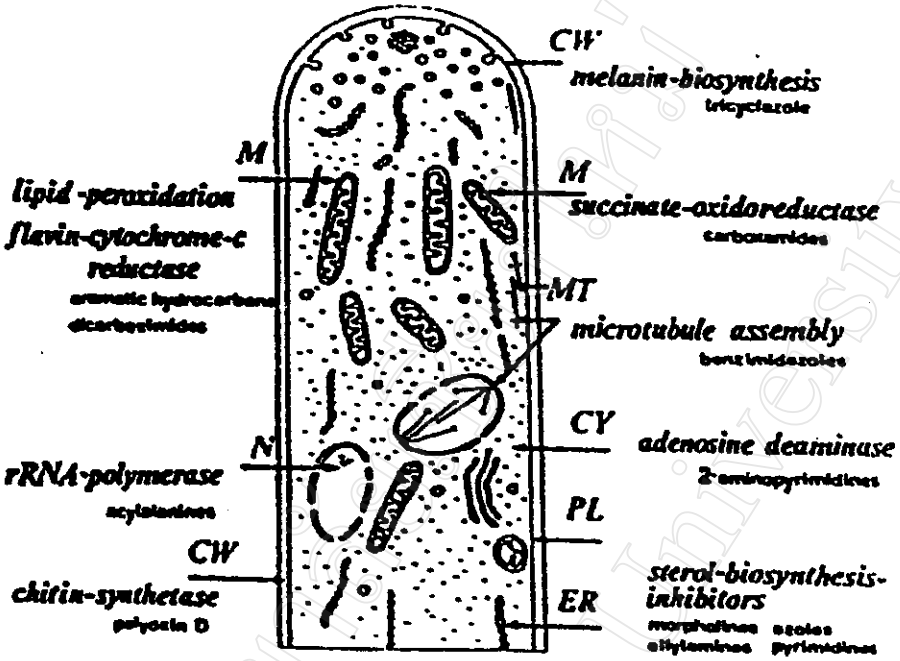


Figure 2.17 Site of action of systemic fungicides on fungal fine structure.

According to available biochemical data on the alteration of sterol structures in membranes and other modifications in membrane lipid composition, fine structural changes have been interpreted as a consequence of the deterioration of membrane function due to alterations in biophysical properties of the lipid bilayer (Sancholle *et al.*, 1988 ; Steel *et al.*, 1989). Changes in membrane composition may modify the activity of membrane – bound enzymes involved in cell wall synthesis, leadind to irregularities in the formation and composition of the cell walls. However, and increase of lipid peroxidation caused by SBI compounds cannot be excluded as an indirect cause for modifications of fungal growth and cell wall formation in SBI sensitive and resistant fungal strains under long term stress (Lyr, 1987, Hippe' and Giesen, 1988). Further evidence for specific alterations in membrane fine structure were provided be freez – fracture analysis of triazole - treated sporidia of *U. avenac* (Hippe', 1985). Triadimenol is thought to change the elasticity and fluidity of

biological membranes , since rearrangements of proteinous intramembrane particles (IMP's) leading to the formation of 2 , 4 – D hexagonal cluster of IMP's was demonstrated . The crystalline hexagonal ordering of IMP's was explained by a simple physical model based on the length of the mismatch between the lipid bilayer and the proteinous particles (Hippe and Liith, 1986). It is proposed that the liquifying (decreasing order) effect of SBI fungicides on the lipid bilayer strengthens the IMP interaction and thereby induces a phase transition of the IMP's from a random to a hexagonal distribution. Biophysical measurements on the fluidity of liposomes prepared from morpholine and azole – treated fungi ; support that idea (Steel *et al.*, 1989). Investigations of our understanding of the mode of action of SBI fungicides. In powdery mildew – infected plant tissue most of the fungicide triadimenol was found in epidermal cells containing haustoria (Howard and Aist, 1980). The formation of haustoria (Hippe', 1985) was severely inhibited by triazole fungicides (Propiconazole , triadimenol) , whereas the development of germ tubes and appressoria was not affected (Heller *et al.*, 1990 ; Smolka *et al.*, 1988). In particular , fungicide – induced degeneration of haustoria of powdery mildew included swelling of the sheath membrane, damage and vacuolization of the haustorial cytoplasm , accumulation of electron dense material, and thickening of the haustorial cell wall. The accumulation of plant material at the haustorial neck appeared to extend and to completely encapsulate damaged haustoria (Heller *et al.*, 1990 ; Smolka *et al.*, 1988). Effects on the growth, sterol metabolism , and cellular fine structure of plants have also been described, and might partly contribute to antifungal action in a still unknown way. The prime target of SBI agents is, however, believed to be fungal sterol biosynthesis.

