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ANALYTICAL PROCEDURES FOR 1-TRIACONTANOL AND ITS PRESENCE IN PLANTS AND THE ENVIRONMENT

By

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ABSTRACT

ANALYTICAL PROCEDURES FOR 1-TRIACONTANOL
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Several procedures for the extraction and analysis of triacontanol were compared and a standard procedure was developed using Soxhlet extraction, preparative TLC and GLC analysis of the fluoracyl derivative. Triacontanol was found to be present throughout the environment as a natural constituent of many plant waxes and plant residue samples, including leaf waxes of corn, rice, alfalfa, soybean and broad bean, soils, cow manure, jojoba seed oil, and cotton fiber.

The external wax was compared to the inner plant tissue in the jade leaf, potato tuber, and apple fruit to determine whether triacontanol is in plant parts other than the surface wax.

Labelled palmitic acid was used to trace the site of triacontanol synthesis. Jade leaf sections were incubated with palmitate-1-^{14}C and TLC of chloroform extracts from this tissue was performed. Bands which co-chromatographed with triacontanol standards contained about 1% of the total label recovered.

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INTRODUCTION

The study of plant waxes has developed in the last 50 years in response to questions concerning water use efficiency and the use of agricultural pesticides. A continuous cuticle forms the plant's protective boundary with its environment, reflecting and trapping materials in the angles of crystalline wax exuded from the inner cells. Penetration of the plant cuticle is a necessary feature of agricultural chemicals and pursuing this application has brought further interest in understanding the structure and function of the leaf and fruit cuticle.

1-Triacontanol, a 30-carbon primary alcohol (MW 438) is a natural component of plant and insect waxes. Recently, it has been shown (68) that the exogenous application of triacontanol at low rates increases growth of vegetable and cereal crops grown in nutrient culture and under greenhouse and field conditions. Triacontanol as a naturally occurring leaf constituent is therefore of increased agricultural interest.

This study has been done in an attempt to describe the normal location of triacontanol within plant tissue both as a component of the wax material, and as a free alcohol with possible growth-promoting properties. As a wax component, triacontanol is one of a class of secretions manufactured close to the plant surface. Since a system exists for manufacturing and transporting these secretions, it is likely that wax components, like the long chain alcohols, could be shuttled

elsewhere than to the cuticle, to perform functions other than that of protecting the plant. This study of the location of triacontanol in plant tissue provides information which may eventually answer some of these questions pertaining to the function of individual wax elements and their potential for exploitation in agriculture.

In order to study the natural function of triacontanol, a sensitive analytical procedure was developed for extracting and analyzing triacontanol in plant, soil, and animal material. This procedure may be used for following the translocation of applied triacontanol as well as for observing the synthesis and movement of the endogenous alcohol.

LITERATURE REVIEW

Triacontanol as a constituent of plant leaf wax

Plant waxes are crystallized lipids predominately composed of free fatty alcohols, free fatty acids, esters, hydrocarbons and in some cases, aldehydes (44). Plant waxes are found primarily on the surface of the cuticle and embedded within the cuticular layers. The epicuticular and cuticular waxes help form the nearly continuous envelope which encloses all aerial parts of the plant. Waxes occur on leaves, stems, fruits, flowers and seeds. There are reports of finding small amounts of wax in nearly every part of the plant including the roots and secondary xylem and phloem (44). Wax materials may be as much as 4% of the fresh weight of a leaf and 15% of the dry weight (14). The plant waxes serve several distinct functions involving prevention of water loss, defense of the plant against disease organisms and growth regulating activity. In addition, the waxes help determine the wettability and absorptivity of the plant surface.

The effect of plant waxes on the prevention of water loss has been studied with whole plants, with isolated cuticles and with plant models. In one study (70), the permeability of isolated cuticular membranes to water was determined before and after solvent extraction. Extraction of the waxes increased the water permeability by a factor of 300 to 500. The study concluded that water permeability is completely determined by the amount of waxes present.

Grape berries (Vitis vinifera) which do not have stomates were used to examine the effect of wax on cuticular transpiration alone (63). The rate of cuticular transpiration of grape berries was found to be related to qualitative differences in wax composition rather than cuticle thickness. The drying rate of grape berries was measured after solvent extractions removed either the 'hard wax' (mostly oleanolic acid) or the 'soft wax' (containing long chain alcohols, aldehydes, esters, acids and hydrocarbons). The drying rate was found to depend only on the amount of 'soft wax' fraction present (63).

Since the isolated cuticle is very fragile and difficult to work with, models of the plant cuticle have been devised which enable the study of water loss. Grncarevic and Radler (24) utilized a plastic membrane (cellulose triacetate film) coated with various fractions of grape berry wax to study water evaporation. The hydrocarbon, alcohol and aldehyde fractions of grape wax were responsible for the highest reduction in evaporation, with chain length being an important parameter. Fractions containing the longer chain alcohols controlled evaporation to the greatest extent.

The wettability and absorptivity of the plant surface has been studied specifically in regards to the application of pesticides. The affinity of surfaces for water (as measured by the contact angle of water droplets) is influenced by the amount of wax, its composition and its physical configuration (33). The degree of uptake of compounds through the cuticle depends on the presence of wax and on the molecular structure of the compound. Bukovac et al. (3) studied the isolated cuticle of tomato (Lycopersicon esculentum) and found a positive relation between the degree of chlorination of phenoxyacetic and benzoic acids and the rate of their transfer across the cuticle. When the waxes were removed from

the cuticle, the transfer of materials was greatly enhanced.

The role of the cuticle in disease has been discussed by Martin (54) with the conclusion that the role is not a large one. A few fungi are known to degrade cuticles (such as Penicillium spinulosum) by attacking the waxy materials and the degree of penetration of fungi appears to be related in some cases to the thickness of the cuticle. The waxy surface may repel water which is required by a pathogen, and substances inhibiting to fungi may exude to the surface from the cellular tissue or may form a part of the wax. However no difference has been noted in the wax and cutin composition of susceptible and non-susceptible plants (54).

Growth regulating activity has been observed for some cuticular waxes and wax components. Vlitos and Cutler have shown that the cuticular waxes from sugarcane promote elongation of Avena first-internode sections (80). With the same bioassay they have shown growth promoting effects of long chain primary alcohols isolated from tobacco (cv. Maryland Mammoth) (11).

Ries et al. (68) described the extraction and identification of triacontanol from a biologically active fraction of alfalfa (Medicago sativa). Triacontanol, which was isolated from a chloroform extract of the water soluble fraction of alfalfa, was found to increase dry weight and water uptake of rice seedlings grown in nutrient culture and dry weight of corn, barley (Hordeum vulgare), tomatoes, cucumbers (Cucumis sativa), and wheat (Triticum aestivum) grown in soil, in greenhouse and field studies.

Much of the foundation for the study of plant waxes was contributed by Chibnall and co-workers beginning in the 1920s. The majority of their work dealt with the purification and analysis of wax material from insect and plant extracts. Fatty alcohols were isolated from the total wax by

ether and acetone extraction of the fresh tissue. X-ray determinations of the long crystal spacings coupled with melting point determinations of the alcohols enabled Chibnall to separate mixtures of alcohols which had been thought to be pure (9). In 1961 Chibnall reviewed his previous work using mass spectrometry and found that his initial analyses were substantially correct (81).

According to Chibnall and Piper's investigations, most leaf waxes consist basically of primary alcohols with lesser amounts of fatty acids and hydrocarbons (8). These primary alcohols are predominately even-numbered, saturated long chain compounds with 24 to 36 carbons (9). The most common primary alcohols are hexacosanol (C_{26}) and octacosanol (C_{28}) (43). The esters found in wax are composed of fatty acids and alcohols corresponding to those in the free materials (43).

Triacontanol has been found in many plant waxes which are used commercially. In the wax from the Brazilian palm (Copernica cerifera) which is used for the manufacture of polishes (Carnauba wax), triacontanol accounted for 20% of the alcohols. Candelilla wax (Euphorbia antisyphilitica) which is used to manufacture candles has an alcohol component labelled myricyl alcohol which contains 35-40% triacontanol.

In other plants of economic value, triacontanol has been found to represent a considerable portion of the alcohols present. The primary alcohols in wheat leaf have been shown to contain 0.7% triacontanol; apple peel, 21%; white clover (Trifolium repens), 59%; runner bean leaf (Phaseolus multiflorus), 24%; cactus leaf (Cactacea sp.), 32%; lucerne leaf, 95% and white mustard leaf (Spinapis sp.), 35% (81). The alcohols isolated from a benzene extract of Loblolly pine pollen (Pinus taeda) contained 0.7% triacontanol (71). Several trees of the Eucalyptus family had a triacontanol composition of 9% of the total wax (32). Alcohols obtained

from saponification of the esters of Eucalyptus wax yielded 8% triacontanol and 52% octacosanol.

Triacontanol has been noticeably absent in most alcohol fractions of the different varieties of Brassica oleracea which Kolattukudy found to contain alcohols of carbon lengths C_{12} to C_{28} only (38,40). Tulloch reports a slight amount of C_{30} alcohol in Brassica oleracea (78). Likewise, work with tobacco (Nicotiana tobaccum), spinach (Spinacia oleracea), and pea (Pisum sativum) has turned up no triacontanol in primary alcohol fractions (9,46).

The relative amounts of each fatty alcohol present show considerable variation from species to species. Hexacosanol was isolated by Chibnall as the major alcohol from cocksfoot (Dactylis glomerata), octacosanol from wheat and triacontanol from lucerne (9, 10). Tulloch, comparing drought resistance in rye (Secale cereale) and wheat, noted that the percentage of their free alcohols differed from 8 to 14% respectively (79). In rye, the major alcohol was hexacosanol while in wheat, octacosanol comprised 80% and triacontanol 3% of the alcohols.

Even within the same species different researchers have provided divergent analyses of wax composition. Kolattukudy (44) found 3% of the free alcohols in grape berries and 18% in grape leaves were triacontanol. In a petroleum extract of the cuticle wax of grape leaves, Radler and Horn (65) found that 6% of the free alcohols and 7% of the esterified alcohols were triacontanol. Both researchers agree that the major alcohols of grape wax, regardless of concentration are hexacosanol and octacosanol. In another instance, Kranz, Lamberton and Murray (50) established that sugarcane (Saccharum officinarum) wax alcohols contained 6% C_{30}; Kolattukudy (44), also working with sugarcane, found that 4% of the free alcohols was triacontanol.

The total wax structure and appearance may differ within a species due to such differences in the proportions of each material (9). While Chibnall reported that the Connecticut cultivar of tobacco contained only hydrocarbons, Crosby and Vlitos (11) found an absence of hydrocarbons in Maryland Mammoth tobacco and the occurrence instead of the corresponding long chain alcohols, possibly representing a significant biochemical difference between the Mammoth and other cultivars.

Regardless of these variations, researchers have tried to examine waxes for taxonomic purposes. Eglinton, examining species of Crassulaceae isolated on the Canary Islands, concluded that within the limits of his investigation, a single species was found to possess a fairly constant hydrocarbon distribution pattern (13).

At different sites on the same plant there may be large differences in wax composition. Tulloch (76) compared wax of the leaf blade and leaf sheath in Spring and Durum wheat cultivars. He found that, while alcohols were the major components of the leaf blade wax, beta-diketones were the predominant material in the sheath.

A comparison of leaves on the same plant also show differences in wax content. Some species like corn show surface wax on some leaves but not on others (69). Surface wax was found to accumulate on young growing corn leaves; when non-expanding leaves were dewaxed, new wax was not found. The quality and quantity of leaf wax extracted from members of the same species or from different parts of individual plants are dependent on many factors including the environment as well as the genetic make-up and maturity of the plant.

Studies dealing with leaf wax composition and level over time give some clues as to the metabolic role of wax materials. These studies suggest that the wax is deposited in young expanding leaves, undergoes several

transformations (such as the formation of esters) and eventually remains metabolically inert for the rest of the life of the plant (8,35).

Chibnall and Piper (8) followed the wax composition of brussel sprout at various stages of growth. The seeds were found to contain no wax, but upon germination rapid wax synthesis took place, continuing throughout the life of the plant. In this case they found no apparent change in the composition of the wax over time. Chibnall and Jordan monitered the runner bean leaf over a 10 week period (35). As with brussel sprout, the amount of wax was found to increase with time suggesting that the wax materials are by-products of metabolism which slowly accumulate.

In attempts to see whether wax material would be metabolized under starvation conditions, Jordon and Chibnall detached leaves of runner bean and placed their petioles in water in the dark for over 8 days. Wax and unsaponifiable material remained unchanged during starvation, confirming the view that they are end-products of metabolism (35).

Kolattukudy (44) examined the constancy of wax levels and their function in metabolism and showed that the very long wax components appear to undergo little turnover or interconversion except to disappear due to weathering. Even under conditions of starvation, wax was not utilized by the plant in any way (44).

Kurtz (52) correlated the yields of wax of 13 species of plants with age. In general the total wax content was found to increase with age. Waxy acids were found to decrease and then increase parallel to ester formation.

The level of free alcohols within leaf wax also seems to be correlated with ester formation. Tulloch (76) examined the variation in leaf wax composition with age and noted that the wax esters were apparently formed from a free alcohol pool which was not replenished. In the 3 wheat

cultivars he tested, the free alcohol content was 40-50% of the total wax for the first 52 days following germination. After 52 days a sharp drop in alcohol content to 20% coincided with an increase in ester content as well as beta-diketone content. The alcohol content continued to drop, reaching a 5% level after 100 days.

The variation in free alcohol content of the wax with time may provide some explanation for the discrepancies noted in wax composition data from study to study. It is clear that much work has been done in terms of cataloging wax material but the research is often full of ambiguities which make generalizing from the literature difficult, especially regarding individual wax components such as triacontanol.

Some of the discrepancies in data are obviously due to the fluidity of the material involved. Plant wax is not a static structure, but is responsive to the environment, particularly to light and moisture conditions. The mechanisms of this responsiveness offer a worthwhile area for future research.

Besides the nature of the materials, discrepancies in wax analyses may stem from the methods of extraction which are employed. Figures based on the extraction of whole plants are found to differ markedly from extractions of the epicuticular wax alone (23). For example, how does one compare an ether extraction of whole plants for 8 hr to an analysis achieved by dipping the leaves in chloroform for 30 sec? The literature in wax analysis makes only vague use of standard definitions of wax fractions based on extraction procedures. Part of the present study was concerned with interpretating methods of wax extraction in terms of the different wax fractions they may represent.

The location of triacontanol within plant tissue

There has not been much research conducted on the presence of long

chain alcohols within plant tissue and even less on describing triacontanol. The method and site of triacontanol synthesis can be understood from studies involving other long chain alcohols and wax components.