2.10.2 Benzimidazoles : inhibition of microtubules assembly

The Group of bezimidazole fungcides together with the thiophanates, which are transformed to benzimidazoles, contain a large number of chemically related compounds (e.g., benomyl, carbendazim, nocodazole) which act by binding to β-tubuling and thus disrupting microtublue function. In agriculture and medicine many bezimidazole derivatives have been developed for practical plant disease control and cancer chemotherapy, respectively. The biochemical basis for their selectivity is due to a differential binding affinity of the tubulin from the various organisms. Microtubules, which are ubiquitous cytoskeleton constituents of the cell, are dynamic structures that continually undergo assembly and disassembly. Inhibition of microtubule assembly in fungi by benzimidazoles disturbs cellular processes dependent on microtubules, such as mitosis and meiosis, the intracellular transport of molecules, and the movement of organelles. Interference of bezimidazoles and thiophanates with nuclear processes in certain plant pathogenic and nonphytopathogenic fungi, as well as in other biological systems, has been convincingly demonstrated by electron microscopy. Therein effects of these agents are manifested by a disorganization of the cellular contents of fungal spores and hypha, mammalian and animal cells. Metaphase arrest of mitosis, the aggregation of cytoplasmic vesicles and the ER as well as an irregular thickening of the cell wall have been reported in benzimidazole-treated tissues . The effects of carbendazim, the degradation product of benomyl, on the fine structure of *Fusarium acuminatum* have been documented in detail using advanced low temperature preparation techniques in conjunction with electron microscopy. The ability of carbendazim to disturb specifically the cell structure and influence cell development directly after application was clearly demonstrated by the examination of freeze-substituted hyphal tip cells in a short-term analysis. In untreated cells the Spitzenkörper region contained a large number of apical vesicles and microvesicles, lying within a network of microfilaments. This accumulation of intracytoplasmic vesicles at the hyphal apex containing material necessary for cell wall synthesis in dicates the direction of cell expansion. Microtubules which are generally orientated parallel to the long axis of

the cell are believed to maintain the SpitzenkÖrper integrity. Carbendazim altered the tip cell ultrastructure and the SpizenkÖrper integrity within ten minutes of exposure. Microtubules were no longer observed as ubiquitonus components in the apical cytoplasm. The fungicide caused a simultaneous redistribution of apical vesicles, including a gradual disappearance of the Spizenkö rper. Presumably, the reduction of cell expansion is a result of the disruption of microtubule-mediated transport of cell wall material. Prolonged exposure to carbendazim was associated with increased cytoskeleton destruction, an irregular deposition of cell wall layers, and an appearance of autolytic vacuoles and multivesicular bodies in the cytoplasm. These bodies have also been observed in other fungal organisms after treatment with bezimidazoles. The relative insensitivity of both cytoplasmic microtubules in subapical cells and of spindle body-associated microtubules to carbendazim has been explained on the basis of different rates of metabolic turnover of cytoplasmic and nuclear microtubules. Inaccordance with biochemical results, these elecron microscopic data support the hypothesis that microtubules are the primary target for benzimidazoles. However, the disruption of microtubule assembly may also result from alterations to some other target important in microtubule regulation and microtubule functioning.

2.10.3 Microscopic studies of ultrastrcture

Electron microscopic studies can be seen as an important step to a better understanding of the mode of action of fungicides on the fungal pathogen and host parasite interface. Ultrastructural data provide leads biochemical and biophysical investigations and help to resolve the relationship between the influences of fungicides on the function and structure of treated bilogical systems.

2.11 Formulation

The formulation is the final physical condition followed by application of pesticides. Formulation is the processing of a pesticidal compound by any method that will improve its properties of storage, handling , application, effectiveness, or safety. The term formulation is usually reserved for commercial preparation prior to actual use and does not include the final dilution in application equipment (Ware, 1983).