The site of synthesis of a chemical and its function are often thought to be interwoven characteristics except for substances like hormones which are transported to their active sites. The designation 'site' can describe both a physical location within the plant as well as a biosynthetic pathway. A material synthesized along with wax components will likely become incorporated into the wax layers associated with the cuticle. The presence of a chemical as a wax constituent would also suggest that its functions are limited to functions of the cuticle.

The idea of compartmentation can also be used to distinguish different functions of the same material. It is possible that when a cuticular material is not found in the cuticle it may serve some function other than cuticle formation.

The problem of the location of triacontanol within the plant was approached in several different ways in this study. A general review of what is known about the synthesis and location of wax components will give the proper context for these studies relating to triacontanol.

Fatty alcohol synthesis

Acetate is the precursor for both short chain and longer chain fatty acids typically found in the cuticular and wax layers (37). Kolattukudy has demonstrated the incorporation of labelled acetate into the long fatty acids of apple skin wax (Malus communis) (44). Labelled C_2 to C_{18} acids were fed to leaf tissue slices of broccoli, pea, spinach and groundsel (Senecio odoris) and the label was recovered in the C_{20} through C_{28} acids (38,42), indicating that the synthesis proceeds from acetate through short chain fatty acids to long chain fatty acids. In a divergent pathway, acetate also

gives rise to the straight chain hydrocarbons (paraffins) of the wax material (42).

Some disagreement has existed about whether the conversion of short to long chain acids is by way of an elongation or head-to-head condensation mechanism. Incorporation of radioactive acetate and fatty acids into the branched long chain hydrocarbons of tobacco has suggested that hydrocarbons are produced from the condensation of 2 long chain fatty acids (37). However studies with fatty acids labelled at various sites along the carbon chain have shown that head-to-head condensation would not account for the equal incorporation of the carboxyl and inner carbons in straight chain hydrocarbons (41,42). Elongation followed by decarboxylation has been shown to be the major path of hydrocarbon synthesis, while for alcohols, elongation of fatty acids is followed by reduction through an aldehyde intermediate (38,44). These reductions have been shown to be enzymatically controlled. Working with cell free systems from broccoli, Kolattukudy has demonstrated the ability of 2 distinct protein fractions to catalyze the reduction of the acid to the aldehyde and the aldehyde to the alcohol respectively (44).

Labelled C_2 to C_{18} fatty acids and alcohols are, in turn, incorporated into wax esters (38,39,40). The chain length distribution of alcohols in the wax esters parallels that of the free alcohols indicating they are from the same pool (39,44,50).

Site of synthesis and function

Wax material is presumed to be synthesized in the epidermal cells and then immediately secreted to the cuticular layer and outer wax surface. Evidence for the location of wax materials has been obtained from studies which compare the presence of fatty acids and paraffins in the chloroplast, excised epidermis and mesophyll cells to the presence of these lipid materials

in the leaf as a whole. The synthesis of fatty acids and wax components has been followed as a function of the incorporation of labelled precursors in various leaf fractions.

Lettuce (Lactuca sativa) chloroplasts have been shown to incorporate labelled acetate into chloroplast lipids including palmitic, stearic, myristic and oleic fatty acids (74,75). The possible occurrence in the chloroplast of some longer chain cuticular components (hydrocarbons, in particular) has been documented (22). However, in another study the waxes, hydrocarbons and hexacosanol present in the whole spinach leaf were described as absent in the chloroplasts alone (83). Small quantities of the above that were detected in the chloroplasts were accounted for as cuticular contamination due to the chloroplast isolation procedure.

Kolattukudy discusses the fact that cuticular components may be found within plant cells (44). Much earlier Channon and Chibnall (5) suggested that 12% of the ether soluble "cytoplasmic" materials are hydrocarbons. Chibnall (8) describes the appearance of wax materials inside plant cells: "The function of the third class of waxes, namely those which are an integral part of the fat phase of plant cells, is less clearly defined, but they are not secretions...these waxes are made up in large part of primary alcohols and paraffins...their function may be to help control the liquidity of the fat phase. But not many of these waxes have yet been systematically investigated".

An analysis of the leaf wax of several species of Argentine palm showed the presence of wax in mesophyll tissue (23). The surface wax was extracted from palm leaves by dipping in solvent and then the remaining tissue was extracted by Soxhlet to give the "mesophyll" extract. The wax of the palm Copernicia alba was completely extracted by surface extractions, but in 3 Trithrinax species (T. campestris, T. schyzophylla and T.

biflabellata) 34-54% of the wax was extracted from mesophyll tissue.

Hill and Mattick (30) investigated the occurrence of hydrocarbons inside the leaf. Ninety percent of all hydrocarbons in cabbage was extracted from the leaf surface. The remaining leaf pulp also contained alkanes, but the authors point out that they did not differentiate between inner cuticular layers and the inside of the leaf so it is not clear where the 'inner' hydrocarbons are actually located. This criticism may also be applied to the Argentine palm wax study mentioned above.

Isolated epidermal layers incorporated as much acetate into wax esters and hydrocarbons as did intact leaf discs showing that the mesophyll tissue synthesizes very little of these materials (40,42). Again by separating epidermal tissue from the leaf the synthesis of fatty acids was observed (71). Acetate-1-^{14}C was incorporated into fatty acids in both the mesophyll and epidermal tissue of groundsel, but only the epidermis incorporated acetate into fatty acids longer than C_{18} (71). This suggests that the epidermis is the principal site of synthesis of the very long fatty acids.

The site of incorporation of labelled fatty material can be measured using the length of the extraction procedure as a gauge. More than 98% of labelled paraffins was extracted by a 10 sec dip in chloroform after incubation of broccoli leaves with labelled acetate (38). Since the major portion of the paraffins was located in the outer cuticle, the experiment lends support to the hypothesis that wax materials are synthesized very close to the surface and arrive on the surface of the leaf as soon as they are synthesized.

Besides physically manipulating the leaf to distinguish the epidermis from mesophyll sections, other techniques are available for determining the compartmentation of wax synthesis. It has been known for some time that many of the herbicides and agricultural chemicals owe much of their effective-

ness to causing a reduction in epicuticular wax. Flore and Bukovac (17,18) studied the effect of S-ethyl dipropyl-thiocarbamate (EPTC) and trichloroacetic acid (TCA) on the cuticle of cabbage. Both soil and foliar applications of the 2 herbicides were found to reduce surface wax production by 50-60%. Such chemicals provide a tool for examining the mechanisms of wax synthesis and for differentiating the synthesis of the internal and surface plant lipids.

Fatty acid synthesis in the chloroplast is light dependent and coupled to photosynthetic reactions, stimulated by light and inhibited by 3-(4-chlorophenyl)-1,1-dimethyl (Monuron), an inhibitor of photosynthesis (43). Alkane synthesis is not affected by light and is found to be insensitive to Monuron (43). Since radioactive precursors that label alkanes also label long fatty acids (39,42), what applies to the alkanes may also apply to the other long chain wax materials.

The herbicide TCA inhibites alkane synthesis without affecting short chain fatty acid synthesis in the chloroplast (38) by inhibiting decarboxylation. For this reason, TCA was shown to have little effect on the primary alcohols although severely inhibiting hydrocarbon, ketone, and secondary alcohol synthesis and altering the surface structure of the leaf (1). Thiocarbamates also reduce formation of the cuticular waxes by inhibiting chain-elongating enzymes (46). The thiocarbamate diallate was found to inhibit the biosynthesis of pea epicuticular lipids, most particularly by decreasing the total and relative amounts of primary alcohols (73). The reduction in primary alcohols was found to have a direct effect on the structure of the surface wax as noted in EM studies, although none of the growth parameters for the whole plant were changed. By noting the ratios of wax components before and after diallate treatment, the synthesis of long chain alcohols could be distinguished from that of hydrocarbons, and

secondary alcohols.

Studies of wax synthesis indicate that most of the waxes are not synthesized in the chloroplast, or in the mesophyll cells but in the epidermis where they are readily exuded to the outer layers. However, cuticular contaminants are found inside the leaf and whether they are due to techniques of extraction and isolation alone is not clear. The important issue is one of approach and definition. At what limit of concentration can a material definitively be said to exist? If 90% of all alkanes are found in the cuticle, what about the missing 10%; should the 10% be considered error, and under what circumstances? On the other hand, if the presence of small concentrations of wax material inside the leaf is of interest, a level of 10% might be considered substantial. In this case, the problems of contamination have to be dealt with as thoroughly as possible especially in the isolation of mesophyll material.

It may be concluded that previous studies have not provided an answer to the question of whether or not there are small quantities of wax material within the leaf. This omission may be due to the accepted concept of wax function. In general it is thought that wax components contribute only in a structural manner and that they have no purpose beyond the external wax layers. It has been shown that the concentration of various components in the wax helps determine the waxes overall structural characteristics and this description in turn serves to restrict the theoretical role of wax materials to defensive and passive functions. If it can be shown that various wax constituents do exist within leaf cells, then a new definition of their functions may be in order.

The appearance of plant waxes in the environment

Plant waxes, particularly the longer chain compounds, are stable materials which do not break down easily and which are consequently found

throughout the environment. As plants decay in the soil, wax material accumulates and is slowly degraded by microorganisms which are able to metabolize plant waxes as a carbon source. That plant wax and cuticle components are not easily utilized is evident from data showing that they have been found in 5000 year old sediment from a fresh water lake (15) and are believed to be the precursors for the n-paraffins found in crude petroleum oils (55).

Soil humus, that part of the soil formed from vegetative growth, contains about 20% lipid substances (72). The types of lipid present include hydrocarbons, phospholipids, fatty acids, fatty alcohols, esters and terpenoids. Organic soils have been shown to have a greater lipid content than mineral soils with an additional correlation of low pH and lipid content (72).

Meinschein and Kenny (58) analyzed benzene-methanol extracts of 6 different soils by mass spectroscopy. They compared the spectra of the soil samples to the spectra of beeswax and concluded that soil contains the same fractions found in the insect wax. Various other reports in the literature have described the composition of beeswax, showing that it contains the basic fractions associated with plant waxes as well as 34% triacontanol (9,77).

Galoppini and Riffaldi (20) took ether extracts of 6 soils from woodland zones. They found that the wax materials constitute the largest fraction of lipids present in soil organic matter.

Microorganisms have been identified which can utilize the long chain compounds as a source of carbon. A pseudomonad isolated from orchard soil has been shown to utilize cutin as a sole cource of carbon (27). Products obtained from the bacterial culture by ether extraction contained the same hydroxy acid products found with alkaline hydrolysis of cutin.

Pseudomonas sp. was also found to utilize ursolic acid, a major component of apple fruit wax (26).

Another soil bacterium, Micrococcus cerificans, has metabolized a major paraffin of cabbage wax, nonacosane, under experimental conditions (25). A mold, Aspergillus versicolor, grew slowly on odd and even-numbered paraffins up to a chain length of 34 carbons (31). CO_2 was the only product found indicating that substantial metabolism of the paraffins had taken place. In this case, some growth also occurred on a medium containing primary alcohols - specifically "myricyl" alcohol from Carnauba wax - although growth on alcohols was not as good as on paraffins.

Over 100 yeasts, bacteria, fungi and actinomycetes are capable of growth on wax components (57). It is thought that the process of degradation of the long chain materials is by beta- and alpha-oxidation (25,57).

Studies have been conducted involving the metabolism of wax materials by higher forms of life. Octadecane-1-^{14}C was fed to goats, rats and chickens which converted the hydrocarbon to free fatty acids and esters. Eighty percent of the label was recovered in C_{18} fatty acids (56). When labelled 2:2-Dimethylstearic acid was fed to rats, 90% of the label was found excreted in the urine after considerable beta-oxidation (2). A longer chain wax material, nonacosane, was fed to rats (47) and C_{17} acid was found as the major product with 75% of the alkane excreted unmetabolized. In the same experiment, labelled C_{28} acid was metabolized to C_{16} and C_{18} acids also suggesting a beta-oxidation chain-shortening system. Radioactivity in the rat was found distributed throughout all classes of lipids and throughout all tissues and organs with the most concentration in the liver.

These studies indicate that animals have the ability to absorb and

metabolize long straight chain acids and alkanes, at least to a limited extent. However, the majority of the long chain wax components tend to be excreted without being metabolized. A study of the accumulation of long chain hydrocarbons in cow manure, concluded that they were not a product of animal or bacterial metabolism but were derived from the cow's diet (60). The distribution and relative concentrations of paraffins in the leaf wax of the pasture plant spotted bur clover (Medicago arabica) was the same as that in cow manure and soil samples from the same area. Nonacosane was the predominant alkane in all three (60).

Sewage sludge from a domestic plant was examined for cutin content (48) with the idea of utilizing such plant residues as a source of carbon for bacteria and fungi. The sludge was found to contain 6-14% lipid materials. Upon hydrogenolysis of an organic solvent extract of the sewage, fatty acids unique to cutin polymers were identified by GLC-mass spectroscopy. Fatty alcohol fractions were also found.

Analytical methods for studying wax materials

The quantitative analysis of wax components depends on the separation techniques which are employed. The 'outer' or epicuticular wax is commonly designated as that material which is extracted from whole plants by dipping in organic solvent for 30 sec or less (16). To obtain the inner cuticular wax requires a longer extraction time. More thorough methods of extraction such as Soxhlet extraction, are used to extract the total soluble wax (16).

The long chain alcohol (and acid) components of the wax exist in both free and esterified forms. The extent of interchange between the 2 forms is not known although the free alcohol 'pool' has been shown to provide the substrate for esterification with fatty acids (38,40,50). The determination of long chain alcohol concentration in a given plant

tissue will vary depending on whether the free alcohols or total alcohols are being extracted. The present study was primarily concerned with the free form of triacontanol which increases plant dry weight with exogenous application (67,68).

One of the earliest extraction methods reported in the literature was that used by Channon and Chibnall in 1927 to determine the composition of cabbage wax (7) and to extract triacontanol from alfalfa (10). Fresh leaves were "minced" in water and squeezed 2 times through silk. The green juice was coagulated by heating to 70 C followed by rapid cooling. The material was pressed in a cloth until no liquid appeared and then ground in a mortar and extracted with ether in a Soxhlet apparatus for 40 hr. The ether soluble substances were then extracted with boiling acetone to obtain the non-phosphatide material including the long chain alcohols. The substances soluble in boiling acetone were saponified to remove all fatty and phosphatide substances (primary alcohols are not saponifiable). Of the dry weight of cabbage and lucerne leaf, .35% was wax material and of this, 28% was triacontanol in the case of lucerne. The concentration of total triacontanol (free and esterified) in lucerne was determined as 1000 ug/g.

Subsequent studies comparing wax extraction by ether versus other organic solvents, have shown that the nonpolar wax constituents are only slightly soluble in ether and are completely soluble in chloroform (4,16). The most common method of extracting the surface wax is by immersing the fresh plant material in chloroform for 30 sec or less (13,29,43).