Formulation trends shift with time and need. Traditionally, pesticides have been applied as water sprays, water suspensions, oil sprays, dusts and granules. Spray formulations are prepared for insecticides, defoliants and desiccants. Consequently, more than 75 percent of all pesticides are applied as sprays. The bulk of these are currently applied as water emulsions made from emulsifiable concentrates, sometime abbreviated as EC.

2.11.1 Emulsifiable concentrates (EC)

Emulsifiable concentrates, synonymous with emulsible concentrates, are concentrated oil solutions of the technical grade material with enough emusifier added to make the concentrate mix readily with water for spraying. The emulsifier is a detergentlike material that makes possible the suspension of microscopically small oil droplets in water to form an emulsion (Ware, 1983). Although they are the most widely applied liquid formulation, ECs also have disadventages. Some of the organic solvents used in ECs may be harmful because of their toxicity and their flammablility. ECs are also coming more and more under regulatory pressure due to the organic solvents, may of which contribute to volatile organic compounds (VOC) emissions. As a consequence, formulation chemists have to look for new solvent systems. These alternatives do not always have same performance characteristics, such as active ingredient loading capacities, miscibility with water , and stability profiles (Foy and Pritchard, 1996)

1) Formulation tools – components and methods. (Nayayanan , 1966)

1.1) Components

An agricultural formulation as an emulsifiable cencentrate (EC) or microemulsifiable concentrate (MEC) will contain the following typical components:

* Active ingredient (s)
* Solvent (s)
  - primary solvent
  - secondary solvent, cosolvent
* Emulsifier (s)
  - primary emulsifier (dispersing agent)
  - secondary emulsifier (secondary dispersing agent, wetting agent)
  - additional wetting agent
* Polymeric stabilizers (crystal inhibition)
* Preservatives (optional)
* Others

These concentrates should produce stable emulsions microemulsions on dilution. Stability criteria depends on the use pattern.

1.2) Active ingredients

The general trend is to formulate active ingredients of wide biological spectra in a single formulation. The active ingredient should not chemically react in the medium. The formulators choice of the active ingredients is limited. The choice is dictated by the history of biological performance. Thus, the formulation matrix must be robust in being capable of formulating structurally different active ingredients.

1.3) Solvents

Choice of the solvent is based on physical properties such as solubility of active ingredients. Flash point , toxicity (Becher, 1985), vapour pressure, compatibility with emulsifiers, solubility in water, thermal/chemical stability, and phytotoxicity. Most of the data are available from the suppliers. The solvent to be chosen must show high solubility for the active ingredients. On dilution of the concentrate, it is the solvated molecules of the active ingredients that are emulsified , and therefore higher solubility generally results in higher loading of the active ingredients (Narayanan and Chaudhuri, 1992). Flash point of a formulation will generally be higher than the lowest flash point of the component. However all interactions of the components are to be considered in judging the flash point of a formation. It is best to detemine the flash point of a formulation experimentally (Catanach and Hampton, 1992 ; Myers, 1988). Use of solvents with low vapor pressure is advantageous in preventing crystallization of solid active ingredients form the spray droplet after deposition on the target. Most solvents can evaporate as azeotropes with water. Solvents with low vapor pressure not only provide low VOC in the formulation, but may keep the active ingredients in the micellar form, when most of the water evaporate. Figure 2.17 show relative evaporation rates of different solvent systems and Figure 2.18 for the relative advantage of using a nonevaporative solvent system in the formulation (Narayanan, 1993).

Solubility characteristics of the solvents can be evaluated from the Hanson's fractional solubility parameters (Barton, 1983 ; Narayanan and Chaudhuri, 1993). The use of solubility parameters to optimize the emulsifier system was attempted by Meusbuger (1983) in formulating an o/w emulsion for DDT. A computer systems for the given actives ingredients from group contribution (Meusburger, 1983). Phytotoxicity of a number of hydrocarbon and oxygenated solvents is published (Krenek and King, 1978). The general trend is that xylene range solvent show highest phytotoxicity (Krenek and Rohed, 1988; Manthey and Nelewaja, 1992). Aliphatic hydrocarbons showed lower phytotoxicity to host crops (soybean, corn, wheat, and cotton) compared to aromatic hydrocarbon. Oxygenated solvents that have higher polarity are generally more phytotoxic. Surface tension of the solvent and the evaporation rate would also affect its phytotoxicity. As some of the

solvents, particularly these that are surface active, can enhance the biological activity of the formulated pesticides, and consequently will require a lower dose of the active ingredients, phytotoxicity has to be evaluated with the formulation at appropriate dose level for reduced spray volume rate (Login *et al.*, 1992; Narayanan and Chaudhuri, 1993).

Solvent – based formulations are generally preferred for foliar-applied pesticides, as they tend to enhance uptake and translocation *via* penetration, cuticular solubilization and stomatal entry. The uptake is also influenced by the surfactants *via* cuticular diffusion (Bauer and Schonherr, 1992; Riederer and SchÖnherr , 1990; SchÖnherr and Bauer, 1992) or stomatal infiltration (Field and Bishop, 1988).

Some of the newer solvents that are relatively safe and approved by the U.S. EPA and/or approved in Europe are alkylpyrrolidones (Narayanan and Chandhuri , 1992) alkylbiphenyls (Vincent, 1992), and tetra hydrofuran derivertives (Doyal *et al.*, 1992).

Use of mixed solvents is an alternative approach for formulating EC's and microemulsion concen trates (Narayanan and Chaudhuri, 1993; Narayanan and Chazdhurion, 1994 ; Narayanan and Chazdhuri, 1994 b; Narayanan *et al.*, 1995 ; Narayanan *et al.*, 1993a ; Narayanan *et al.*, 1993 b; Narayanan *et al.*, 1992 ; Narayanan *et al.*, 1991).

The solvent combination is such that one of the solvents is a highly polar solvent (with Hansen's fractional solubility parameter : polar component f_p is > 0.3 and dipersive component $f_d < 0.5$)., the second solvent being hydrophobic (with $f_d > 0.6$ and $f_p < 0.25$) and surface active and optionally a third highly hydrophobic solvent with $f_d > 0.8$. Mixed solvent approach is useful to simulate several existing solvents (often toxic) by using a limited number of well-studied and environmentally safe solvents. Solvent mixtures. Figure 2.18 summarizes the Hansen's fractional solubility parameters for some of the common solvents. Appropiate mixture of solvents, for example, solvent 1,2 and K can simulate many other solvents listed (Narayanan and Chaudhuri, 1992).

The solvent combination is so chosen to provide excellent solubility for the active ingredients. The ECs prepared by using appropiate surfactants provide excellent stability on dilution, especially if one of the hydrophobic solvents is surface active.

1.4) Emulsifiers

Choose of the emulsifier in a formulation, particularly as applied to ECs and microemulsion concentrates or microemulsion, depends upon the properties of the emulsifiers. The following properties of an emulsifier will assist the formulation in making an appropriate selection in a particular formulation: the structure, polydispersity, HLB value, PIT, solubility in water and oil phase, Kraft point, surface tension, interfacial tension with model system (oil/water) dynamic surface tension, critical micelle concentration (CMC), synergy with other emulsifiers, wetting efficiency, capacity to form complexes with solvents and polymeric stabilizers. Generally, supplys will provide some model formulations as guidelines. (Nayayanam, 1996)

The emulsifiers are one of the most important innert components in agricultural formulations. The major effect in formulating is to identify the most appropriate combinations of emulsifiers for a given system of active ingredients (a.i.s) and solvent (oil or water), (Nayayanam,1996).

The criterion is to obtain the required stability of the concentrate, even on dilution with water that has a normally high salt content. Since the composition is very complex, the choice of emulsifier is not obvious , and a background, knowledge and experience in the field of formulation will be very helpful. A screening process is essential before optimization of the emulsifier system, Another criterion for the end-use formulation is compatibility with other commercial pesticide formulations in the tank mix (Becher, 1992 ; Collin and Munie, 1992).

Hypohilic - Lypophilic Balance (HLB) surfactants.

The HLB value gives a measure of the relative simultaneous attraction of the emulsifier for water and oil (the two phases present in an emulsion). HLB values can be either calculated in term of empirical group contributions given in Table 2.6 - using Equation as HLB = 7 + hydrophilic group number - hydrophilic group number , or by experimental

determination by comparing performance of the experimental emulsifier with standard mixtures of known HLB (e.g. sodium oleate HLB = 20 , and oleic acid HLB = 1).

Expensive bibliographies are available on HLB of several surfactants, their trade names, along Mc Cutcheon (1993). Figure 2.19 shows the effect of HLB of the emulsifier and the type of resulting emulsions.