To extract the total wax, chloroform-methanol 2:1 v/v is used as suggested by the Folch method for lipid extraction (19). After plant tissue is washed with chloroform to extract the surface wax, it is then extracted for 6 to 24 hr with chloroform-methanol to obtain the internal

waxes (28,46,83). Before extraction of the internal lipids plant material is usually dried in an oven at 100 C and then crushed so that greater bulk can be extracted (38).

Further isolation of the lipid extracts to determine wax composition is usually accomplished by TLC and GLC with the most accurate analyses achieved by mass spectroscopy. There are many referrences in the literature to the use of analytical and preparative TLC using silica gel G as the solid phase for wax materials (34,78). Often a hexane-ether-acetic acid mixture (70:30:1) is utilized as the liquid phase particularly when hydrocarbons and fatty acids are being investigated. Plates are developed with a 0.1% alcohol solution of 2,7 dichlorofluroescein and marked under UV light (34,46,78). Wax analysis by GLC is often performed with a non-polar column such as a 3-5% SE 30 liquid phase and temperatures ranging from 230 to 260 C (30,40,41). Derivatives are employed to form esters or ethers such as trifluroacetates or trimethylsilyl ethers to give more rapid analyses and better peak symmetry.

The use of radioactive precursors may be used to study the synthesis of wax material and the specific pathways associated with individual wax components (37,43). Although acetate is often used as a labelled precursor, stearic and palmitic acids have been found to be incorporated into the long chain wax materials at a much faster rate (38,39,41).

Fatty acid precursors were incorporated into the wax of both whole leaves and leaf slices (40,46). Young, expanding leaves are used since that is the stage at which there is rapid cuticle synthesis (41,45). For 2 hr prior to incubation, leaves are placed in water in the dark in order to make them fully turgid (41). Leaves are incubated by placing their cut petioles in small vials containing the radioactive solution for 3 or 4 hr, at 30 C in the light (40,42). Kolattukudy describes using a

minimum of 10 μCi of fatty acid precursor for every g of fresh leaf material (53). The fatty acid solution is dispersed in a small amount of water (usually about 1 ml or less per leaf) with a few drops of tween-20 and sonication (37,41,46).

After the incubation period, the surface wax is extracted by dipping the fresh leaves in chloroform for 30 sec (41,40). Internal lipids are extracted by stirring tissue in a 2:1 mixture of chloroform and methanol for 2 hr (37,46). The chloroform extract is evaporated and brought up to a known volume and samples are then applied to TLC. Wax fractions are scraped and counted by liquid scintillation (37,45,46) and by radio gas-liquid partition chromatography.

Sources of contamination

There are two major types of contamination associated with studies of wax material. The first type is one of artifact contamination; the unexpected recovery of trace amounts of wax material from leaf mesophyll sections may be an example of an analysis open to question concerning techniques and definitions employed (how one distinguishes the inside from the outside of a leaf, for example).

The second type of contamination is the problem resulting from the widespread use of plant fibers in common laboratory materials. Mandava and Mitchell (53) extracted biologically active chemicals which they termed "plant hormones" from Whatman #1 filter paper, which is composed of cotton fibers. The "hormones" consisted of a mixture of sterol derivatives containing fatty acid esters, fatty alcohols and hydrocarbons (docosanol was identified among the alcohols).

Gaskin and MacMillan (21) suggest using "Parafilm" as a source of alkane standards for GLC. The extraction procedure they employed was simply to wash the parafilm with a hydrocarbon solvent such as benzene

or hexane. Especially of interest is the predominance of longer chain alkanes such as C_{35} (pentatriacontane). Their message is also one of warning to those using parafilm around GLC samples.

As an illustration of the pervasiveness of the problem, Hill and Mattick (30), while investigating the distribution of alkanes on the leaf surface and within the leaf of cabbage, extracted and analyzed a number of samples as likely sources of contaminants: "Polyethylene films, teflon-covered bar magnets, parts of the rotary evaporator and waring blendor, lubriseal, human skin oils, nivea creme hand lotion and residues on evaporation of distilled hexane...". The polyethylene and hexane residues alone contained hydrocarbons and were used as the control analyses (30).

MATERIALS AND METHODS

Extraction procedures

Successive extraction was utilized to obtain the epicuticular wax and the total wax from the plant leaf. Surface wax was obtained by dipping fresh leaves in chloroform for 30 sec after which the plant material was dried and weighed. Soxhlet extraction of dried, ground plant material provided a total lipid extract from which the alcohol components were further fractionated by thin layer chromatography (TLC) and gas-liquid chromatography (GLC).

Several different methods of extraction were employed and their triacontanol yields compared using alfalfa hay. The conditions for GLC analysis will be described in a later section.

The first extraction method employed was the procedure which had provided the biologically active fraction from alfalfa later identified as triacontanol (68). This method involved grinding alfalfa in a basic buffer (pH 9) in a Waring blender, followed by 2 extractions with equal volumes of chloroform in a separatory funnel. The chloroform extracts were combined, filtered and evaporated under vaccum.

Fifteen g of oven-dried alfalfa were ground in 500 ml buffer, extracted twice with 500 ml chloroform and then evaporated to dryness. The residue was taken up in 500 µl chloroform. A sample of 100 µl was taken for GLC analysis. The yield was 30 µg of triacontanol per g dry weight alfalfa, considerably less than that noted by Chibnall (10).

Hydrolysis of the esters from the dried alfalfa (15 g) was achieved by saponification with sodium hydroxide (2 N NaOH) at 30 C for 12 hr. This was followed by 2 chloroform extractions (1:1) in a separatory funnel and 2 subsequent water washes. The chloroform phase was evaporated for GLC analysis. The yield of triacontanol by this method was 400 µg triacontanol per g alfalfa. The higher yield by this method compared to the previous one, is reasonable since saponification allows the determination of the alcohol content of the wax esters as well as the free alcohol concentration. Saponification was not used further except in the case of soy bean seed because this study deals only with the free alcohol content of plant tissue.

The most efficient extraction of the free alcohols was achieved using Soxhlet extraction. Dried alfalfa (7 g) was extracted for 10 hr with chloroform and the extract then brought to a volume of 58.5 ml from which 1 ml was taken for GLC analysis. The final yield was 562 µg of triacontanol per g alfalfa. Since it gave a higher yield of triacontanol than the other methods described above, Soxhlet extraction was chosen as the most efficient and thorough method for extracting samples. The extraction procedure was modified after examining the literature (37,46) and in further Soxhlet extractions a chloroform-methanol 2:1 solution was used as the extracting solvent.

Two experiments were conducted to establish the length of time necessary for total Soxhlet extraction and the percent recovery of triacontanol using this method. In both cases the plant material used was corn cv. Pioneer 3780 grown under greenhouse conditions, harvested at 1 week and dried in a 40 C oven.

Ten g samples of the dried corn were extracted by Soxhlet with 150 ml of chloroform-methanol for 6, 12 and 24 hr. Four samples were run for each

extraction time. The solvents were evaporated and the material transferred to 50 ml volumetric flasks. Samples were applied to preparative thin layer plates, eluted from the plates, derivatized with heptafluorobutryic acid anhydride and analyzed by GLC using electron capture detection (ECD) on a 3% SE 30 column. Retention time was measured in cm and peak areas were calculated by triangulation.

Table I. Soxhlet extraction of corn tissue for different lengths of time

Extraction time (hr)	Peak area[a/] (cm^2)	Triacontanol (µg/g)
6	2.77 ± .19	220
12	3.01 ± .16	241
24	3.00 ± .25	240

a/ F value for different sampling times not significant at 5% level.

Increasing the length of extraction time over 6 hr did not show a significant increase in the yield of triacontanol. Six-hour Soxhlet extraction is therefore sufficient for most determinations.

A percent recovery experiment was designed to establish the rate of recovery of triacontanol from plant tissue by Soxhlet extraction when samples were spiked with pure triacontanol. Five g of dried corn tissue was extracted as above. Two corn tissue samples were spiked with 3 mg of triacontanol; 2 samples were extracted of corn alone, and 2 samples of 3 mg of triacontanol were extracted. Two subsamples from the triacontanol extracts were taken to determine the recovery from TLC. One subsample was applied to TLC, developed, eluted and derivatized with heptafluorobutyric anhydride for GLC along with the corn samples, and the other subsample was derivatized directly without TLC. All samples were Soxhlet extracted for 12 hr with 150 ml of solvent. The solvent was

evaporated under vacuum and brought up to 25 ml from which 100 µl was taken for TLC and GLC analysis. GLC was performed using a column of 3% SE 30 at 250 C with ECD. Peak areas were calculated by triangulation.

Table II. Percent recovery of triacontanol by Soxhlet extraction

Sample	TLC	Peak area (cm^2)	Triacontanol	Recovery (%)
Triacontanol, Soxhlet	0	7.49 ± .37	2934 µg	98
Triacontanol, Soxhlet	+	5.22 ± .26	2040 µg	68
Corn	+	3.40 ± .68	336 µg/g	
Corn + 3 mg triacontanol	+	7.50 ± .76	590 µg/g	54

Corn samples were spiked with triacontanol and compared by GLC to samples of triacontanol extracted by the same method. Recovery from TLC was calculated using samples of the triacontanol extract.

The triacontanol spike (3 mg) added to the corn and the triacontanol extracted alone were intended to give a maximum peak area corresponding to a 20 mg/L solution of triacontanol. A 20 mg/L triacontanol standard gave a peak area of 7.66 cm^2. The percent recovery of triacontanol from the spiked corn was calculated based on the difference between the spiked and the unspiked corn sample (4.10 cm^2) compared to the expected peak area for a 20 mg/L solution. A large portion of the loss may be attributed to the percent recovery from the TLC plate which was 68% in this case. The remainder of the loss, 30% of the initial triacontanol extracted, may be due to the interaction of the triacontanol with the plant material.

An examination of this and the previous experiment with corn show a discrepancy in the calculated triacontanol concentration of the dry material. Since the samples were taken from the same batch of dried, ground corn, the variation must be accounted for by the extraction or

analytical procedures. It may be inferred that one extraction run is not sufficient to determine the precise amount of triacontanol present, although the concentration range may be established.

Gas-liquid chromatography

Samples were derivatized for flame or electron capture detection and run on one of the several GLC instruments used throughout the study. Data reported are averaged from a minimum of 2 injections per sample.

Most of the earlier samples were run on a Packard gas chromatograph (7300 series) with a flame ionization detector. The column was 1.8 m x 2 mm and was packed with 80/100 mesh Gas-Chrom Q coated with 1.25% SE 30. Samples were run at a column temperature ranging from 230 to 250 C, an inlet and detector temperature of 250 C and a carrier gas flow of 400 ml/min. The peak areas were calculated by triangulation according to the formula Area = Ht x base at ½ Ht and compared with known triacontanol standards (Figure 1).

Trifloroacetic acid anhydride was used to form a derivative with triacontanol for flame ionization. The trifloroacetate ester was made by adding methylene chloride to the dry sample and refluxing with TFA (1 part TFA:5 parts methylene chloride) for 15 min. Two ml of solvent were used for a mg of sample. The solvents were evaporated to dryness with nitrogen gas or purified air. Two ml of methylene chloride were added to the dry sample and 2 - 3 µl applied to the GLC.

The major portion of the GLC analyses was performed on a Beckman GC-65 with electron capture detection interfaced with a Digital PDP 8/e-based data system for peak retention time and peak area integration. A 1.8 m x 2 mm glass column was used containing 80/100 mesh Chromasorb Q with a 3% SE 30 liquid phase. Samples were run at a column temperature between 240 and 250 C, with inlet and detector temperatures of 300 C and a helium

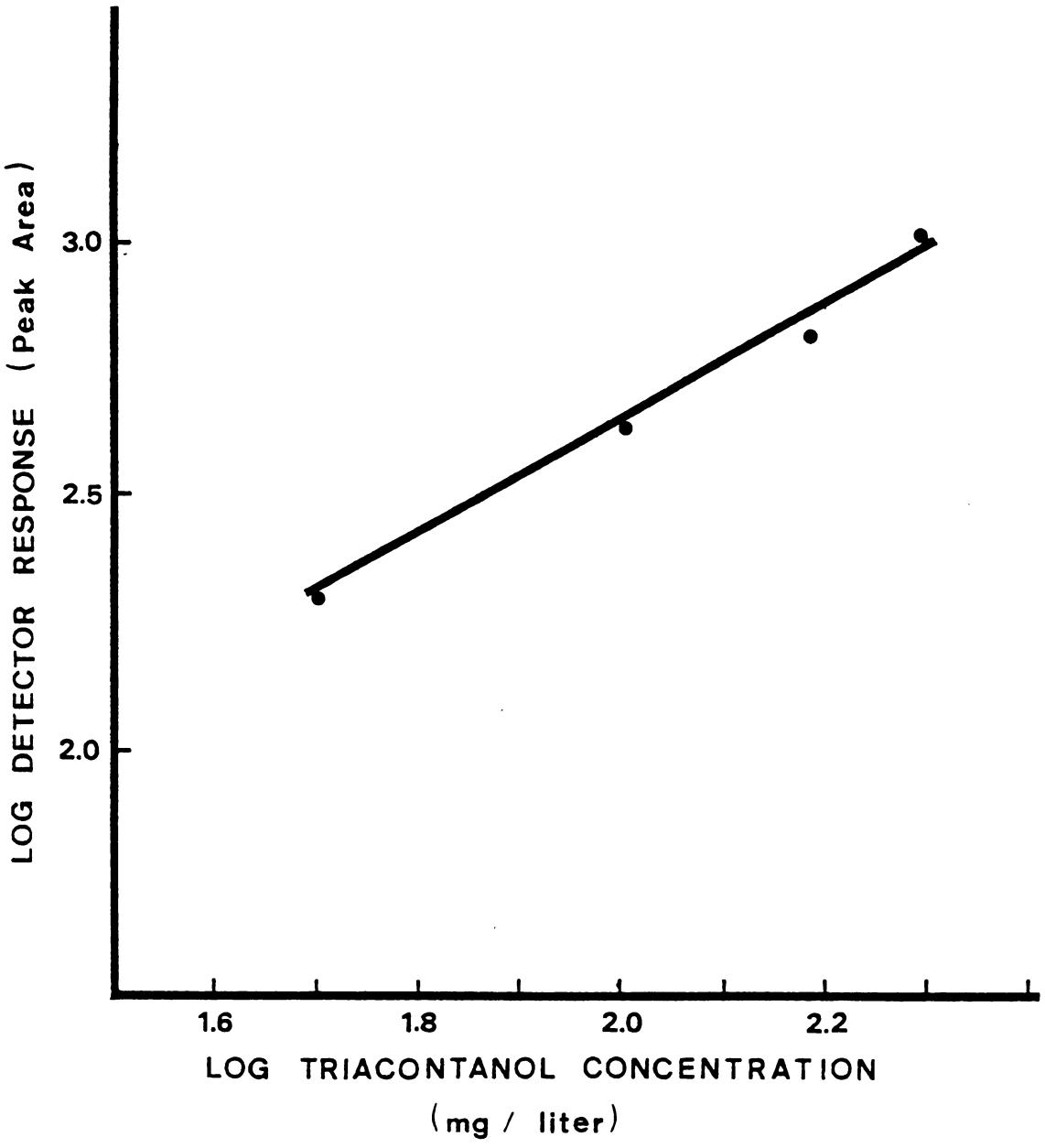

Figure 1. Linearity of flame ionization detection for triacontanol standards. The log of triacontanol concentration is plotted versus the log of the GLC detector response using flame ionization detection and a 1.25% SE 30 column at 240 C. The linearity of response is described by the equation: $y = .409 + 1.12\ x$ with $r = .98$ for 2 samples each of triacontanol concentrations of 50, 100, 150 and 200 mg/L. GLC peak areas were determined manually by triangulation.

gas flow of 45 ml/min.