HLB values, which are helpful in screening emulsifiers as a first-step process to determine the type of emulsions, are not predictive of emulsion stability. Emulsion stability is a complex function of drop size, interfacial viscosity , interfacial film elasticity, magnitude of electrostatic and steriorepulsion internal phase volumn, and the cross sectional area of the micellar component. Therefore, application of the HLB concept cannot solve the practical emulsion problems, but can be used as the first step in the screening process (Valkenburge, 1973 ; Foy and Pritcharcl, 1996)

Choice of emulsifiers on term of cohesive energies has been attempted for formulating ECs (Meusburger, 1983). A formulator typically chooses the best solvent option for the active ingredients (taking into consideration cost, toxicity, environmental impact) and emulsifying agents that optimize the desired end result. Optimization is therefore done by manipulating surfactants, usually a mixture of nonionics and anionics. Triangular coordinate diagrams are useful in setting up mixture design for composition - based on optimization (Figure 2.18), (Becher, 1995; Maokerjee, 1983; Skelton *et al.*, 1992)

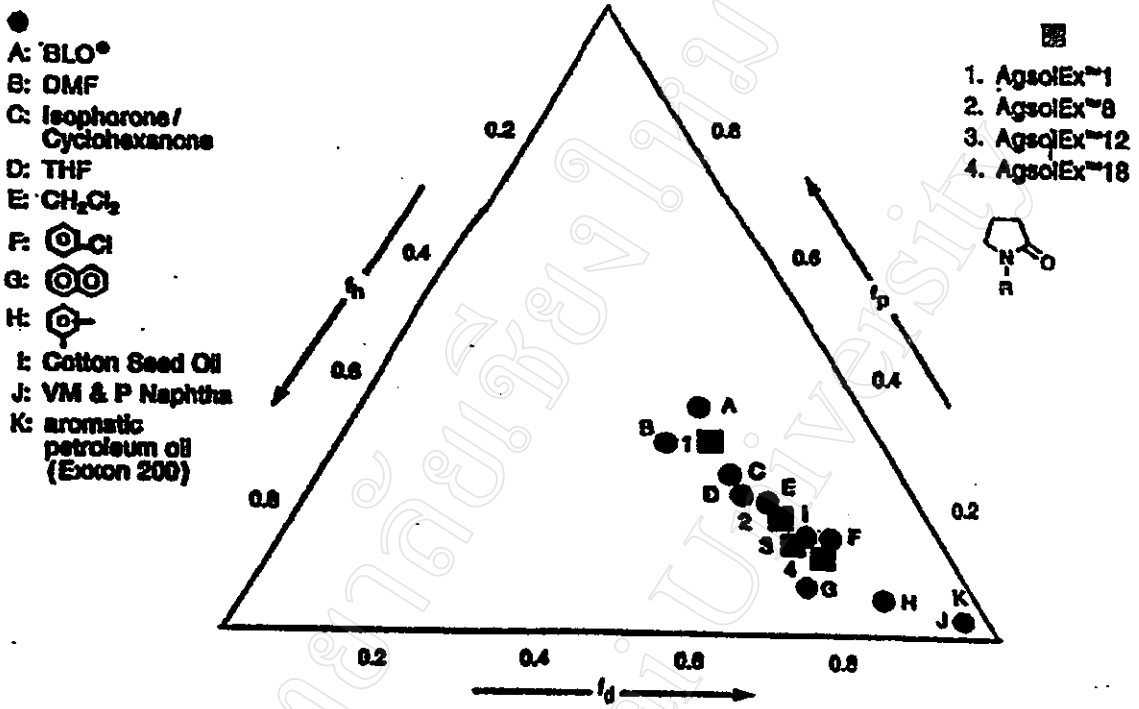


Figure 2.18 Hansen's solution parameter .