The sensitivity of the EC detector allowed the detection of the fluoroacyl derivative of triacontanol at a concentration of 1 mg/L or 3 ng per injection. Most samples and standards were prepared in the 30-80 ng per injection range.

The derivative formed for EC was made with heptafluorobutyric acid anhydride which forms an ester of the alcohol containing 7 fluorines. The procedure was as follows: 0.5 ml benzene was added to the dried elutant from TLC in a small teflon-lined screw-top vial. To this was added 0.1 ml of a 0.05 triethylamine in benzene solution followed by 10 µl of heptafluorobutyric acid anhydride. The vial was capped and placed in a 50 C water bath for 15 min. After cooling, 1 ml of water was added and the vial shaken for 1 min. One ml of 5% aqueous ammonia solution was then added and again shaken for 5 min. A sufficient amount of time was allowed for the water and benzene phases to separate (usually about 1 hr). Samples were taken from the upper benzene phase for GLC analysis (62). Although less than 50 ng samples should be used for the derivatization proportions described above (62), triacontanol samples of 60 µg were run using 1/2, 1 and 2 times the reaction mixture, and showed that the reaction mixture is in excess in the above proportions and does not limit most determinations.

The linearity of the ECD response to triacontanol concentration was determined by plotting the log of the detector response versus the log of the concentration according to the formula, Log R (detector response) = log K_2 + log dm/dt. The relationship is shown to be a linear one for the concentrations used (Figure 2).

The retention time of triacontanol standards was examined in relationship to a series of long chain alcohols to verify that the number of carbon

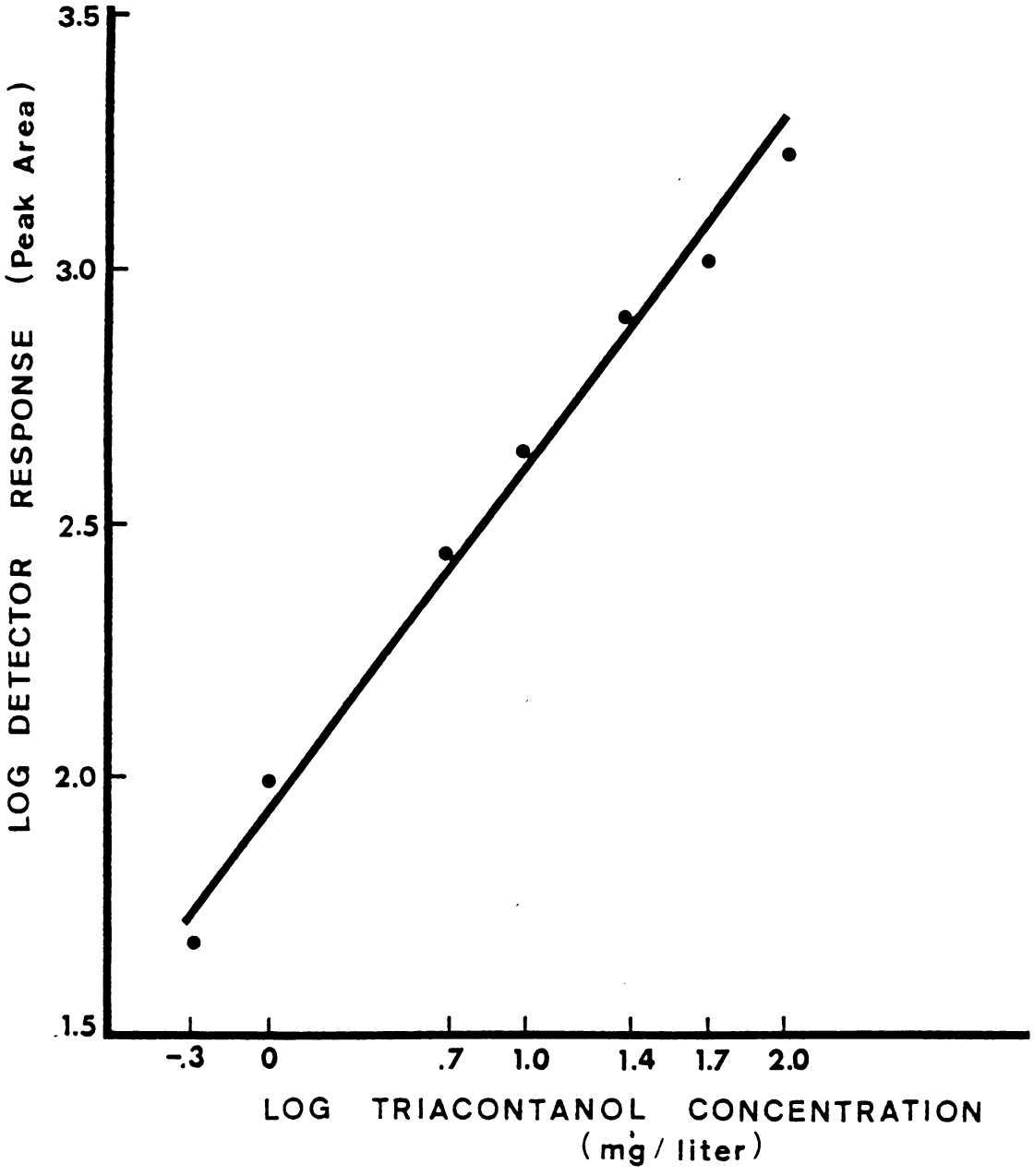

Figure 2. Linearity of electron capture detection for triacontanol standards. The log of triacontanol concentration is plotted versus the log of the GLC detector response using electron capture detection and a 3% SE 30 column at 250 C. Data points are averages of 2 different standard runs of triacontanol concentrations from 0.5 to 100 mg/L. R = .98 for the line equation: $y = 1.94 + .687 x$. GLC peak areas were calculated by computer.

atoms versus retention time could be defined in a linear manner (the log of the retention time versus the number of carbon atoms per molecule should give a linear relationship). The derivatives of the primary alcohols C_{20} to C_{30} were formed with heptafluorobutyric acid anhydride and analyzed by GLC using ECD on a 3% SE 30 column at 250 C (Figure 3).

Several samples were analyzed using two different GLCs, different columns and temperatures, different methods of derivatization and mass spectroscopy in order to further validate the presence of triacontanol in unknown samples. The wax from jade leaves, an extract of cow manure and an extract of sandy loam soil were all derivatized using N,O-Bis-(TMS)-acetamide to form the trimethylsilyl ether of the alcohol. The TMS derivatives were analyzed by GLC using flame ionization detection on a 5% OV-1 column at 300 C and by mass spectroscopy using direct probe (Figure 4). In addition, the fluoracyl derivative was formed on a separate sample and analyzed by the GLC-ECD system described previously, at 230 and 250 C. Data for the jade leaf wax is presented as representative of the samples analyzed.

Table III. A comparison of retention times of triacontanol standards with triacontanol peaks in jade leaf wax using two different columns and different temperatures

Sample	Retention time		
	5% OV-1, 300C	3% SE-30, 250C	3% SE-30, 230C
Jade leaf wax	9.83	4.69	12.82
Triacontanol standard	9.78	4.69	12.90

The mass spectrum of triacontanol shows a peak at the mass-to-charge ratio (m/e) of 510 for the molecular ion and a peak at m/e 495 which is the typical M^+ - 15 pattern for TMSi derivatives. The jade leaf wax, soil and manure samples all show the same peak at m/e 495. The mass spectrum

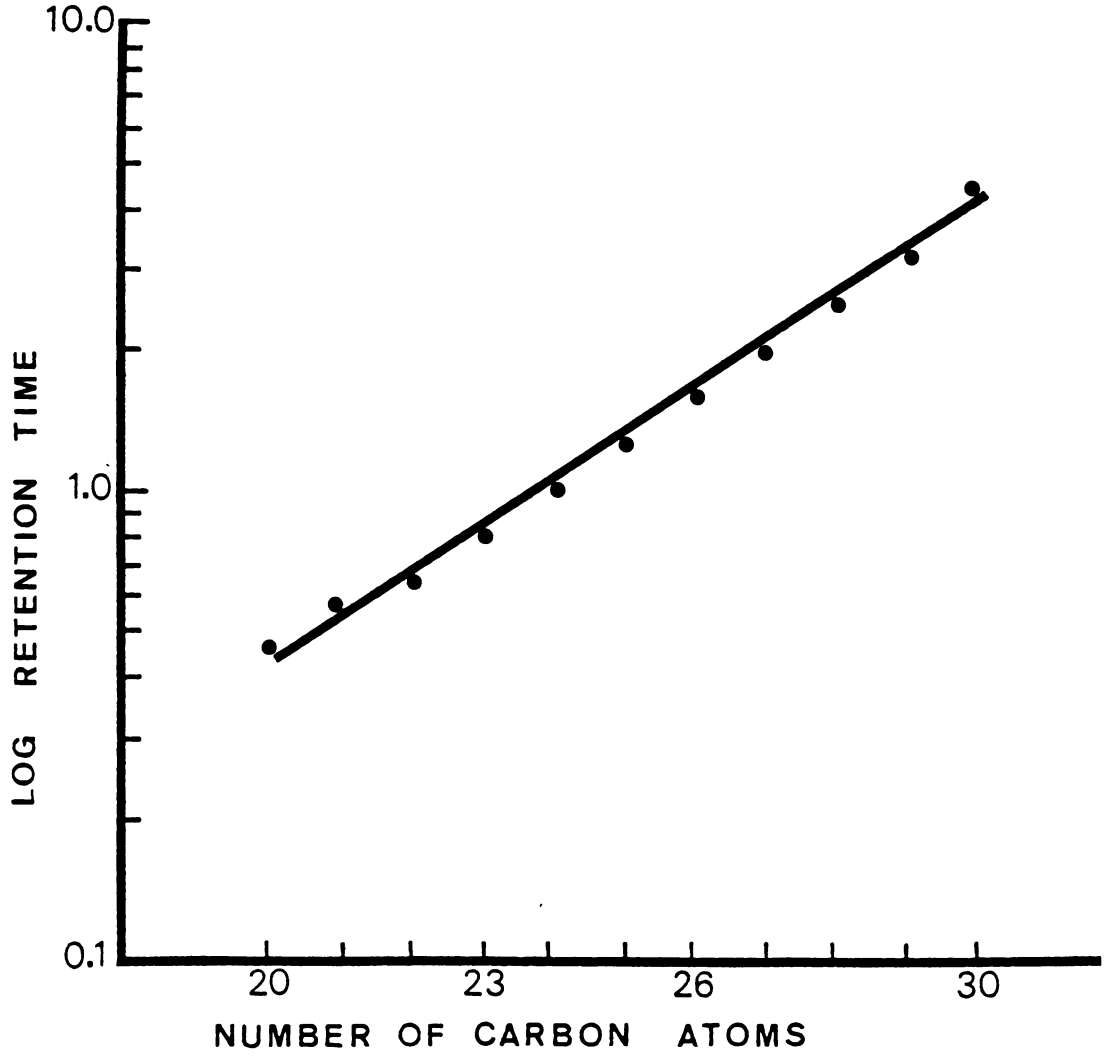

Figure 3. Linearity of GLC retention times for an homologous series of long chain primary alcohols. The log of GLC retention times are plotted versus the number of carbon atoms. Replicated samples of each alcohol were run on a 3% SE 30 column at 250 C with ECD. The linearity of response is described by the equation: $y = -2.31 + .097\ x$ and $r = 0.99$. GLC retention times were calculated by computer.

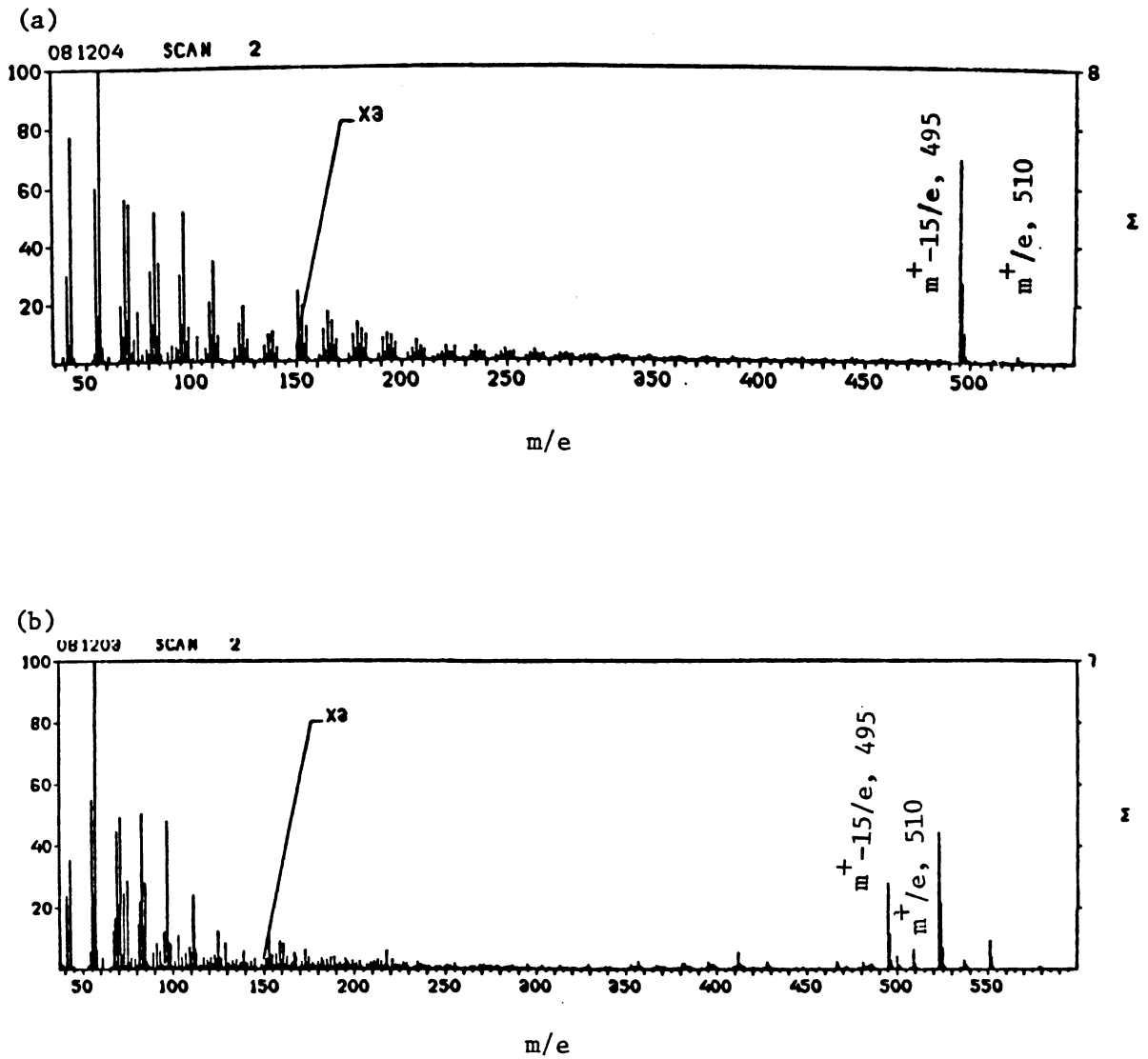

Figure 4. Mass spectrogram of jade leaf wax chloroform extract. Electron impact (70 ev) mass spectrum of TMSi derivative of triacontanol (a) and jade leaf wax extract (b). The analysis was carried out with a direct probe inlet, using a Varian CH-5 double-focusing mass spectrometer and a PDP - 11/40 minicomputer system for data acquisition and reduction. The right ordinate shows percentage of total ionization, the left ordinate the normalized intensity.

of the samples shows that they contain only long chain alcohols indicating that the preparative TLC procedure is efficient and specific. In the example spectrum of jade leaf wax (Figure 4), the peaks are 14 m/e units, or 1 CH_2 atom apart representing the primary alcohol series from C_{28} through C_{33} with major peaks at C_{30} and C_{32}, with the $M^+ - 15$ peaks being the major peaks in all cases.

Thin layer chromatography

Following the extraction process, samples were applied to thin layer plates for preparative chromatography. It was found that the long chain alcohols could be separated from the other wax components quite successfully using silica gel G, 250 microns thick, as the stationary phase and 2% ethanol in chloroform as the solvent system.

Several different solvent systems were tried in an attempt to get the best possible separation of the fatty alcohols from the rest of the wax material on the plate. A ketone (C_{35}), a fatty acid (C_{16}) and a hydrocarbon (C_{30}), all straight chains, were run on thin layer plates using 3 different solvent systems and their Rf values compared to that of triacontanol.

Table IV. Rf values for triacontanol and various wax components with different solvent systems for thin layer chromatography

Solvents	C_{35} ketone	C_{30} alcohol	C_{16} fatty acid (Rf value)	C_{30} alkane
Ether:hexane:methanol (40:10:5)	.95	.89	.69	.96
Chloroform, 6% acetic acid	.92	.63	.69	.92
Chloroform, 2% ethanol	.79	.54	.12	.78

Chloroform + 1% ethanol and ether:hexane:methanol are reported in the literature for use in separating wax components (28,40). The later improved

the fatty acid and alcohol separation while the first did not give much alcohol movement on the gel. Increasing the percent ethanol to 2% gave good separation of the alcohols from the acids, as well as adequate solubility in the liquid phase (Figure 5).

After drying, the plates were developed by spraying with a 0.1% alcoholic solution of 2,7 dichloroflorescein followed by a water spray. This method of developing TLC plates is non-destructive and allows the further elution of material off the plate (79). Sections of the plate were scraped and eluted in test tubes in a warm water bath (50 C) with several ml of a chloroform-methanol 2:1 solution for 3 successive elutions. The tubes were shaken intermittantly to give gel-solvent contact. The solvents were evaporated with filtered air and derivatized for GLC.

The percent recovery from the TLC plates was established by 4 different runs in which 2 samples each of triacontanol were either applied to a plate or derivatized directly without TLC.

Table V. Percent recovery of triacontanol standards from TLC

Date	TLC	Triacontanol (µg)	Peak area	Recovery (%)
7-18-77	0	18	330	85
	+	18	281	
7-21-77	0	18	2093	73
	+	18	1528	
11-25-77	0	6	3616	94
	+	6	3379	
3-11-78	0	12	7.49	70
	+	12	5.22	

Peak areas were calculated by computer except for runs on 3-11-78 which were calculated by triangulation. GLC was performed using a 3% SE 30 column at 250 C with ECD. Samples were derivatized with heptafluorobutyric acid anhydride.

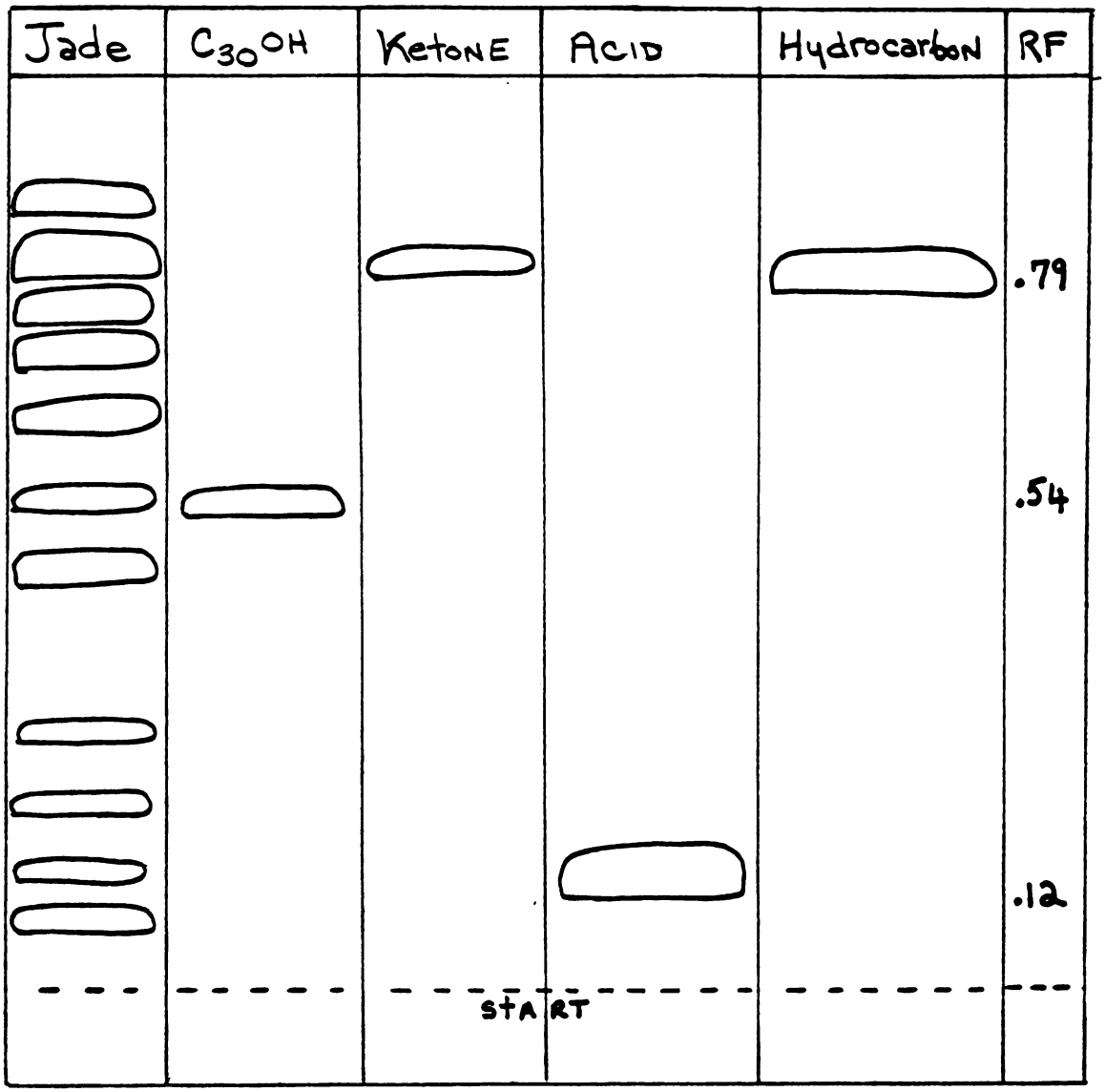

Figure 5. TLC chromatogram comparing Rf value of triacontanol with other wax components. The Rf value of the C_{30} alcohol is compared to the Rf values of a C_{35} ketone, a C_{16} fatty acid and a C_{30} hydrocarbon using silica gel G as the stationary phase and chloroform + 2% ethanol as the liquid phase. The plate was developed with 0.1% 2,7 dichloroflorescein followed by a water spray and viewed under uv light.

An extract of jade leaf wax was applied to a thin layer plate and sections above and below the Rf value corresponding to triacontanol were analyzed for triacontanol content in order to determine whether there was any drag or diffusion of the triacontanol on the plate. One cm sections above and below the triacontanol Rf value did not contain peaks which co-chromatographed with the standard when analyzed by GLC.

Sources of contamination

During the course of this study, various sources of contamination were found when blanks were run through the extraction and analytical process. The most serious contaminant came from the cotton fiber thimbles (Gossypium sp.) used to hold the sample for Soxhlet extraction. Whatman extraction thimbles (cellulose, single thickness, 26x60 mm) were found to contain triacontanol when they were extracted alone.

The fiber thimbles were extracted by two different methods: by Soxhlet extraction for 8 hr and by soaking them in chloroform-methanol 2:1 for 24 hr. The amount of triacontanol in each sample was calculated per thimble to give an estimate of the material which might possibly leach into a given plant sample. Unfortunately the thimbles varied in triacontanol concentration so that samples could not be corrected accurately for thimble concentration. A glass wool mat was found to be free of triacontanol and a practical material to hold the sample for extraction.

After examining the extraction thimbles, Whatman #1 filter paper was also analyzed for triacontanol content since filter paper was used for filtering extracts prior to TLC application. In addition, filter paper is used as the major method of triacontanol application for hydroponic and tissue culture bioassays. Since filter paper is also composed of cotton fiber, the recovery of triacontanol was predicted. A sample of 3.38 g of 5x9 cm Whatman #1 filter paper was soaked in 200 ml of chloroform-

methanol solution for 24 hr. The total extract was dried and prepared for GLC analysis using ECD.

The presence of triacontanol in plant, soil and manure samples

A survey of some common crop plants was taken to establish the general occurrence of triacontanol at detectable levels. Samples were analyzed by TLC and GLC equipped with ECD at 250 C unless otherwise specified. Samples were replicated and the GLC peak areas were calculated as the average of two runs of each sample.

Corn cv. Pioneer 3780 was extracted by dipping the leaves in chloroform and by Soxhlet extraction of the total leaf tissue. One week old corn leaves were dipped in chloroform giving a final 10 ml extract of 4.8 g dry weight of leaves. For Soxhlet extraction, 10 g dried, ground corn leaves were used for each of 12 samples. Each extract was brought up to 50 ml of solvent after evaporation. In the case of the chloroform dip 100 µl samples were taken for analysis while 50 µl samples were taken from the Soxhlet extracts.

Another study was designed to see whether there was any noticeable difference in the amounts of triacontanol in corn leaves over time and whether such a difference would bear any relation to the amount of triacontanol in the outer leaf wax compared to the total extract. Corn leaves were harvested in successive weeks and 1000 cm^2 of leaf area were dipped in chloroform. The leaf material was dried and weighed and the solvent evaporated and prepared for GLC using flame detection. The dried material was re-extracted using the saponification method described previously for total lipids. Corn seed was germinated for 24 hr and 20 g of dry material was extracted by Soxhlet. The seed extract was transferred to a 10 ml volumetric from which 200 µl was taken for GLC analysis using ECD.

Soy bean leaves (Glycine max) and the ungerminated soy bean seed were extracted to determine the presence of triacontanol. Soy bean leaves were extracted by dipping in chloroform, which was evaporated to 10 ml. After drying, the weight of the leaves was 13 g. A sample of 100 µl was taken for analysis.

Ground soy bean seed was extracted using the saponification method. The extract of 19 g dry weight seed was brought up to a total volume of 13 ml after evaporation. Analysis was performed by GLC equipped with flame detection at 235 C.

Five different samples of alfalfa cv. Vernal stems and leaves were extracted by Soxhlet extraction. Each 5 g sample was transferred to a 10 ml volumetric after evaporation from which 100 µl was analyzed.

Rice roots and shoots were analyzed separately for both triacontanol and octacosanol concentration. Duplicate samples of shoots and roots were Soxhlet extracted for 6 hr. The rice shoot extract was brought up to a 5 ml volume in chloroform and the root extract to 1 ml for 0.6 g and 0.25 g dry weight samples respectively and 100 µl samples taken for analysis.

A chloroform extract of coconut milk was taken by shaking 375 ml of coconut milk with equal volumes of chloroform in a separatory funnel. The extract was evaporated to 25 ml from which 100 µl was taken for analysis.

Xylem exudate was obtained from field corn seedlings grown under field conditions by slicing the stem with a razor blade and collecting drops of exudate with a disposable pipette. The total exudate of 150 ml was extracted with equal volumes of chloroform; the chloroform was evaporated and the total sample was analyzed.

Twenty g of Thomas Agar used for agar media was extracted for 12 hr by Soxhlet extraction. The total extract was evaporated and analyzed.

Several non-plant samples were analyzed for triacontanol. Beeswax was analyzed as an example of the biosynthesis of triacontanol by an insect (9) and as a possible organic source of the alcohol. The occurrence of free triacontanol in beeswax has been noted by several researchers although there have been differences in the analyses reported (9,77). One g of beeswax was dissolved in 10 ml of chloroform from which 50 µl (.005 g) was analyzed.

Two other types of non-plant materials were analyzed for triacontanol content. Soil and cow manure which contain plant residues are both components of the crop environment and might influence the application of triacontanol to crops in the field.

Two soils were analyzed, a Spinks sandy loam and a Miami silt loam, both from tomato test plots where increased yields were measured from triacontanol treatments (66). Samples of 60 g of each soil type were extracted by Soxhlet for 8 hr. Each extract was evaporated to 5 ml from which 100 µl was analyzed.

Extracts of manure from a Holstein cow and from an unborn calf were analyzed in a similar manner to the soil samples. Each analysis was performed on 100 µl samples taken from Soxhlet extracts of the 2 manures. For the cow manure 10 g was extracted and the extract evaporated to 11.5 ml. A calf manure sample of 10 g was likewise extracted and brought up to 25 ml.

The location of triacontanol within the plant

Plant parts other than the surface wax were extracted to see if triacontanol could be found inside the plant.