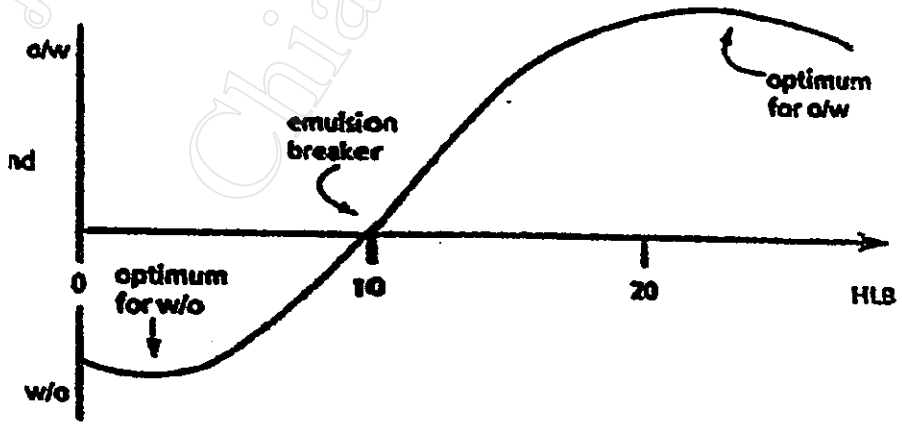


Figure 2.19 Variation of type and amount of residue emulsion.

Table 2.6 Davis's HLB group Number.

Groups	Group Numbers
Hydrophilic Groups	
$-OSO_3^-Na^+$	38.7
$-COO^-K^+$	21.1
$-COO^-Na^+$	19.1
N (tertiary amine)	9.4
Ester (sorbitan ring)	6.8
Ester (free)	2.4
-COOH	2.1
-OH (free)	1.9
-O-	1.3
-OH (sorbitan ring)	0.5
Lipophilic Groups	
-CH-	
$-CH_2-$	0.475
CH_3-	
=CH-	
Derived Groups	
$-CH_2CH_2O-$	0.33
$-CHCH_3CH_2O-$	-0.15

2.11.2 Preservatives

The use of preservatives is sometimes necessary in agricultural liquid formulations. Even though the concentrate contains a pesticide which is a toxicant (for example, fungicide) , the matrix could act as a growth media, especially in aqueous systems containing nutrients for bacterial growth. Some of the oxygenated solvents / cosolvents and ethoxylated surfactants can act as a growth media for fungal/bacterial growth. Some of the common preservatives are parabens (alkyl ester–of p – amino benzoic acid, or methylol derivertives of glycine, hydration, and others).

2.12 Technology to be Developed for Formation of Crude Extract from Galanga, Sweetflag and *Rhinacanthus nasutus*

A lot of researches have been carried out on Thai herbs and medicinal plants since the last decades and by many institution . The works covered various plant biodiversity. Active ingredient were searched for beneficial functions in pharmaceutical and cosmatic industries. Only few studies have been under taken on the usfullness of plant biodiversity for agricultural use. One of the most successful plant extract for agricultural application is the neem tree, which produce azadirachtin (Wink, 1993) effective as insecticide. For which the success of field application still hawever under pro and contra discussion. Beside neem tree, a lot of plants have been screened for the effectiveness to control fungi, bacteria and insect, although most of the work are carried out only at laboratory scale. Galanga, sweetflag and *Rhinacanthus nasutus* are also reported to be containing fungicidal active substance (Korpraditsakul *et.al.*,1990, 1991; Bhassabuttra, 1997). Vry fear researchs and development up to the end product have been rerely carried out.

In the case of galanga, detail study from extraction up to structural elucidation by GC-MS were performed by Srisornkampol (1996) and Lertviraswat (1997). The major active ingredient in galanga was lastly confirmed as 1'-acetoxychavicol acetate, but no further study on the fungicidal efficiency and even the formulation has yet been carried out.

In the case of sweetflag and *Rhinacanthus nasutus,* Korpraditsakul *et.al.* (1991) confirmed the positive efficiency to control *Colletotrichum gloeosporioides* (Penz.) Sacc. (mango anthracnose fruit rot) by the ethanol crude extract at the concentration of 5,000 ppm. The further study on this matter has been however never being reported elswhere.

In these studies galanga, sweetflag and *Rhinacanthus . nasutus* (Kurz.) were selected out for a complete research and development up to product development (formulation) to use for control *Colletotrichum gloeosporioides* (Penz.) Sacc. (anthracnose fruit rot in mango). The works will cover extraction study, purification, structural elucidation, mode of action, and formulation. According to the literature review there are many methods and techniques possible applied for the studies, but due to the limiting financial support and lacking of sophisticated instrument for some experiment, the studies will firstly pararell carried out on all the three plant species. Only the plant

with highest antifungal efficiency will lastly selected out to complete the research program up to formulation.

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