The oil of jojoba seed was diluted 1:1 with chloroform and a sample equivalent to 12.5 µl of the oil was analyzed.

Dried potato peel cv. Norchip was extracted using a chloroform soak

for 2 hr and an extract of 0.43 g was taken for analysis. The potato tuber was peeled with a razor blade and dipped briefly in chloroform to rid any chloroform-soluble contamination from the peel. After peeling the tuber was dried and ground and 60 g was extracted for 8 hr by Soxhlet. A sample equivalent to 0.6 g was analyzed. Apple fruit cv. McIntosh was peeled in a similar fashion, dried and extracted by Soxhlet. A sample equivalent to 1.2 g was analyzed by GLC.

Broad bean (Vicia faba) leaves were used in a first attempt to isolate and analyze epidermis and mesophyll leaf sections separately. A 2% pectinase solution containing segments of broad bean leaves was swirled in an erlenmeyer flask under vacuum. Releasing the vacuum after a few minutes causes a rapid infiltration of the solution into the leaf tissue. The epidermis was then stripped easily from one side of the leaf, although it was found to be almost impossible to remove both the upper and lower epidermis from the same leaf section. Broad bean leaves were dipped in chloroform to obtain an extract of the outer leaf wax. Sections of the epidermis were also extracted with solvent.

The leaves of jade (Crassula argentea) were used for more thorough studies because they have a thick mesophyll portion which is easy to separate mechanically from the epidermis. To avoid contamination leaves were broken by hand and the mesophyll sections were cut from the interior of the leaf without contact with the epidermis. While this method produced pure mesophyll tissue, the epidermis could not be obtained free from mesophyll cells so that the best method of extraction of the outer wax was dipping the whole leaf in solvent.

Fresh jade leaves (11.7 g dry weight) were dipped in chloroform for 30 sec. Mesophyll sections were dried in an oven at 40 C overnight and

then extracted twice with a 2:1 mixture of chloroform and methanol in a heated (50 C) water bath. The solvents were evaporated to dryness and brought up to volume. The entire mesophyll extract of 0.47 g and a sample equivalent to 1.95 g of whole leaf material were analyzed.

Labelled fatty acid incorporation into leaf tissue and recovery from leaf wax

The following experiments were designed to determine the uptake and incorporation of labelled palmitic acid: whole leaves of broad bean or whole leaves and mesophyll sections of jade leaves were incubated with the fatty acid and the surface wax was analyzed for the presence of ^{14}C label. Rf zones from the TLC plates were scraped and counted by liquid scintillation on a Packard model 3003 liquid scintillation spectrometer with PPO and POPOP as the primary and secondary fluors in toluene.

Palmitate-1-^{14}C, 4.03×10^{-4} mM with a specific activity of 54.6 millicuries/mM, was obtained after the solvent had evaporated. The purity of the palmitic acid was tested by plating .44 µCi on silica gel G and developing the plate in hexane:ether:acetic acid (70:30:1 v/v/v). Bands of 1 cm were scraped and counted with nearly all the counts found in a 1 cm section.

For experiments with plant tissue, between .05 and 1 µCi of the labelled fatty acid was dispersed per ml of water with a few drops of tween-20 and sonication with an aerograph ultrasonic cleaner (Branson Instrument Co.). Jade and broad bean leaves were selected which were close in size and relatively young in age so that they would still be growing rapidly. They were kept in the dark with their petioles in water for 2 hr prior to incubation with palmitate. Leaves were incubated in the light for 3 hr after which the soluble wax was extracted by dipping in chloroform and samples of the extract taken directly for radioactivity determination.

An experiment with broad bean and jade leaves was conducted in order to determine whether the label would be incorporated into the leaf waxes and to what extent. Approximately 1 μCi per ml for the broad bean and .25 μCi for the jade leaves were dispersed in water. Leaves were placed in 1 ml each for 3 hr incubation.

Jade mesophyll and epidermal tissue was incubated in small (4.5 cm) petri dishes containing 1 ml water, 1 ml palmitate solution and enough fresh leaf material to cover the dish. The palmitate solution was prepared to give .25 μCi/ml. Leaf material was incubated for 3 hr. Following the incubation, the leaf material was washed thoroughly with distilled water, freeze-dried and extracted with hot chloroform and methanol. Samples of each extract were applied to TLC and 1 and 2 cm sections were scraped and counted. As a check, the palmitate stock was applied to a TLC plate and developed under the same conditions as the leaf extract samples. Some counts were detected in the long chain alcohol region of the plate but not enough to account for the amounts detected in the plant samples based on the initial palmitate incubation solutions applied.

RESULTS AND DISCUSSION

The presence of triacontanol in plant, soil and manure samples

Corn, broad bean, rice, alfalfa, soy bean and jojoba seed were among the crop samples which showed peaks on the GLC which co-chromatographed with authentic triacontanol standards (Table VII).

The corn leaf dip extract of one week old corn yielded an average of 30.9 µg/g triacontanol while the corn leaf Soxhlet extract gave 234 µg/g showing that more triacontanol was extracted from the total leaf than could be accounted for in the surface wax alone (Figure 6). Compared to other crop leaf waxes, corn contains a relatively large amount of triacontanol. Since most of the alcohol is not found in the waxes on the leaf surface it may be bound within the cuticular matrix or synthesized and stored in large quantities in the epidermis.

Triacontanol appears to be synthesized in greatest amounts by 1 to 14 day old corn seedlings. The extreme difference in triacontanol concentration which is noted between the surface and total leaf extracts of 1 week old corn do not continue in the older plants where the difference is minimal after 7 weeks. The percentage of triacontanol extracted from the surface wax compared to the total plant is 46% at 2 weeks and 81% at 7 weeks; the change in percentage over time may indicate a redistribution of triacontanol, a lack of any new synthesis of the alcohol, or simply a change in proportion of the amount of triacontanol synthesized to the increased leaf weight.

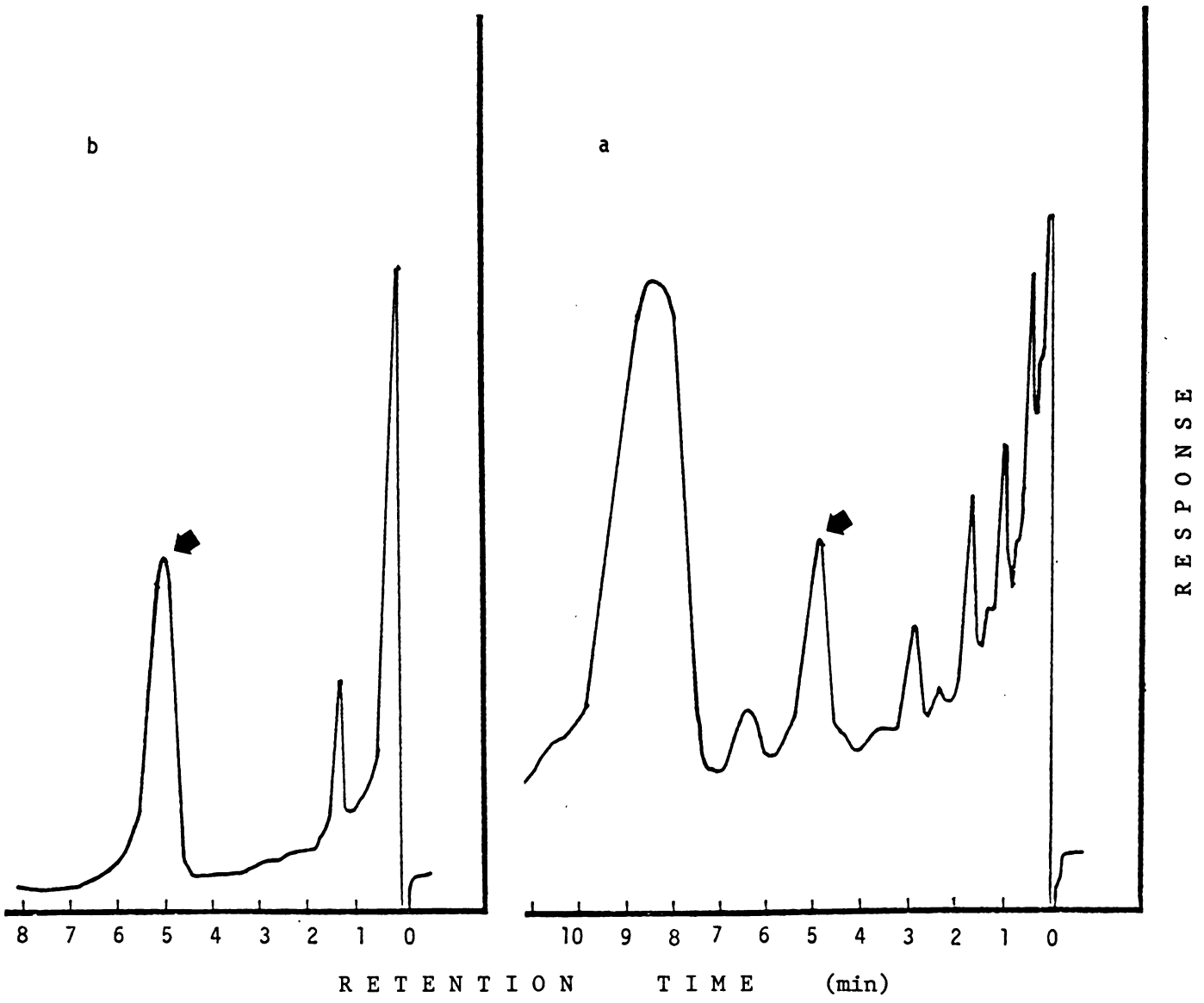

Figure 6. GLC chromatogram of a chloroform-methanol extract of corn leaves. Chromatogram a) extract of corn cv. Pioneer 3780 and chromatogram b) triacontanol standard co-chromatographed with the corn sample. Samples were extracted using Soxhlet extraction for 24 hr. Samples were derivatized with heptafluorobutyric acid anhydride and run on a 3% SE 30 column using ECD at 250 C.

Table VI. Corn leaf wax analysis for triacontanol composition with age of plant

Age (days)	Triacontanol (µg/g) Outer wax	Total leaf	Outer wax as percent of total
1	0	0	
14	42	91	46
28	21	68	31
35	15	42	36
42	13	43	30
49	13	16	81

The shoots and roots of rice when analyzed separately were found to contain 481 µg and 40.7 µg of triacontanol respectively per g of dry tissue. On the other hand, more octacosanol was found in the roots (73 µg/g) than in the shoots (48 µg/g). The ratio of fatty alcohols reverses for the shoot and root with triacontanol predominating in the shoot and octacosanol in the root. Other workers (69,76) have noted variations in the composition of waxes from different parts of the same plant. The information which is available is not sufficient to be able to suggest a pattern for this variation but it may reflect different wax structures and functions. In the case of rice, the surface lipids of the root are probably contained in the polymer suberin rather than wax; the gross structural differences between the 2 materials could easily account for the shift in alcohol ratio.

Another leaf wax which is high in triacontanol concentration is that of alfalfa (Figure 7). The almost exclusive presence of triacontanol among the long chain alcohols is probably responsible for the fact that the triacontanol effect on plant growth was first observed using ground alfalfa as a soil treatment (68).

In addition to plant waxes and polymers, several non-plant samples, soil, manure and beeswax, were found to contain triacontanol at high levels. The triacontanol concentration in the soil provides a background for the

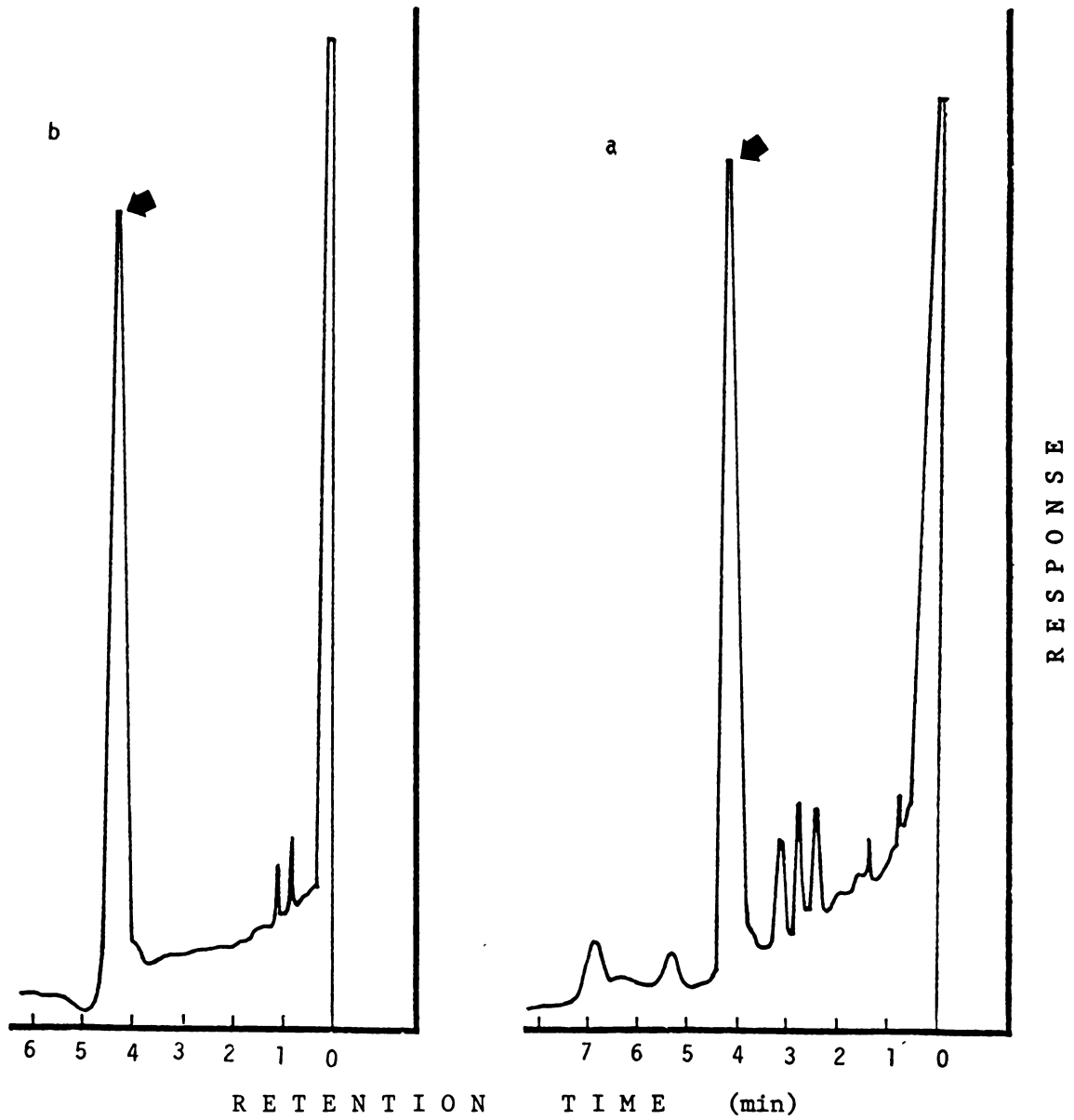

Figure 7. GLC chromatogram of a chloroform-methanol extract of alfalfa leaves and stems. Chromatogram a) Vernal alfalfa leaf and stem extract, and chromatogram b) triacontanol standard co-chromatographed with the alfalfa sample. Samples were extracted using Soxhlet extraction for 24 hr. Samples were derivatized with heptafluorobutyric acid anhydride and run on a 3% SE 30 column using ECD at 250 C.

application of triacontanol to crop plants as a growth stimulator. It is interesting that the amount of triacontanol existing already in the soils which were analyzed does not seem to prevent plants from responding to applied triacontanol. Of the 2 soils which were analyzed, the Miami silt loam contains more organic matter than the sandy loam, and was found to contain twice as much triacontanol (Figure 8).

Another plant residue sample was analyzed in the form of the manure of a Holstein dairy cow and the colon contents of her unborn calf. The calf was fed directly from its mother so that the contents of its colon would have been absorbed through the placental walls or synthesized by the calf. The quantity of triacontanol found in the manure suggests that, unless mammals can synthesize triacontanol, a major portion of the triacontanol ingested is excreted without being metabolized. This agrees with studies by Kolattukudy and Hankin (47) and Oro et al. (60) who conclude that the majority of the long chain acids and alkanes in a mammal's diet are excreted. Manure is known to contain many materials beneficial to plant growth and as such is universally used as a soil supplement. One of the benefits of manure could possibly be due to its concentration of available triacontanol (Figure 9).

In 1934 Chibnall et al. analyzed the alcohol constituents of beeswax from the bumble bee (Apis mellifica). It was shown that the alcohols which had been termed "neoceryl", "ceryl", "montanyl" and "myricyl" by previous workers were actually mixtures of long chain primary alcohols. Montanyl alcohol and myricyl alcohol were both shown to contain 40% triacontanol (9).

Tulloch (77) reported in 1971 that triacontanol constituted less than 10% of the free alcohols in beeswax. With ethanolysis of the wax esters, however, the yield was increased to 34% triacontanol. Downing et al. (12)

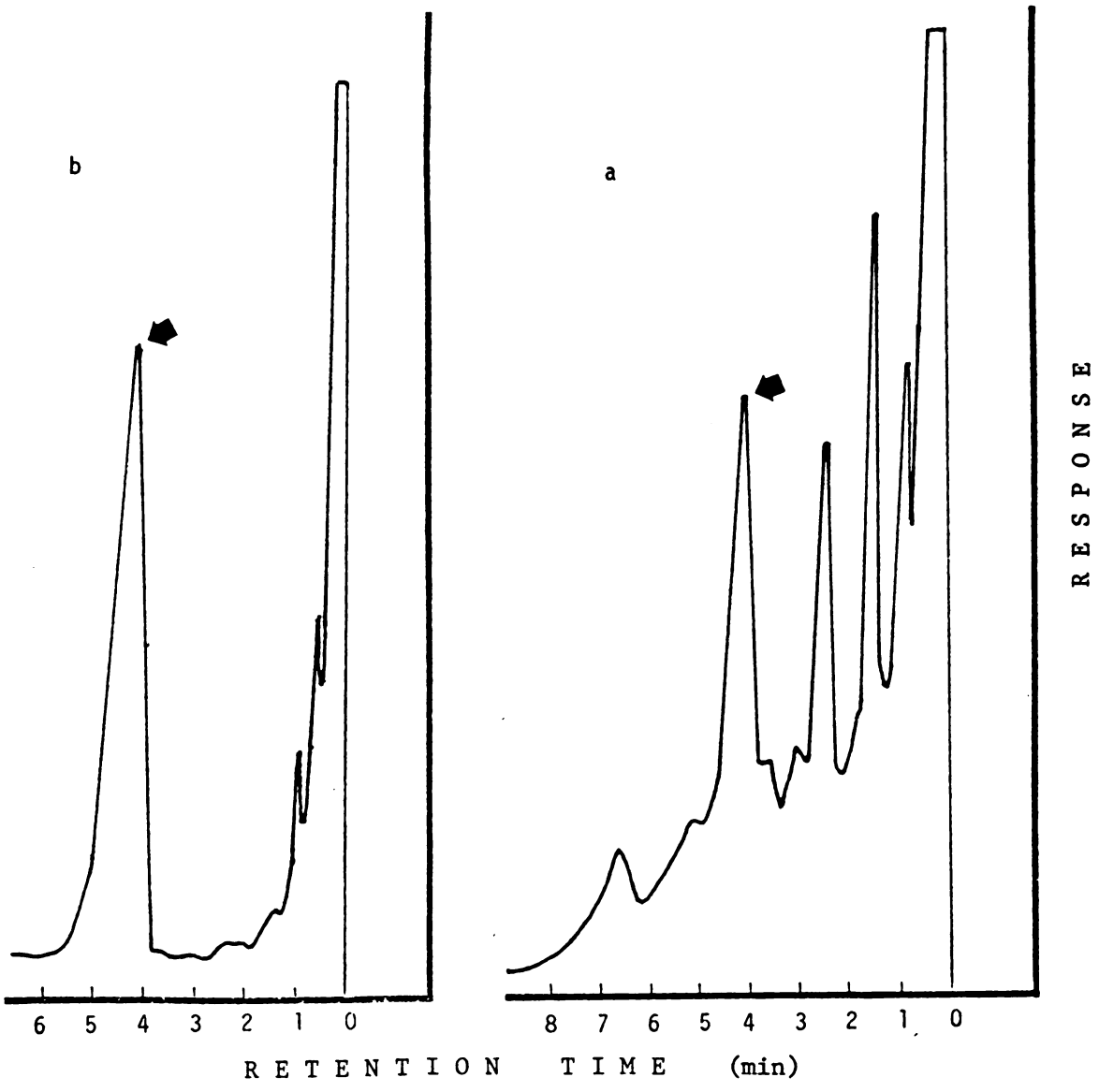

Figure 8. GLC chromatogram of a chloroform-methanol extract of soil. Chromatogram a) extract of Miami silt loam soil and chromatogram b) triacontanol standard co-chromatographed with the soil sample. Samples were extracted using Soxhlet extraction for 8 hr. Samples were derivatized with heptafluorobutyric acid anhydride and run on a 3% SE 30 column using ECD at 250 C.

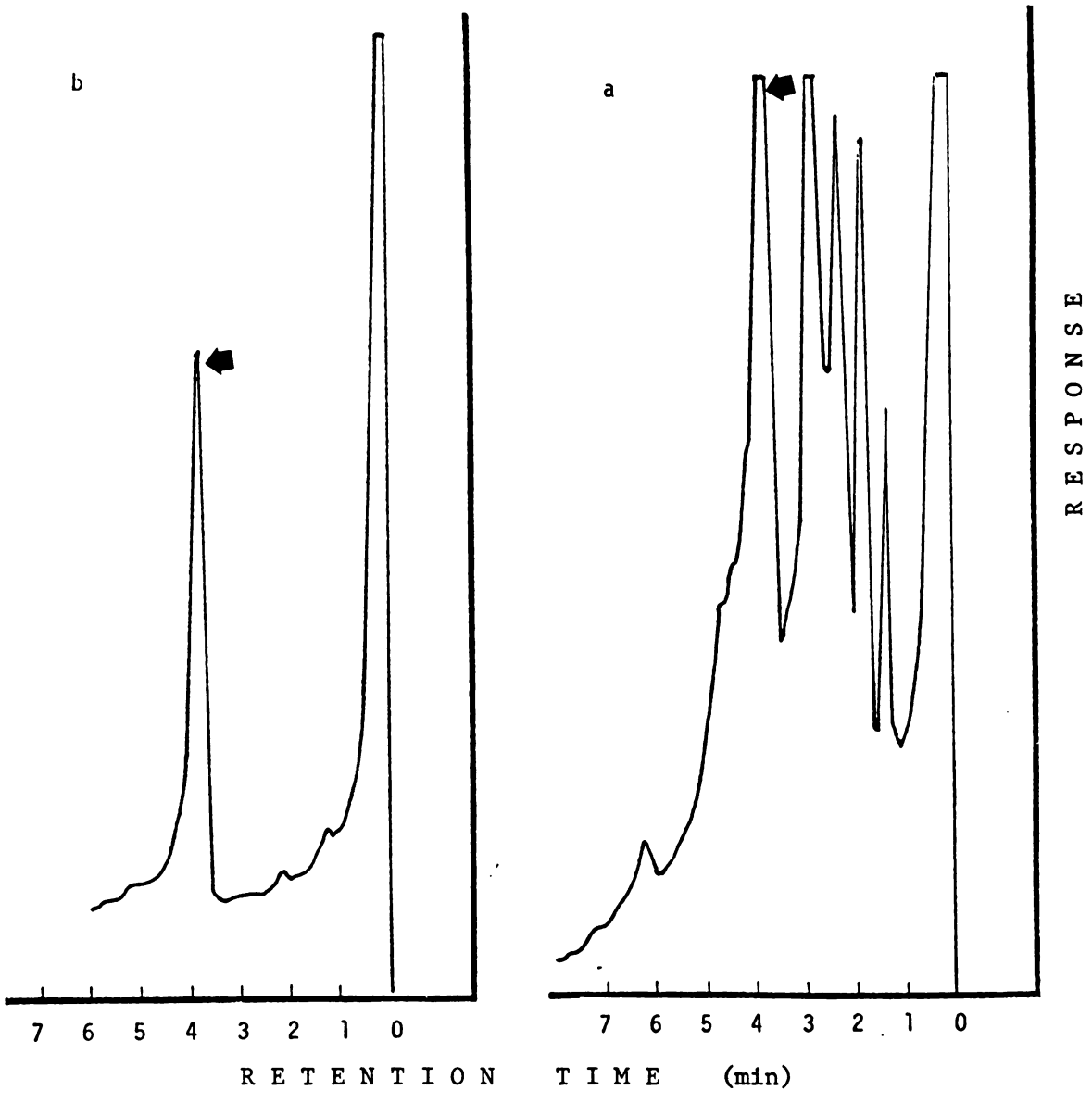

Figure 9. GLC chromatogram of a chloroform-methanol extract of cow manure. Chromatogram a) extract of Holstein cow manure and chromatogram b) triacontanol standard co-chromatographed with the manure sample. Samples were extracted using Soxhlet extraction for 8 hr. Samples were derivatized with heptafluorobutyric acid anhydride and run on a 3% SE 30 column using ECD at 250 C.

Table VII. The presence of triacontanol in extracts of plant wax, soils and manure

Source	Size of extract	Sample size	Extraction method	Triacontanol (µg/g)
Soy bean leaf	12.9 g	.130 g	chloroform dip	70
Soy bean seed	18.7 g	.144 g	saponification	8
Alfalfa leaves	5.0 g	.050 g	Soxhlet	173
Rice leaves	0.6 g	.012 g	Soxhlet	481
Rice roots	0.3 g	.025 g	Soxhlet	41
Jojoba oil	0.5 ml	.013 ml	chloroform	54
Corn leaf	10.0 g	.048 g	Soxhlet	234
Corn leaf	4.8 g	.010 g	chloroform dip	31
Broad bean leaf	1.4 g	.028 g	chloroform dip	13
Corn xylem exudate	150.0 ml	150.000 ml	chloroform	0
Coconut milk	375.0 ml	1.500 ml	chloroform	0
Thomas Agar	20.0 g	20.000 g	Soxhlet	0
Beeswax	1.0 g	.005 g	chloroform	234
Spinks sandy loam	10.0 g	1.200 g	Soxhlet	1
Miami silt loam	10.0 g	1.200 g	Soxhlet	2
Cow manure	10.0 g	.087 g	Soxhlet	97
Calf manure	10.0 g	.040 g	Soxhlet	36
Whatman thimbles	3 thimbles	1 thimble	Soxhlet	0.86
Whatman #1 filter paper	3.4 g	3.400 g	solvent soak	0.40

The detection limit is 10 µg/g on a dry weight basis when analyzed by GLC using ECD and a 3% SE 30 column at 250 C.

found that triacontanol was the major alcohol, constituting 32% of the alcohols after hydrolysis of Apis mellifica wax. The extraction process in the present study gave a very high yield of triacontanol even though hydrolysis of the esters was not employed. It appears that there is a natural variation in beeswax composition much as there is with the plant waxes.

Among samples which were analyzed which did not show any triacontanol at detectable levels were coconut milk, agar and corn xylem exudate.

In conclusion, a variety of plant species and plant residue materials are found to contain free triacontanol in detectable quantities. The general survey presented here illustrates the relative abundance of the long chain alcohol in common crop plants and in the crop environment.

Changes in triacontanol content of a plant over time, and differences in content from similar plant material show the considerable variation in plant wax components that has been found by other researchers. Some variability is accounted for by the extraction procedures used, but much is also due to natural fluctuations in triacontanol level. In order to delineate the amount and causes of this variation, a single plant species should be investigated more thoroughly including the analysis of different cultivars as well as repeated analyses of the triacontanol content throughout the life of the plant.

The location of triacontanol within the plant

The appearance of wax material within the plant is usually ascribed to specialized plant organs such as seeds which may contain waxes as a storage material to be utilized during germination. The seed from the jojoba plant (Simmondsia californica) contains an oil which is a liquid wax-ester mixture, commercially important in the manufacture of comestics, lubricants, candles and pharmaceuticals (82). Soxhlet extraction of the

ground seed followed by saponification has shown that the mature seed contains 35% wax by dry weight (59). The major alcohols of jojoba oil esters have been identified by other workers as eicosanol, docosanol, and tetracosanol with no report on the minor alcohol composition given. In this study the triacontanol content of 54 μg/g indicates the presence of the free alcohol in a seed storage wax believed to contain only wax esters (Table VII).

Several other plant parts were investigated to determine if triacontanol could be found in the inner tissue. Potato tubers (Solanum tuberosum) and apple fruit were peeled and the insides analyzed for triacontanol concentration.

Table VIII. An analysis of the inner tissue of potato tuber and apple fruit

Source	Amount extracted (g)	Size of sample (g)	Triacontanol (μg/g)
Potato periderm	43	0.4	0.68
Potato parenchyma	60	0.6	1.15
Apple fruit parenchyma	60	1.2	0.30

Samples were extracted by Soxhlet for 8 hr and analyzed by GLC at 250 C by ECD on a 3% SE 30 column.

Both the potato and apple parenchyma contained detectable quantities of triacontanol although the concentrations were very low and not adequate for more in depth investigation.

In other attempts to separate inner from outer tissue, extracts of the outer leaf wax of broad bean were analyzed by GLC giving 13 μg of triacontanol per g leaf tissue (Table VII) while extracts of the peeled epidermal sections gave barely detectable peaks when analyzed by GLC due to the difficulty of separating enough epidermal material for an adequate analysis.

More promising experimental leaf tissue is that of jade which can be snapped in half to give uncontaminated mesophyll tissue. The yield of triacontanol expressed per gram dry weight of leaf tissue is 42.3 µg from the surface wax while the mesophyll tissue contained roughly 1/10th or 4.58 µg/g dry weight (Figures 10 and 11). This result is not expected and indicates that triacontanol is found within jade mesophyll cells or within surface waxes which are embedded between the mesophyll cells. If triacontanol is found within the mesophyll cells as distinct from the surface waxes, then this is a clue that the chemical may be affecting cell growth and metabolism.

To follow the question of triacontanol's presence in jade mesophyll tissue, several experiments were designed using a labelled fatty acid to investigate the hypothesis that triacontanol may be synthesized in mesophyll cells. Since it has been shown conclusively by other workers that wax components are synthesized in the epidermis, the synthesis of additional triacontanol in the mesophyll would suggest that its presence in jade mesophyll is not due to embedded wax alone.

Palmitate-1-^{14}C was used as the labelled precursor since it has been shown that the C_{16} and C_{18} fatty acids are readily incorporated into the long chain wax components. An initial study showed that radioactivity was recovered in the surface wax of both broad bean and jade leaves when whole leaves were incubated in palmitate solution.

Table IX. Labelled palmitate incorporation in broad bean and jade leaf wax

Source	Total extract of leaf (cpm)	Incorporation in wax (%)	TLC % alcohols
Broad bean	14599	1.12	
Jade	6667	1.11	.88

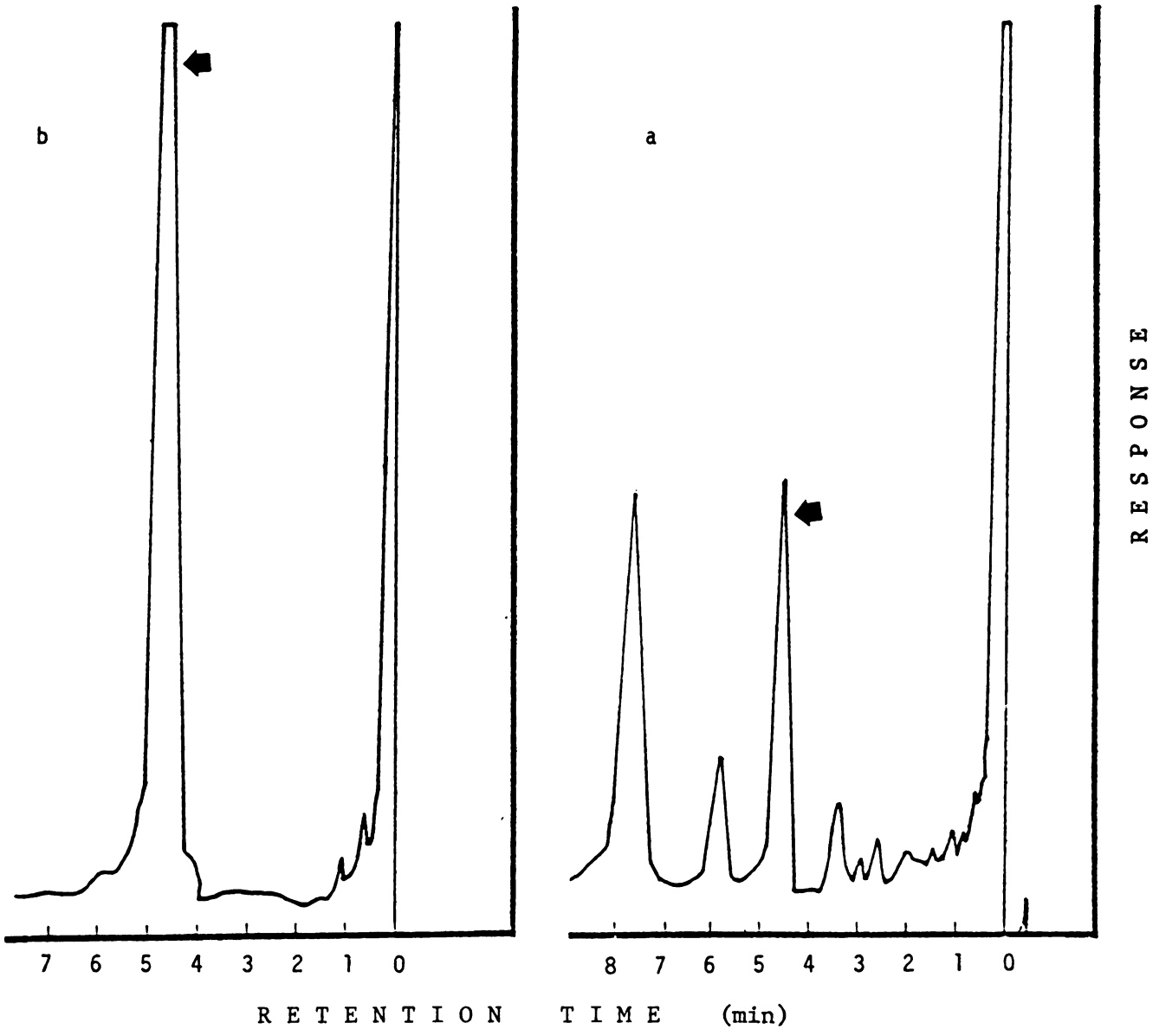

Figure 10. GLC chromatogram of a chloroform extract of jade leaf wax. Chromatogram a) extract of jade leaf wax obtained by dipping in chloroform for 30 sec and chromatogram b) triacontanol standard co-chromatographed with the jade leaf extract. Samples were derivatized with heptafluorobutyric acid anhydride and run on a 3% SE 30 column at 250 C using ECD.

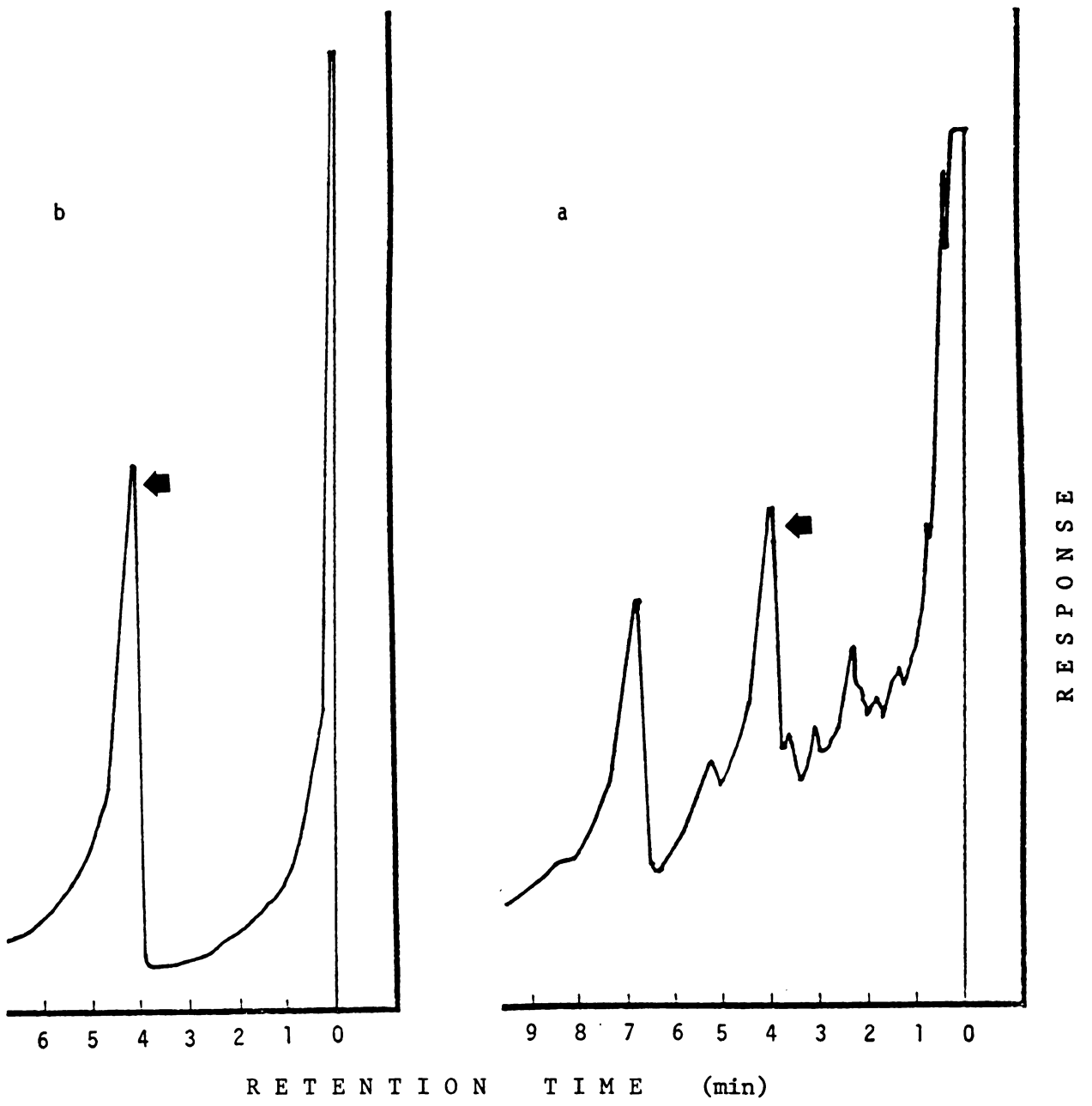

Figure 11. GLC chromatogram of a chloroform-methanol extract of jade leaf mesophyll. Chromatogram a) extract of jade mesophyll section and chromatogram b) triacontanol standard co-chromatographed with the jade mesophyll extract. Samples were derivatized with heptafluorobutyric acid anhydride and run on a 3% SE 30 column at 250 C using ECD.

Whole jade leaves were compared after 1 and 4 hr of incubation with labelled palmitate to see if incorporation of the label into leaf wax would increase with time. Repeated chloroform extraction of the leaf surface was done to determine whether the label could be found below the surface wax.

Table X. Labelled palmitate incorporation in jade leaves after 1 and 4 hr

Length of incubation (hr)	Leaf dip #1 (cpm)	Incorporation in wax (%)	Leaf dip #2 (cpm)
1	476	1.20	33
4	359	0.88	15

The incorporation of labelled palmitate into the outer wax layer of the jade leaf appears to occur within the first hr of incubation. Following the depletion of some substrate within that first hour there is no noticeable increase in incorporation with additional time. Since no additional label was recovered with repeated chloroform extractions it may be concluded that the extraction of surface wax is complete within 1 min (the length of extraction times used here) and that palmitate is incorporated into the epicuticular wax before the other surface layers of the leaf such as the cuticle. Following this evidence, long chain wax components would seem to be transported to the surface immediately upon synthesis.

Subsequent experiments with isolated leaf sections of jade provide an indication that there may be a distinction between those wax constitutents which are synthesized in the epidermis and are transported immediately to the leaf surface, and those long chain compounds which are synthesized in mesophyll tissue.

Three sections of jade leaf, mesophyll material cut from the inside of

the leaf, portions of epidermis peeled from the leaf and remaining leaf material referred to as the 'remainder', were incubated separately in palmitate-1-^{14}C and chloroform extracts analyzed by TLC for the presence of label in the long chain alcohol section.

Table XI. ^{14}C label recovered after solvent extraction of jade leaf tissue

Sample	Total cpm	Tissue dry wt (mg/sample)	TLC alcohol section[a/] (cpm total)	(cpm/mg)	(% total)
Mesophyll	30,744	69.2	397	5.74	1.29
Epidermis	26,801	7.2	170	23.65	0.63
Remainder	17,188	129.2	553	4.27	3.22

[a/] Chloroform-methanol extracts of jade leaf sections were applied to TLC and sections with the same Rf as triacontanol were eluted from the silica gel and radioactivity determined by liquid scintillation.

A second experiment, also with jade leaf sections, gave similar results to those in Table XI:

Table XII. Radioactivity recovered from jade leaf mesophyll and epidermis extracts in TLC sections after incubation of 1-^{14}C palmitate with leaf segments

Rf	Palmitate	Mesophyll Counts per minute Total	per mg	Epidermis Total	per mg	Remainder Total	per mg
0.00	11,826	8,284	136.0			2,831	44.0
0.12		10,139	166.0			1,746	27.0
0.24	127	1,858	31.0			139	2.0
0.29	45	195	3.2			56	0.9
0.35		168	2.8			67	1.0
0.41	64	319	5.2			161	2.5
0.47*	23	421	6.9	51	22.2	191	2.9
0.53	91	161	2.6			76	1.2
0.59		290	4.8			132	2.0
0.65	167	1,056	17.3			233	3.6
0.76	64	1,154	18.9			444	6.9
0.88	0	63	1.0			519	8.0

*Rf value of triacontanol standard

For the second experiment (Table XII), 1 and 2 cm sections from the entire TLC plate were counted in order to determine the label distribution and to check that the label found in the alcohol section was not drag from higher on the plate. Since, for all leaf extracts, the section directly above the triacontanol Rf shows less cpm, a drag effect does not account for the label recovered in the alcohol section.

Radioactivity recovered in the alcohol fraction of the TLC plates suggest that some of the label fed to mesophyll tissue may be incorporated into long chain alcohols. The radioactivity incorporated into the mesophyll cells was likewise not expelled immediately into the surrounding medium as observed for epicuticular wax synthesis. These observations support the hypothesis that there are two distinct mechanisms for triacontanol synthesis, one which involves surface wax formation and one which involves the stock-piling of triacontanol in mesophyll cells. However no conclusions can be drawn from this data regarding the incorporation of radioactive palmitate into triacontanol. To show that the label is in long chain alcohols at all, a sample would have to be run on either a GLC with a splitter for liquid scintillation counting or a high pressure liquid chromatograph. The preliminary experiments reported here could be elaborated to determine whether triacontanol is present (or synthesized) within mesophyll tissue.

The objective of this study was to determine whether triacontanol can be found in mesophyll tissue in order to examine possible roles of triacontanol in plant growth and development. An assumption followed here was that the site and function of a molecule are interrelated. Researchers have reported that long chain wax components originate from the epidermis and are secreted outwards, although a few studies hint at the possibility of finding wax components within plant tissue. Should it

be shown that any wax material, including the long chain alcohols, are indeed found at sites within the plant then new functions would have to be investigated which relate to their new location. This possibility is of interest in the case of triacontanol which has been shown to be biologically active. Much future research is required to unravel the question this suggests, whether triacontanol has a natural role like that of applied triacontanol in the growth of the plant. It is hoped that this study will lay the foundation for future research on this subject.

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